DOCTOR'S FORMULAS

ΦΟΡΜΟΥΛΕΣ ΥΓΕΙΑΣ

βιβλιογραφία

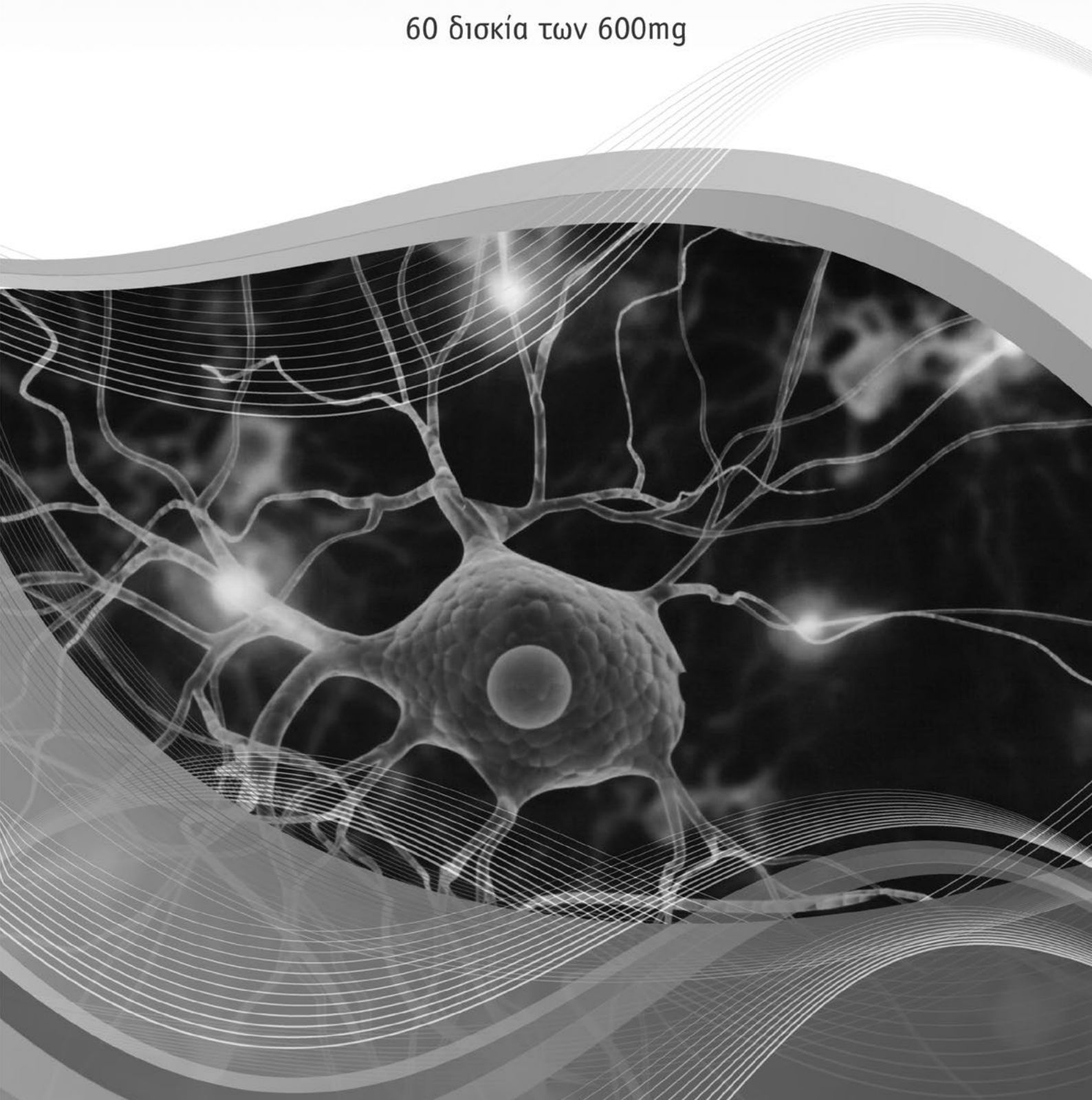
SEDAR

περιεχόμενα

SEDAR

ΦΟΡΜΟΥΛΑ
ΎΠΝΟΥ
για τη χαλάρωση
και τον ύπνο

60 δισκία των 600mg



SEDAR

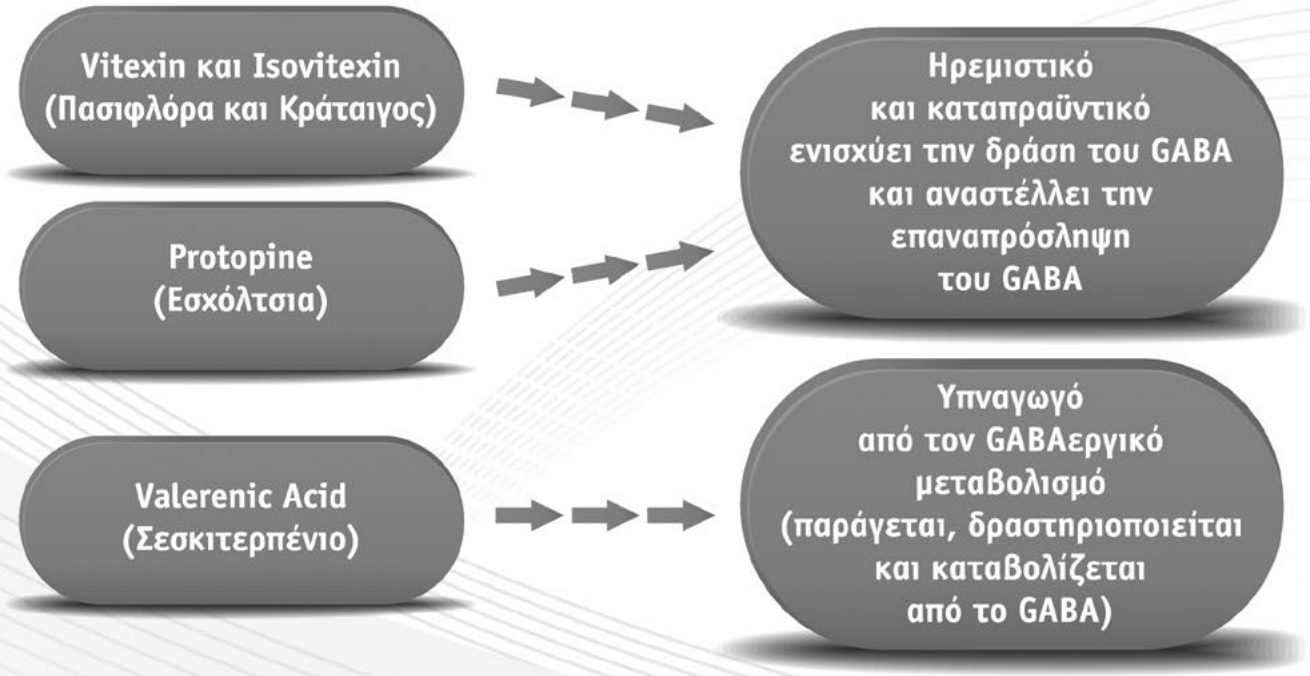
Επιφέρει ένα φυσιολογικό ύπνο σε αντίθεση με τις βενζοδιαζεπίνες, που αναστέλλουν την κατάσταση REM (rapid eye movement) κατά την διάρκεια του ύπνου.

οι νευροδιαβιβαστές που ευθύνονται για τον ύπνο:

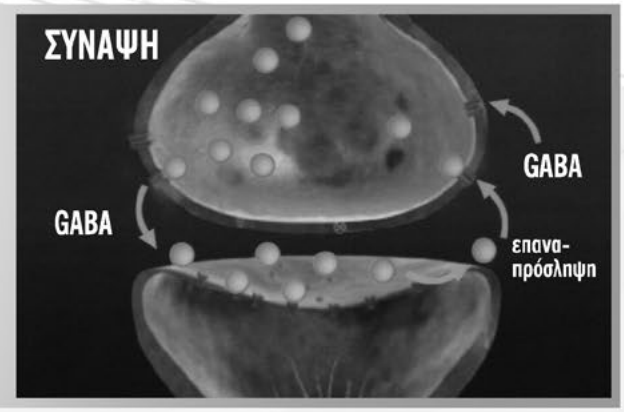
- **GABA** - (γαμμα-αμινο-βουτυρικό οξύ) - Αναστολέας
- **ΣΕΡΟΤΟΝΙΝΗ** - (5-υδροξυτρυπταμίνη) - Αναστολέας
- **ΜΕΛΑΤΟΝΙΝΗ** - Φυσιολογική ροή του ύπνου

ΑΝΑΣΤΑΛΤΙΚΗ ΔΡΑΣΗ ΤΩΝ GABA-ΕΡΓΙΚΩΝ ΝΕΥΡΩΝΩΝ

συστατικά και δράση:



- Περιέχει **ISOVITEXIN** (Pasiflora και Biancospino) - **PROTOPINE** (Escolzia) - **PAPAVERRUBINA** (Papavero rosso και Rosolaccio) συστατικά που παρατείνουν τη δράση του GABA (γ-αμινοβουτυρικό οξύ = νευροδιαβιβαστής που συνδέεται με τον κύκλο του ύπνου και υπάρχει σε φυσιολογική κατάσταση στον εγκέφαλο) μέσω της αναστολής της επαναπρόσληψης.

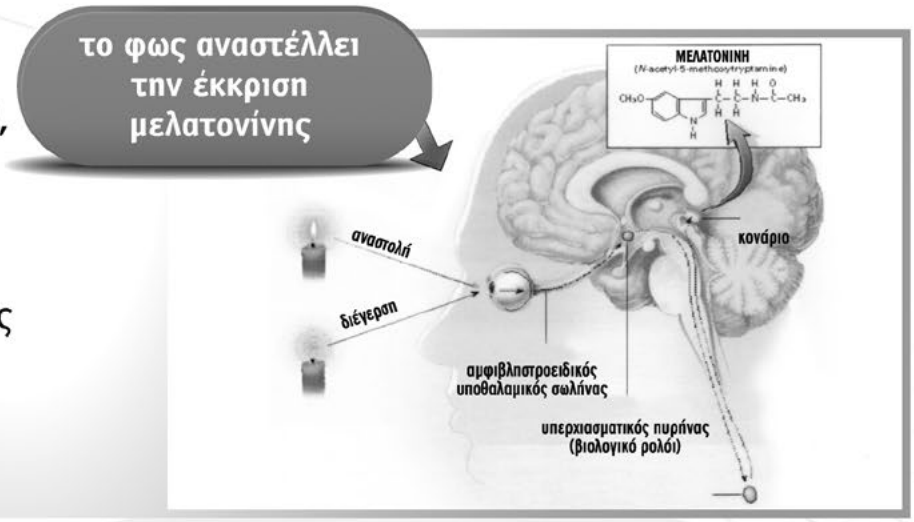


- Περιέχει **L-ΤΡΥΠΤΟΦΑΝΗ**, ένα σημαντικό αμινοξύ που μετατρέπεται μέσα στον εγκέφαλο σε **ΣΕΡΟΤΟΝΙΝΗ** (νευροδιαβιβαστής που λειτουργεί φυσιολογικά στον κύκλο του ύπνου) και που εν συνεχεία μεταβολίζεται σε **ΜΕΛΑΤΟΝΙΝΗ.**

Η ΒΙΟΣΥΝΘΕΣΗ

- L-ΤΡΥΠΤΟΦΑΝΗ (SEDAR)
- ↓
- 5-ΥΔΡΟΞΥ ΤΡΥΠΤΟΦΑΝΗ
- ↓
- 5-ΥΔΡΟΞΥ ΤΡΥΠΤΑΜΙΝΗ
- ↓
- N-ΑΚΕΤΥΛΣΕΡΟΤΟΝΙΝΗ
- ↓
- ΜΕΛΑΤΟΝΙΝΗ

▪ Η Μελατονίνη που υπάρχει στο SEDAR, καθώς και εκείνη που επιτυγχάνεται από την **L-ΤΡΥΠΤΟΦΑΝΗ**, ρυθμίζουν τους ρυθμούς του ύπνου/αυπνίας και προσφέρουν έναν φυσιολογικό ύπνο.



ΔΡΑΣΗ

- **Υπναγωγό**
- **Ρυθμίζει την κατάσταση ύπνου**
- **Αγχολυτικό**
- **Μυοχαλαρωτικό**

ΣΥΝΙΣΤΑΤΑΙ ΣΕ ΠΕΡΙΠΤΩΣΕΙΣ ΟΠΩΣ

- **Διαταραχές ύπνου**
- **Άγχος**
- **Υπερένταση**
- **Εκνευρισμός**
- **Αδυναμία ύπνου**

ΔΟΣΟΛΟΓΙΑ

- Ένα δισκίο τη νύχτα ή σύμφωνα με την συμβουλή του γιατρού.

ΔΙΑΤΡΟΦΙΚΕΣ ΠΛΗΡΟΦΟΡΙΕΣ	ανά 100g	ανα ημερήσια δόση 1 δισκίο
Πασσιφλόρα ξηρό εκχύλισμα σε ποσοστό 3,5% ισοβιτεξίνης	16,67g	100mg
Βαλεριάνα ξηρό εκχύλισμα 0,42% βαλερενικών οξέων	16,67g	100mg
Κράταιγος ξηρό εκχύλισμα 1% βιτεξίνης	16,67g	100mg
Κόκκινη παπαρούνα ξηρό εκχύλισμα (Φυτό Papaver rhoeas) E/D:1/4	8,33g	50mg
Εσχόλτσια (Παπαρούνα της Καλιφόρνιας) ξηρό εκχύλισμα 0,35% πρωτοπίνης	8,33g	50mg
Τρυπτοφάνη	3,33g	20mg
Μελατονίνη	0,17g	1mg

PLOS | ONE

Meta-Analysis: Melatonin for the Treatment of Primary Sleep Disorders

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Abstract

Study Objectives: To investigate the efficacy of melatonin compared to placebo in improving sleep parameters in patients with primary sleep disorders.

Design: PubMed was searched for randomized, placebo-controlled trials examining the effects of melatonin for the treatment of primary sleep disorders. Primary outcomes examined were improvement in sleep latency, sleep quality and total sleep time. Meta-regression was performed to examine the influence of dose and duration of melatonin on reported efficacy.

Participants: Adults and children diagnosed with primary sleep disorders.

Interventions: Melatonin compared to placebo.

Results: Nineteen studies involving 1683 subjects were included in this meta-analysis. Melatonin demonstrated significant efficacy in reducing sleep latency (weighted mean difference (WMD) = 7.06 minutes [95% CI 4.37 to 9.75], Z = 5.15, $p<0.001$) and increasing total sleep time (WMD = 8.25 minutes [95% CI 1.74 to 14.75], Z = 2.48, $p = 0.013$). Trials with longer duration and using higher doses of melatonin demonstrated greater effects on decreasing sleep latency and increasing total sleep time. Overall sleep quality was significantly improved in subjects taking melatonin (standardized mean difference = 0.22 [95% CI: 0.12 to 0.32], Z = 4.52, $p<0.001$) compared to placebo. No significant effects of trial duration and melatonin dose were observed on sleep quality.

Conclusion: This meta-analysis demonstrates that melatonin decreases sleep onset latency, increases total sleep time and improves overall sleep quality. The effects of melatonin on sleep are modest but do not appear to dissipate with continued melatonin use. Although the absolute benefit of melatonin compared to placebo is smaller than other pharmacological treatments for insomnia, melatonin may have a role in the treatment of insomnia given its relatively benign side-effect profile compared to these agents.

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Introduction

Primary sleep disorders are those not associated with a medical condition, substance use or concurrent psychological disorder. In order to be diagnosed with a primary sleep disorder, the sleep disturbance must cause significant distress or impairment in social, occupational, or other areas of functioning [1]. Nine percent of Americans report having insomnia [2]. Thirty-five to forty percent of Americans report having problems falling asleep or excessive daytime sleepiness [3]. Primary sleep disorders are often comorbid with psychiatric disorders, neurological and cardiovascular diseases [4]. Average medical expenses of individuals with insomnia in the United States is nearly $2000 greater annual than those without sleep problems [5]. Poor sleep is also associated with an increased risk of mortality, hospitalization and traffic accidents [6–8].

First line treatment options for primary sleep disorders often include psychological or behavioral therapies [9]. Sleep hygiene,

sleep restriction, stimulus control, relaxation training and cognitive therapy are examples of such non-pharmacological therapies, all of which have shown some evidence of efficacy [10–12]. Pharmacological treatments for primary sleep disorders, like insomnia, include benzodiazepine receptor agonists, benzodiazepines, sedating antidepressants and other drugs with sedating feature (anxiolytics, antipsychotics, antihistamines). Side-effects vary between these medications and can range from residual daytime sleepiness to dependence [13]. There are many over-the-counter medications and herbal therapies that are used by individuals to treat insomnia. However the efficacy and side-effect profile of these substances are not as well known or studied as prescription medications [14].

Many trials have been performed to assess the efficacy of exogenous melatonin in treating primary sleep disorders. Melatonin is a hormone secreted primarily by the pineal gland in response to variations in the circadian cycle and has been used for the last two decades for the treatment of sleep disorders in adults and children [15]. In contrast to most available sleep medications, melatonin has little dependence potential, is not associated with habituation and typically produces no hangover. Given its reported hypnotic effects, relatively benign side-effect profile and over-the-counter availability, melatonin has been widely utilized in the United States [16].

A previous meta-analysis demonstrated that melatonin was beneficial in treating most primary sleep disorders over the short-term (4 weeks or less) [17]. However, since this meta-analysis, 7 trials have been published with an additional 1258 subjects. These additional trials nearly triple the sample size of previous meta-analyses. The additional power provided by these new trials will (1) allow us to more precisely estimate measures of treatment effects and (2) examine moderators of melatonin efficacy. Specifically, we will examine melatonin's effects on sleep latency, total sleep time and sleep quality. We will also examine the moderating effects of measure type, dose and duration of melatonin treatment.

Methods

Selection of Studies

PubMed was searched by two reviewers (AQ and EFO) using the terms "Melatonin" and "Sleep Disorder". The search was further limited to include only randomized controlled trials and meta-analyses. The bibliographies of related reviews, meta-analyses and included articles were searched for additional eligible citations. All studies included were published before or on March 2012.

Inclusion Criteria

Trials were included if they (1) analyzed primary sleep disorders as defined by the DSM-IV, (2) examined the effects of melatonin, (3) were randomized placebo controlled trials, (4) had at least 10 participants for parallel designs or 5 participants for crossover designs and (5) were published in English. Disagreements regarding the inclusion of studies were discussed between the two reviewers (AQ and EFO) and ultimately decided by the third reviewer (MHB) when necessary.

Meta-analytic Procedures

Data was extracted using Microsoft Excel spreadsheets. Extracted data included sleep onset latency, total sleep time, sleep quality, age of sample, dose, duration, drug formulation. Sleep onset latency, total sleep time and sleep quality data were also classified in objective measures (polysomnography or actigraphy) and/or subjective (scales, questionnaires, sleep logs).

Our primary outcome measure was mean improvement in sleep onset latency, total sleep time and sleep quality. In this meta-analysis, we considered sleep efficacy the same as sleep quality. We examined the difference between melatonin and placebo by calculating the weighted mean difference (WMD) using Comprehensive Meta-Analysis (Biostat, Englewood, NJ) for sleep latency and total sleep time analysis. Sleep quality was analyzed in Comprehensive Meta-Analysis by calculating the standardized mean difference (SMD). SMD was favored over WMD for measuring sleep quality because rating scales assessing sleep quality differed between the included studies. A fixed-effects model was used for this meta-analysis with the results for a random-effects model presented as a sensitivity analysis.

Publication bias was assessed by plotting the effect size against standard error for each trial (funnel plot) [18]. In addition, publication bias was statistically tested by the Egger's test and by determining the association between sample size and effect size in meta-regression [18]. Heterogeneity between trials was determined by means of two separate statistical estimates using Comprehensive Meta-Analysis. First, a Q-statistic was employed to provide a test of statistical significance indicating whether the differences in effect sizes are due to subject-level sampling error alone or other sources. In addition, we estimated heterogeneity using *I-square* statistic, which estimates the proportion of total variance that is attributable to between-study variance.

For secondary analyses we performed several subgroup analyses and meta-regressions. Stratified subgroup analysis was used to assess the effects of type of measure (subjective/objective). We used the test for subgroup differences in Comprehensive Meta-Analysis to determine whether subgroups reduced overall heterogeneity [19]. We initially intended to examine the effects of age group (children younger than 18 years old vs. adults older than 18 years old) on melatonin's effects. However, there were not enough trials in children to conduct this analysis. Meta-regression was performed to examine the association between melatonin efficacy in trials and continuous variables such as (1) dose and (2) duration. Our threshold for statistical significance was selected to be $p<.05$ for the primary analysis, as well as for all subgroups analyses and meta-regression. Forest plots were generated separately on Microsoft Excel using previously published methods to aid in presentation of the results [20]. All data including information on the inclusion/exclusion of studies and extraction of data for meta-analysis is available from the corresponding author by request.

Results

Included Studies

We included nineteen studies involving a total of 1683 subjects in this meta-analysis [21–39]. From the search on Pubmed and related bibliography above described 268 studies were selected. A total of 249 manuscripts were excluded for the following reasons: 123 were not randomized placebo controlled trials, 61 did not examine sleep disorders, 40 did not examine primary sleep disorders, thirteen did not examine the effects of melatonin, four did not have sample size fitting the inclusion criteria, five manuscripts were follow-up studies, two manuscripts were not in a peer-reviewed journal, and one study was retracted. Nineteen studies were included in the analysis, fourteen studies on the efficacy of melatonin for the treatment of insomnia, four studies on delayed sleep phase syndrome and one study on REM sleep behavior disorder. Table 1 depicts the characteristics of included studies in this meta-analysis.

Table 1. Characteristics of Included Trials.

Author	Year	Sample Size	Age	Duration	Dose	Design
Wade AG [22]	2011	746	Adults	21 days/182 days	2mg	Parallel
Kunz D [23]	2010	8	Adults	28 days	3 mg	Cross-over
van Geijlswijk IM [24]	2010	70	Children	7 days	0.05 mg/kg, 0.1 mg/kg, 0.15 mg/kg	Parallel
Luthringer R [25]	2009	40	Adults	56 days	2 mg	Parallel
Garzón C [26]	2009	22	Adults	126 days	5 mg	Cross-over
Lemoine P [27]	2007	170	Adults	21 days	2 mg	Parallel
Wade AG [28]	2007	354	Adults	21 days	2 mg	Parallel
Mundey K [29]	2005	13	Adults	28 days	0.3 mg or 3 mg	Parallel
Smits MG [30]	2003	62	Children	28 days	5 mg	Parallel
Almeida Montes LG [31]	2002	10	Adults	21 days	0.3 mg, 1 mg	Cross-over
Kayumov L [32]	2001	22	Adults	28 days	5 mg	Cross-over
Smits MG [33]	2001	40	Children	28 days	5 mg	Parallel
Zhdanova IV [34]	2001	30	Adults	28 days	0.1 mg, 0.3 mg, 1 mg	Cross-over
Dawson D [35]	1998	12	Adults	8 days	0.5 mg	Cross-over
Nagtegaal JE [36]	1998	25	Adults	28 days	5 mg	Cross-over
Ellis CM [37]	1996	15	Adults	7 days	5 mg	Cross-over
Haimov I [38]	1995	26	Adults	7 days	2 mg	Parallel
Dahlitz M [39]	1991	8	Adults	28 days	5 mg	Cross-over
James SP [40]	1989	10	Adults	14 days	1 mg, 5 mg	Cross-over

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Sleep Onset Latency

Our meta-analysis demonstrated melatonin had a significant benefit in reducing sleep latency. Subjects randomly assigned to melatonin fell asleep 7 minutes earlier on average than subjects receiving placebo (weighted mean difference (WMD) = 7.06 minutes [95% CI: 4.37 to 9.75], Z = 5.15, p<0.001). Figure 1 illustrates a forest plot depicting the estimated efficacy of melatonin from individual trials. There was significant evidence of heterogeneity between trials (Q = 31.9, df = 14, p = 0.004, I^2 = 56%). In the random effects model, sleep latency was reduced by over 10 minutes (WMD = 10.18 minutes [95% CI: 6.1 to 14.27], Z = 4.88, p<0.001). We found no significant evidence of publication bias based on the Egger's Test (intercept = 1.08, [95% CI: −0.35 to 2.52], t = 1.62, p = 0.12). Stratifying trials by objective and subjective measures of sleep onset did not significantly reduce heterogeneity between trials (Q = 3, df = 1, p = 0.08). Melatonin significantly reduced sleep latency on both objective (WMD = 5.50 minutes [95% CI = 2.29 to 8.71], Z = 3.36, p<0.001) and subjective measures (WMD = 10.68 minutes [95% CI: 5.78 to 15.58], z = 4.27, p<0.001). Meta-regression demonstrated that trials of longer duration (parameter estimate (PE) = 0.53 [95% CI = 0.21 to 0.86], p = 0.001) reported greater effects on sleep latency. Trials using higher doses of melatonin also reported greater effects of melatonin on sleep latency at trend levels (PE = 1.95 [95% CI = −0.00 to 3.91], p = 0.05).

Total Sleep Time

Melatonin also significantly increased total sleep time compared to placebo. Subjects randomly assigned to melatonin had on average a total sleep time 8 minutes longer than subjects taking placebo (WMD = 8.25 minutes [95% CI: 1.74 to 14.75], Z = 2.48, p = 0.013). Figure 2 illustrates a forest plot depicting the estimated efficacy of melatonin in individual trials. There was significant evidence of heterogeneity between trials (Q = 21.44, df = 12, p = 0.044, I^2 = 44%). Total sleep time was increased by 8 minutes in the random effects model (WMD = 8.48 minutes [95% CI: −4.02 to 20.98], Z = 1.33, p = 0.184). We found no evidence of publication bias based on the Egger's Test (intercept = 0.3 [95% CI: −0.9 to 1.7], t = 0.6, p = 0.52). Stratifying trials by whether objective or subjective measures of total sleep time were utilized reduced heterogeneity at trend levels (Q = 2.6, df = 1, p = 0.10). Melatonin significantly increased total sleep time on subjective measures (WMD = 11.93 minutes [95% CI: 4.06 to 19.81], Z = 2.91, p = 0.002) but did not on objective measures (WMD = 0.33 minutes [95% CI: −11.19 to 11.87], Z = 0.05, p = 0.95). Meta-regression demonstrated that trials of longer duration (PE = 1.60 [95% CI: 0.50 to 2.69], p = 0.004) reported greater effects on total sleep time, as well as trials using higher doses of melatonin (PE = 7.25 [95% CI: 1.94 to 12.56], p = 0.007).

Sleep Quality

Melatonin demonstrated a significant effect in improving sleep quality. Subjects randomly assigned to melatonin had improvements in sleep quality compared to placebo (standardized mean difference (SMD) = 0.22 [95% CI: 0.12 to 0.32], Z = 4.52, p<0.001). Figure 3 illustrates a forest plot depicting the estimated efficacy of melatonin from individual trials. No significant evidence of heterogeneity between trials was observed (Q = 11.59, df = 13, p = 0.56, I^2 = 0). A random effects model provided the same overall effect. We found no significant evidence of publication bias based on the Egger's Test (intercept = −0.13 [95% CI: −1.08 to 0.81], t = 0.30, p = 0.76). Stratifying trials by objective and subjective measures of sleep quality did not reduce heterogeneity between trials (Q = 0.05, df = 1, p = 0.82). Melatonin improved sleep quality to a similar degree on both subjective (SMD = 0.23

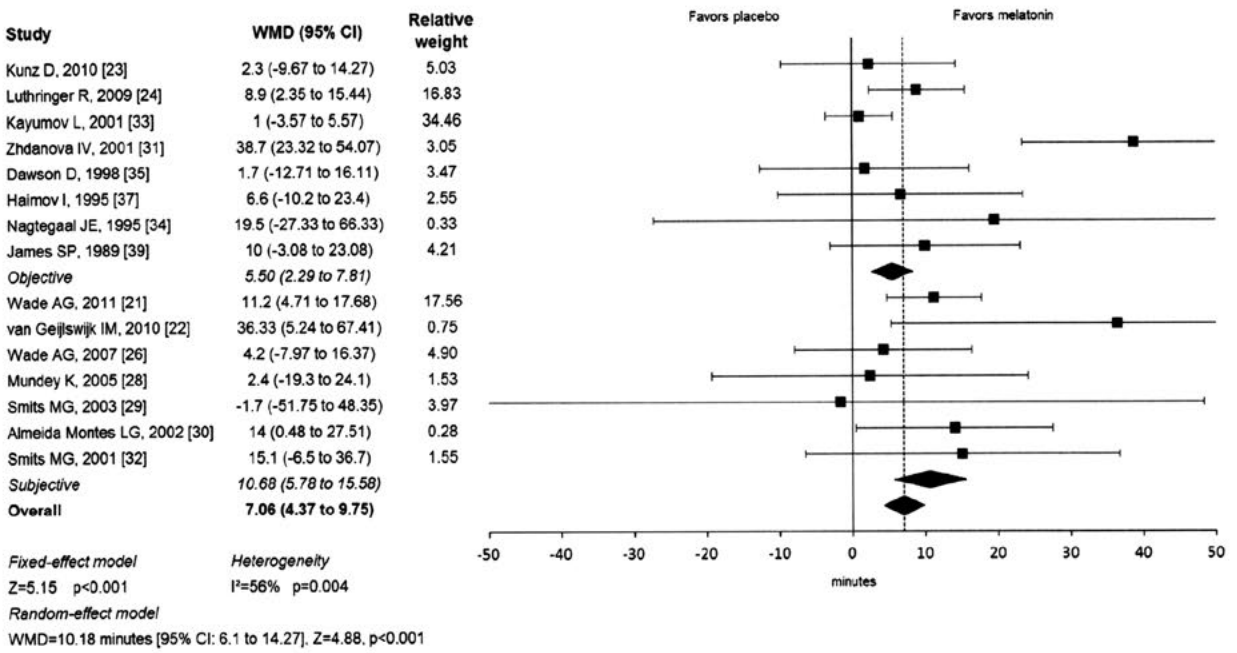

Figure 1. Efficacy of Melatonin in Reducing Sleep Latency. Forest plot depicting reduction of sleep latency in melatonin compared to placebo. Meta-analysis demonstrated a significant benefit of melatonin in reducing sleep latency. WMD = weighted mean difference; CI = confidence interval.
doi:10.1371/journal.pone.0063773.g001

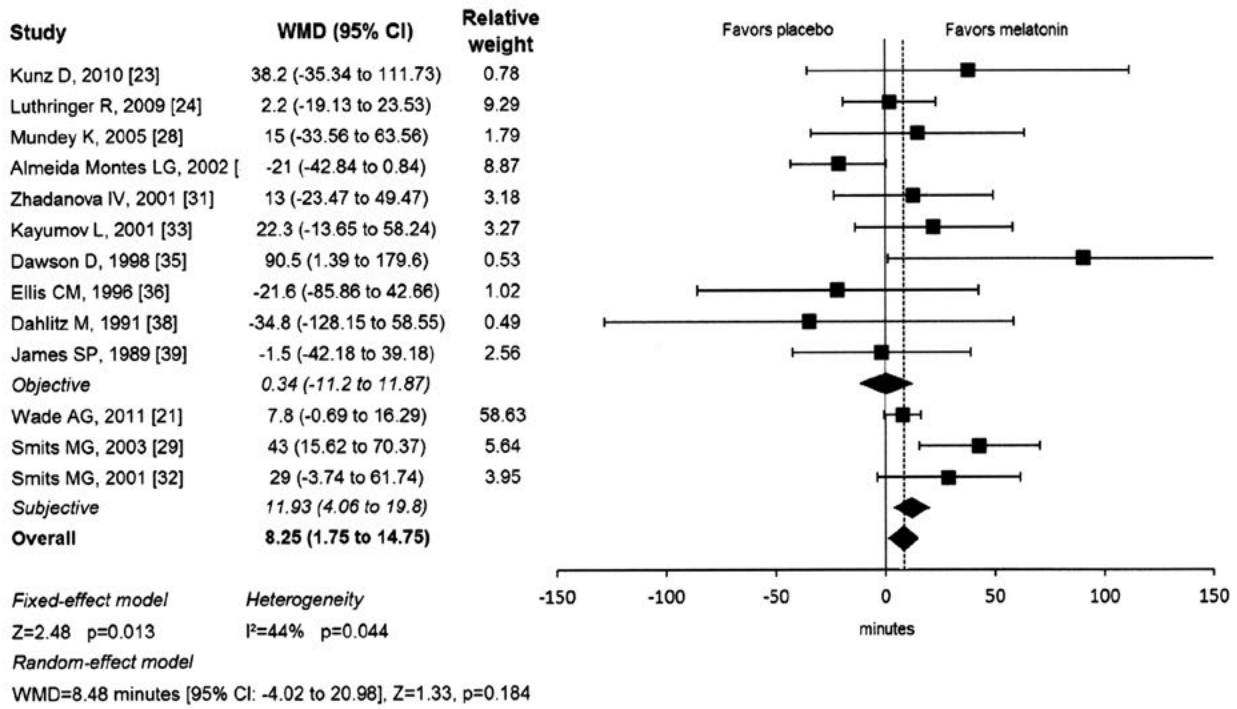

Figure 2. Efficacy of Melatonin in Increasing Total sleep Time. Forest plot depicting change in total sleep time with melatonin compared to placebo treatment. Meta-analysis demonstrated a significant benefit of melatonin in increasing total sleep time. WMD = weighted mean difference; CI = confidence interval.
doi:10.1371/journal.pone.0063773.g002

[95% CI: 0.12 to 0.34], Z = 4.23, p<0.001) and objective measures (SMD = 0.20 [95% CI: −0.04 to 0.44], Z = 1.61, p = 0.10). Meta-regression demonstrated no significant effects of trial duration (PE = 0.005 [95% CI: −0.0006 to 0.012], p = 0.08) or dose of melatonin on sleep quality (PE = 0.011 [95% CI: −0.114 to 0.090], p = 0.81).

Discussion and Conclusion

Our meta-analysis demonstrates that melatonin significantly improves sleep in subjects with primary sleep disorders compared to placebo. Melatonin reduces sleep-onset latency, increases total sleep time and improves overall sleep quality compared to placebo to a statistically significant degree. It should be noted that the improvements in sleep parameters in absolute terms were smaller than previous meta-analyses of benzodiazepines and newer non-benzodiazepine sleep medications. For instance, the reduction in sleep latency observed with melatonin in this meta-analysis, slightly less than 7 minutes, was less than sleep-latency reduction observed in previous meta-analyses of other available sleep medications. A previous meta-analysis demonstrated a significant benefit of benzodiazepines (10.0 to 19.6 minutes) and non-benzodiazepine sleep medicines (12.8 to 17 minutes) in reducing sleep-onset latency for primary sleep disorders [21]. Thus prescription sleep medications are quite likely more effective than melatonin, although head-to-head trials could definitely alleviate any doubts regarding the relative efficacy of these agents.

Meta-regressions were performed to assess the relationship between effect, duration and dose. Higher melatonin doses and longer duration trials were related to significant greater effect sizes on sleep latency and total sleep time. These findings suggest that there is no evidence of the development of tolerance with melatonin use. This stands in contrast to other commonly used hypnotics such as benzodiazepines [13]. No greater effects in sleep quality were observed with melatonin dose or trial duration, suggesting that melatonin effects on sleep quality are constant for any duration or dose.

Given our findings, it is important to note some limitations of this meta-analysis. Due to the relatively small number of trials that met our inclusion criteria, our meta-regression analysis had limited power. This problem was exacerbated by one trial that contributed a very large weight to our overall findings [22]. The relatively small number of trials also limits the ability of the Egger's Test to demonstrate publication bias. However there was no evidence of publication bias for all outcome measures on funnel plot as well. There were relatively few studies examining the efficacy of melatonin in children with primary sleep disorders and this precluded any stratified subgroup analysis based on age. The presence of mostly trials examining primary insomnia limited our ability to perform an analysis based on diagnoses.

Despite these limitations, this meta-analysis demonstrated that exogenous melatonin administered to subjects with primary sleep disorders modestly improved sleep parameters including sleep latency, total sleep time and sleep quality. This finding corroborates the results of a previous meta-analysis conducted in the area several years ago that also demonstrated a significant benefit of melatonin [17]. The benefits of melatonin compared to placebo appear smaller than that of available prescription sleep medications. However, melatonin should be considered in clinical practice due to its benign side-effect profile, cost and limited evidence of habituation and tolerance. Further research is needed to examine the long-term benefits of sleep medications including

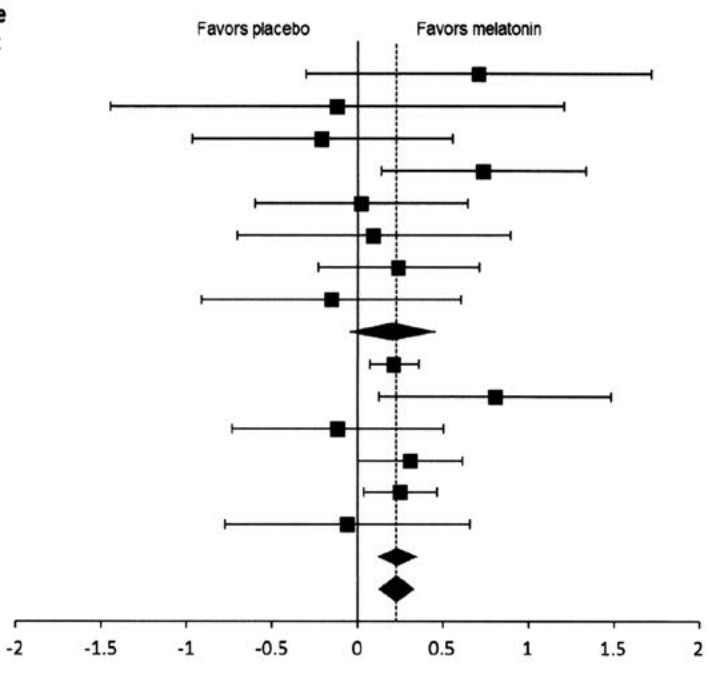

Figure 3. Effect of Melatonin on Sleep quality. Forest plot depicts sleep quality with melatonin compared to placebo. Meta-analysis demonstrated a significant benefit of melatonin in improving sleep quality. SMD = standardized mean difference; CI = confidence interval.
doi:10.1371/journal.pone.0063773.g003

the comparative efficacy of melatonin to common prescription sleep medication.

Supporting Information

Document S1 PRISMA 2009 Checklist. Checklist showing what page are each characteristic analyzed.
(DOC)

Document S2 PRISMA 2009 Flow Chart. Flow Diagram. Flow chart showing the selection of studies for this review.
(DOC)

Author Contributions

Conceived and designed the experiments: EFO AQ MHB. Analyzed the data: EFO AQ MHB. Wrote the paper: EFO AQ MHB.

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EXPERT OPINION

Prolonged-release formulation of melatonin (Circadin) for the treatment of insomnia

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Introduction: Insomnia is common among the elderly. The use of hypnotic drugs in elderly patients is frequently criticized owing to dependency, cognitive impairments, falls and withdrawal effects. The production of melatonin, a physiological sleep and circadian rhythm regulator, declines with age. Prolonged-release melatonin (Circadin®), designed to mimic the endogenous pattern of melatonin production, is licensed for insomnia in patients aged ≥ 55 years.

Areas covered: This review summarizes published studies on Circadin's efficacy and safety (Summary of Product Characteristics and Medline search on 'Circadin' and 'insomnia').

Expert opinion: The main significant and clinically relevant benefits are improvements in sleep quality and latency, next-day morning alertness and quality of life. The responses may develop over several days. An oral 2-mg dose once daily, for 3 months, has generally been well tolerated with no rebound, withdrawal or 'hangover' effects and no safety concerns on concomitant therapy with antihypertensive, antidiabetic, lipid-lowering or anti-inflammatory drugs. Untoward effects of hypnotics on cognition, memory, postural stability and sleep structure are not seen with Circadin. Given as a first-line prescription, with 13 weeks' posology and the lack of rebound effects, Circadin has the potential to improve quality of life in insomnia patients aged 55 years and older and avoid long-term use of hypnotics.

Keywords: elderly, hypnotics, insomnia, melatonin, prolonged-release

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1. Introduction

1.1 Insomnia at older age and health consequences

Sleep is a vital brain-orchestrated process that is responsible for the daily restoration of the body and mind. Insomnia is a disorder characterized by difficulties in initiating or maintaining sleep or nonrefreshing sleep (also termed nonrestorative or poor quality) for at least 1 month, and clinically significant daytime distress or impairment in social, occupational or other important areas of functioning. The disorder is categorized as 'primary insomnia' when it has no known physical, mental or environmental causes [1,2]. People with insomnia experience higher rates of relationship difficulties, energy deficiency and depressed mood, irritability, tiredness, anxiety, low concentration and poor memory, amongst other health and wellbeing problems [3,4]. Far from being a minor irritation, insomnia puts sufferers at significantly greater risks of poor mental and physical health ranging from depression, anxiety, immune deficiency and metabolic and cardiovascular disease [5-8]. Insomniacs report frequent medical problems and have twice as many doctors' visits yearly and a higher hospitalization rate compared with good sleepers [9-14].

The prevalence of the condition varies widely depending on how insomnia is defined. However, up to 15% of adults complain of persistent symptoms of insomnia

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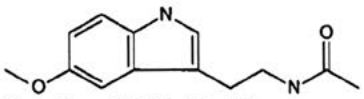
Box 1. Drug summary.

Drug name	Prolonged-release melatonin (Circadin)
Phase	Launched
Indication	Insomnia
Pharmacology description/ mechanism of action	MT1/MT2 and MT3 melatonin receptor agonist
Route of administration	Oral
Chemical structure	C13H16N2O2 
Pivotal trial(s)	Two Phase III trials [93,100] One Phase IV trial [102,103]

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occurring at least 3 nights a week. Insomnia is seen more commonly in women, older people and those with concurrent physical or mental health conditions. Age is a key risk factor for insomnia; from < 10% of those aged younger than 40 years, it is reported in > 40% of individuals aged over 65 years [15-19]. Specifically, the prevalence of poor quality of sleep (sleep described as nonrefreshing) increases with older age [20-22]. Poor sleep quality perception is the strongest risk factor for severely impaired daytime functioning [23-26] and an important risk factor for cardiovascular and coronary heart disease (CHD) incidence [27,28]. Aging is associated with higher incidence of cardiovascular morbidities, particularly hypertension, postural instability and difficulties in memory processing, all of which are exacerbated by insomnia [3,27,29-31].

1.2 Overview of the market

The majority of patients with primary insomnia do not seek medical help for their sleep problem and either continue to suffer or self-medicate with over-the-counter (OTC) medication or alcohol [26,32]. For those that do seek help, initial treatment may be nonpharmacological, consisting of sleep hygiene advice or ideally cognitive behavioral therapy if available. Pharmacological options for the management of insomnia include drugs acting on the GAB_A-A benzodiazepine receptor, mostly short-acting benzodiazepine hypnotics (BZD), such as temazepam, and nonbenzodiazepines commonly referred to as Z-drugs, such as zolpidem, zaleplon and zopiclone. These medicines typically improve sleep onset and total sleep time but do not improve and may even impair daytime functioning [33-39]. Because of concerns about adverse effects including the risk of dependence, in 1988 the Committee on Safety of Medicines (CSM) advised that these drugs should only be prescribed for severe and disabling insomnia or if subjecting the patient to extreme distress. As a general principle, prescribers should always use the lowest effective dose and for a maximum of 4 weeks only [40-43]. If hypnotic drugs are to be used at all they should be used intermittently and gradually tapered off towards the end of the course of treatment to avoid rebound insomnia and withdrawal effects [39,43,44]. Although there is little evidence to support BZD and Z-drugs' efficacy during longer-term use, in particular in the elderly [39], in a recent community survey, more than half of patients who recently used a hypnotic were over the age of 65 years [45,46]. In addition, as insomnia is often long-lasting, especially in the elderly, it is often treated in clinical practice with BZD/Z drugs for long periods [46].

Despite the higher incidence of insomnia in older subjects, little attention has been given to the specific needs of this population in the development of insomnia drugs. Nonrestorative sleep, poor quality of sleep and consequently impaired daytime functioning are the leading and most consistent outcomes of insomnia in older patients [20,22,24,47]. It is now increasingly emphasized that successful treatment of insomnia should not be judged on the basis of sleep quantity or quality alone but, most importantly, should be accompanied by better daytime functioning and quality of life without dependency issues and better safety in the elderly [48-51].

2. Introduction to the compound

2.1 Melatonin – a physiological regulator of sleep-wake cycles

The time, depth and duration of sleep are determined by the homeostatic need (which accumulates during waking hours) and the circadian clock phase (which adjusts sleep to overlap the night hours). Melatonin is a hormone produced naturally in the pineal gland in the dark in response to the night signal from the circadian clock and helps the body to prepare for and maintain sleep [52]. It has a very short half-life and concentrations are maintained by continual production for 8 – 10 h during the night [53,54]. Dim light melatonin onset (DLMO) is considered a valuable marker of the endogenous circadian clock phase [55,56].

Melatonin plays a major role in the circadian regulation of sleep, thermoregulation and blood pressure. First of all, the nocturnal production of melatonin corresponds to the main sleep period in humans. The mean phase relationship between sleep onset and DLMO is constant (~ 2 h) [57]. In totally blind individuals, the circadian rhythms in sleep and wakefulness as well as melatonin production are free running with periods of sleepiness corresponding to peak melatonin production [58]. Certain drugs, (e.g., beta-blockers, typically used to treat hypertension) inhibit melatonin production and impair sleep [53,59]. Accordingly, exogenous melatonin is soporific, which means that it can induce sleep when the homeostatic drive for sleep is insufficient (e.g., upon awakening in the morning) [60]. This effect is mediated by increase in heat

loss [61] and changes in activation patterns of sleep-related brain networks (e.g., the precuneus, parahippocampus) [62,63]. Administration of exogenous melatonin during the day attenuates the wake-promoting signal of the circadian system (chronoquiescent), leading to equal sleep propensity throughout the 24-h cycle [60,64].

In addition to its soporific action, melatonin also acts as a chronobiotic, to phase shift the circadian clock (e.g., in jet-lag or delayed sleep phase syndrome) or to synchronize the free-running circadian sleep-wake rhythm with the 24-h day/night cycle when light is not available (such as in totally blind individuals) [58,65]. The treatment of such disorders (termed circadian rhythm sleep disorders) is aimed at shifting the circadian clock to match the environmental/societal norms [65], but there is at present no medicinal melatonin product approved for such use. A systematic review of published studies on exogenous melatonin (formulations and doses not standardized) for primary insomnia and circadian rhythm sleep disorders showed decrease in sleep onset latency in such patients compared with placebo, but quality of sleep was not significantly improved [66]. Exogenous melatonin simulates/mimics nocturnal circadian physiology (i.e., reduced body temperature, blood pressure and arousal and increased heat loss, fatigue and sleepiness during the night) in patients with low or abnormal melatonin production [61,67-72] but these effects may depend on the formulation used.

Melatonin acts via MT1/MT2 G-protein-coupled receptors (GPCRs) and MT3 receptors located in the brain (e.g., in the circadian clock residing in the suprachiasmatic nuclei, SCN, of the hypothalamus) and peripheral organs (e.g., blood vessels) [73]. MT1/MT2 receptors mediate the soporific and chronobiotic effects of melatonin. The presence of MT1 and MT2 receptors in the SCN and hippocampus, and melatonin's physiological activities in these areas implicate these receptors in the regulation of sleep and circadian rhythms and perhaps memory consolidation. MT1 and MT2 receptors in blood vessels may mediate the peripheral aspects of the circadian rhythms in body temperature and blood pressure.

There is an age-related decline in the robustness of the circadian clock and melatonin production, thus depriving the brain of an important sleep regulator [74-82]. Lower production of melatonin was found in patients aged ≥ 55 years who suffer from poor sleep quality compared with healthy elderly without such complaint [83,84]. Considering the pre-eminence of the circadian clock and melatonin in timing sleep, it is likely that insomnia is linked to abnormalities in melatonin levels. Thus, the decline in melatonin associated with age (or disease) may act in conjunction with other factors (physical and psychological) to impair sleep at advanced age. Melatonin replacement therapy may replenish the deficiency in the endogenous sleep-regulating hormone, thereby improving sleep quality. As part of its therapeutic effect, it can also reinforce the functioning of the circadian clock so that the sleep propensity rhythm is in tune with the societal activity cycle [61] and with the circadian rhythms in metabolism and blood pressure [67,72].

2.2 Prolonged release formulation of melatonin (Circadin) for the treatment of insomnia

2.2.1 Pharmacokinetics and metabolism

Circadin (**Box 1**) is specifically developed to treat insomnia in patients aged ≥ 55 years to satisfy the unmet need for an appropriate treatment for this age group. The rationale for development of Circadin for insomnia in patients aged ≥ 55 years was based on the well-documented decline in an individual's capacity to produce the endogenous hormone, the decline in circadian clock output and the increase in complaints of poor sleep quality at older age. Melatonin undergoes first-pass hepatic metabolism and > 80% is excreted exclusively in the urine as 6-sulfatoxymelatonin [53]. Owing to the rapid absorption and short half-life of melatonin (40 – 50 min) [54,85-88], peak plasma concentrations are reached between 20 and 240 min after ingestion of an immediate-release formulation and decline within 1.5 h, depending on dose [89].

Maintaining effective bodily concentrations of melatonin throughout the night requires either high doses (risk of phase shifts and receptor desensitization, unnecessary burden to liver function) or a prolonged-release formulation. Circadin is a prolonged-release formulation of melatonin, which circumvents the fast clearance of the hormone by releasing the hormone in the gut over an extended period of time, thereby mimicking physiological patterns of melatonin secretion [90]. Thus, peak plasma concentrations occur 2.6 h after ingestion of this formulation and persist over an additional 3.5 h before declining, thus covering the nocturnal period.

Circadin received a new ATC code, N05CH01, the first of a new class of drugs – 'melatonin receptor agonists' – within the subgroup N05C that designates hypnotics and sedatives. This new class of drugs targets MT1, MT2 and MT3 melatonin receptors in the brain, which are distinct from $GABA_A$ receptors that are targeted by traditional hypnotics. Circadin is indicated as monotherapy for the short-term treatment of primary insomnia characterized by poor quality of sleep in patients who are aged ≥ 55 years [91].

2.2.2 Clinical efficacy

Circadin was first licensed in 2007 by the European Medicines Agency (EMA) and later on by other agencies based on a double outcome responder rate (statistically significant and clinically meaningful improvements in quality of sleep and morning alertness) and on a favorable safety profile [90]. Three randomized, double-blind trials with similar designs compared the efficacy of Circadin and placebo for primary insomnia in patients aged ≥ 55 years [92-94] in compliance with Good Clinical Practice (GCP) standards. These trials shared the same basic design including a 1 – 2-week run-in period of single-blind placebo followed by a 3-week period of randomized, double-blind treatment. Patients were

instructed to take either active drug (Circadin® 2 mg, Neurim Pharmaceuticals, Israel-UK) or placebo tablet daily, 2 h before bedtime. Efficacy parameters were measured at the beginning (baseline) and at the end of the 3-week double-blind period.

2.2.2.1 Objective findings

An objective double-blind, placebo-controlled study was conducted in 24 male and 16 female patients aged ≥ 55 years with insomnia [92]. Sleep was assessed objectively using polysomnography and all-night sleep electroencephalography (EEG) spectral analysis. Subjective assessment of sleep was made by the Leeds Sleep Evaluation Questionnaire (LSEQ) [95]. Vigilance and arousal were assessed by the Leeds psychomotor test battery (critical flicker fusion threshold, recognition, motor and total reaction time) [96]. Withdrawal effects were also assessed at 1 day and 3 weeks after treatment.

With Circadin, polysomnography-assessed sleep onset latency decreased by 50% from pretreatment values, 9 min more than with placebo ($p = 0.011$). The duration of wake before sleep onset was reduced by ~ 50% ($p = 0.011$) without negatively affecting sleep structure and architecture. No differences were observed between the groups in duration of awakenings after sleep onset, sleep efficiency and sleep duration. By the end of the double-blind treatment, the Circadin group scored significantly better in the critical flicker fusion test ($p = 0.008$) during daytime. Half of the patients reported substantial improvement in sleep quality at home with Circadin compared with 15% with placebo ($p = 0.018$). No withdrawal or rebound effects were observed for any of the recorded variables 1 day or 3 weeks after stopping the dosing in both groups [92].

2.2.2.2 Subjective findings

During the clinical assessment of Circadin, subjective assessment of sleep quality was based on three measures: the LSEQ, the Pittsburgh Sleep Quality Index (PSQI) and patients' self-reported diaries.

The LSEQ consists of 10 visual analogue scales measuring four domains of sleep: getting to sleep (GTS); quality of sleep (QOS); hangover on awakening from sleep (AFS); and behavioral integrity the following morning (BFW). It has been validated in a number of studies involving the target population of patients aged ≥ 55 years with insomnia [97]. Clinical response was defined as an improvement of 10 mm or more on the visual analogue scales, which is considered to be of clinical importance and relevance [98]. The PSQI comprises nine questions relating to the patients' usual sleep habits during the previous 4 [21] or 2 [99] weeks. An algorithm is used to calculate seven component scores, and these are added to give a global PSQI score.

Lemoine *et al.* [93] included 189 adult male and female outpatients in France and Israel. Circadin was associated with a statistically significant improvement in both the quality of sleep, measured by the LSEQ, and quality of night (QON), measured by a five-point categorical rating scale, compared with placebo (QOS: $p = 0.047$; QON: $p = 0.003$). A statistically significant improvement in morning alertness measured by the BFW domain of the LSEQ was also observed in the Circadin group compared with the placebo group (BFW: $p = 0.002$). The improvement in QOS with Circadin was significantly correlated with the improvement in BFW ($p < 0.01$), indicating a beneficial treatment effect on the restorative value of sleep [93].

Wade *et al.* [100] included 354 primary care patients in the UK. In this study the primary end point was predefined as a responder analysis. A 'responder' was defined as a patient who improved by at least 10 mm on both the QOS and BFW scales of the LSEQ. This double outcome responder rate analysis is contained in **Figure 1** and demonstrates a significant advantage of Circadin over placebo (26 vs 15%; $p = 0.014$). Statistical superiority of Circadin was also demonstrated in each of the variables alone, including QOS as assessed by both the LSEQ ($p = 0.014$) and PSQI ($p = 0.036$), sleep latency as assessed by both the GTS score of the LSEQ ($p = 0.013$) and PSQI (-24.3 min with Circadin vs -12.9 min with placebo; $p = 0.028$) and performance the following morning as assessed by BFW score of the LSEQ ($p = 0.038$). Quality of life, measured by the WHO-5 questionnaire [101] also improved significantly ($p = 0.034$).

2.2.2.3 Clinical relevance of treatment effects

It is interesting to compare the results of the two studies [93,100] in terms of the double outcome responder rate which provides evidence to the clinical relevance of the response. Analysis of Lemoine *et al.* [93] according to this criteria revealed that 47% of patients receiving Circadin had a concomitant improvement in QOS and morning alertness compared with 27% of patients receiving placebo. The difference between the treatment groups was significant ($p = 0.009$) as in the Wade *et al.* study [100] (26 vs 15%; $p = 0.014$, odds ratio = 1.97; respectively). Despite differences in absolute responder rate in the active and placebo responses in the two trials, it is interesting to note that the odds ratio between the Circadin and placebo arms was consistently about 2, indicating that the probability of response was twice as high with Circadin than placebo. This is also seen in the pooled analysis set in which there are 32.4% double outcome responders with Circadin compared with 18.7% with placebo ($p = 0.0003$, odds ratio = 2.08; **Figure 2**). These results confirmed that Circadin 2 mg can produce clinically relevant improvements in sleep quality and morning alertness in older patients suffering from poor-quality (nonrestorative) sleep [100].

A large, long-term (6 months), double-blind, placebo-controlled study was then done with Circadin [102,103], based upon which the treatment was extended from 3 to 13 weeks [91]. Adult outpatients (791, age 18 – 80 years) with primary insomnia were treated with placebo (2 weeks) and then randomized, double-blind, to 3 weeks with Circadin or placebo nightly. Circadin patients continued whereas placebo completers were rerandomized 1:1 to Circadin or placebo for 26 weeks with

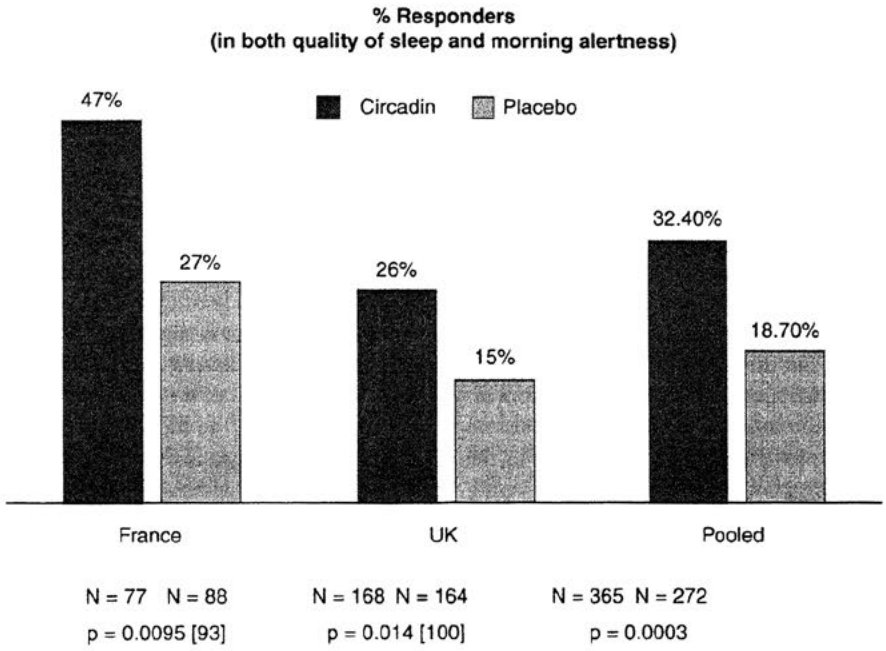

Figure 1. Responder rates in Circadin and placebo-treated groups (3 weeks) in pivotal trials. A 'responder' is a patient who improved > 10 mm on the mean QOS (quality of sleep) and BFW (behavioral integrity the following morning) domains of the Leeds Sleep Evaluation Questionnaire. References to related publications are denoted in square brackets.

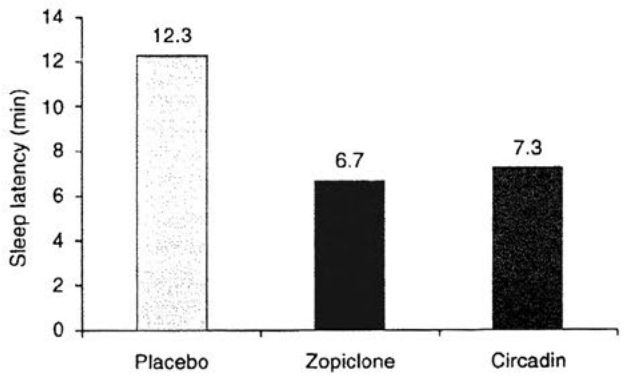

Figure 2. Sleep-promoting effects of Circadin compared with zopiclone and placebo. Objective measurement of sleep latency P=<0.01 for both vs. placebo.
Adapted from [110].

2 weeks of single-blind placebo run-out. Main outcome measures were sleep latency derived from sleep diary, PSQI, quality of life (WHO-5) and Clinical Global Impression of Improvement (CGI-I) recorded at each visit. A total of 746 patients (of which 578 were aged 55 – 80 years) completed the 3 weeks and 555 (421 Circadin 134 placebo) completed the 6-month period. Focusing on the 55 – 80-year-old age group for which the drug is indicated at present, at 3 weeks significant differences in sleep latency as assessed by both the diary (primary variable) and PSQI in favor of Circadin versus placebo treatment (-15.4 vs -5.5 min, p = 0.014 by the diary and -20.9 vs -9.7 min, p = 0.006 by the PSQI) were evident. Sleep quality, total sleep time and quality of life also improved significantly [103]. A behavioral change towards earlier bedtime was also noted with Circadin which was not seen with placebo (p = 0.014). Improvements were maintained or enhanced over the 6-month period with no signs of tolerance. Thus, sleep latency decreased by 8.7 min over placebo at 3 weeks and by 14.1 min over placebo at 3 months (p < 0.001 at 3 months). After 3 months, all of these outcomes (total sleep time, sleep quality, quality of life, behavioral advance of bedtime) were maintained or enhanced, and in addition there were significant improvements in daytime functioning (PSQI and diary) and CGI-I with no signs of tolerance [102,103]. The gradual advance in time going to bed observed in the study with Circadin is not seen with other hypnotics and may represent the activity of Circadin at the circadian clock. These data supported the approval of continuous use of up to 13 weeks [91].

2.2.3 *Clinical safety*

Safety evaluation was based on the four abovementioned double-blind, placebo-controlled clinical trials [92,93,100,102,103], an additional single-blind safety trial [84] and a long-term open-label extension phase of another study [104]. There were no significant differences with Circadin compared with placebo in type and frequency of adverse events. On the contrary, when normalized to duration of exposure in the clinical studies,

Circadin arms reported fewer adverse events than placebo arms, which might reflect a positive impact of better sleep quality on insomnia comorbidities. The most observed adverse effects include headache, pharyngitis, back pain and asthenia. Long-term safety was also established with no deleterious effects on metabolic, hormonal or other safety parameters [103,104]. In the clinical trials no rebound insomnia and withdrawal symptoms were found following discontinuation of Circadin after 3 weeks [92,93] or after 26 weeks (in the double-blind trials) [102,103] or after 26 and 52 weeks (in an open-label trial) [104]. There were no signs of tolerance development (dependency) in the clinical trials after 6 months of double-blind or 6 and 12 months of open-label treatment periods with Circadin [92,102,103]. Circadin can be used in combination with most other drugs that are common in the elderly population including antidiabetic and lipid-lowering drugs [105], and as add-on to antihypertensive therapy [71].

2.2.4 *Comparative efficacy and safety*

The Circadin pivotal trials were all performed versus placebo. The evaluation of comparative efficacy can thus only be done of the basis of historic values from the literature, or head-to-head comparisons in healthy volunteers mostly from a safety perspective. In a published study [106], the effects of acute and repeated doses of zolpidem (5 and 10 mg) given at night for 1, 2 and 7 days on LSEQ-recorded sleep and daytime domains was assessed in a double-blind, placebo-controlled study in 24 healthy elderly volunteers. Statistically significant improvements were found with zolpidem compared with placebo in the QOS and GTS domains of the LSEQ which are also found with Circadin compared with placebo. However, there was no statistically significant difference between zolpidem and placebo in the BFW domain, whereas this domain is consistently improved with Circadin [93,100].

The mean difference between the effects of Circadin and placebo on objectively assessed (polysomnography) sleep onset latency (-9 min) is similar to that obtained with licensed doses of zolpidem (-8.1 min [107,108]), zaleplon (< 8.0 min [42,108]) and the melatonin agonist ramelteon (-10 min [109]). The similarity in sleep-promoting effects can also be evidenced from a published placebo-controlled laboratory study reporting a head-to-head comparison of Circadin 2 mg to zopiclone 5 mg in sleep induction in healthy volunteers [110]. This study indicated similar facilitations of sleep induction as measured objectively (actigraphy) with both drugs compared with placebo (**Figure 2**). Hence, the ability of Circadin 2 mg to facilitate sleep onset, the primary and only parameter on which current hypnotics were licensed, is clinically relevant.

Safety concerns with hypnotics include dependence [111], rebound or withdrawal effects [112-114], alteration in sleep architecture and reductions in deep and REM sleep [115-117], amnesia and cognitive impairment [112-114,118], morning hangover and sedation [35,119,120], increase in risk of falls and fractures [37,39], depression of respiratory function [36,121] and nocturnal hypertension [122]. These are not seen with Circadin. In a head-to-head study of psychomotor functions, driving skills and memory recall in a laboratory setting, zolpidem 10 mg impaired cognitive functioning and driving capacity compared with placebo, whereas Circadin did not have any negative effects on these parameters [118]. Also, memory consolidation as measured by the number of memories recalled the next day was significantly impaired with zolpidem but not with Circadin [118]. Residual effects on cognitive functioning are not observed with Circadin; rather some enhancement of daytime vigilance is suggested [92]. Sleep architecture is not modified with Circadin [92,123]. In particular there is no suppression in slow-wave sleep or REM sleep which is typically seen with the BZD/Z drugs.

Circadin demonstrated comparable efficacy and safety in patients with and without history of classical hypnotic drug use [124]. It was also significantly more effective than placebo in tapering off hypnotic drug use in long-term BZD users [125,126]. Real-life data from a retrospective cohort study in Germany showed that among the 22% previous BZD/Z users in the study, approximately one-third (31%) did not receive any prescriptions of BZD/Z drugs in the 3-month period following Circadin initiation, that is they are presumed to have discontinued BZD/Z drug use [127]. Circadin may thus help to facilitate BZD/Z discontinuation and therefore limit their use in older insomniacs.

Given the favorable benefit–risk ratio of Circadin, the EMA has not restricted its access to severe insomnia, unlike other hypnotics. The usage duration was extended by the EMA in April 2010 from 3 to 13 weeks [91], thus more suitable for use in chronic insomnia, while other hypnotics are restricted to 2 – 4 weeks on account of concerns of safety and potential dependence issues [40-42].

3. Expert opinion

Circadin is a new treatment option for insomnia which seems to address a hitherto unmet need for an effective and safer drug for older patients with insomnia. It has a positive benefit–risk ratio in older patients, including the elderly (65 – 80 years), it improves next-day alertness, an effect that has not been found with classical hypnotics or Z drugs, its use can be extended to 3 months, which better fits the persistent nature of the disorder, and it does not cause insomnia rebound or withdrawal symptoms if discontinued.

Among insomnia sufferers, people aged ≥ 55 years, including the elderly, comprise the major subgroup of patients [128]. Traditional hypnotic drugs have an unfavorable benefit–risk balance for this age group [39]. Circadin was specifically developed for this population. It acts at the roots of primary insomnia pathology by mimicking natural secretion patterns of melatonin that have deteriorated with age; consequently it is able to reinforce the physiological sleep-wake cycle. Patients are thus advised to take the drug 1 – 2 h before habitual bedtime and preferably between 2100 and 2300 h for two

reasons: i) to allow a proper phase position of the replenished melatonin rhythm (and consequently sleep onset [57]) with the external night-day cycles; and ii) to allow full night coverage of the active drug. Circadin has shown efficacy in multiple sleep and daytime parameters in patients aged 55 – 80 years (average age 65.8 years), including improvements in sleep quality, morning alertness and quality of life, as well as sleep latency, and this efficacy profile was specific to this age band [103]. Most safety concerns with use of hypnotics do not occur with Circadin. Therefore, the British Association for Psychopharmacology consensus statement on evidence-based treatment of insomnia, parasomnias and circadian rhythm disorders recommends Circadin as a first-line therapy in insomnia patients aged ≥ 55 years [51]. Given as first-line prescription, and 13 weeks' posology, without rebound effects, Circadin has the potential to avoid long-term, hazardous use of hypnotics.

A limitation in the use of this drug is in the relatively slow onset of action that makes it unsuitable for treatment of transient insomnia. The increase in benefits with time of treatment may represent the activity of melatonin at the circadian clock, or reinstatement of responsiveness through a hitherto undiscovered mechanism. Another limitation is the age restriction on the target population. Also, the absence of 'hammer effect' with this drug may be perceived as weak efficacy by patients seeking such effect.

Declaration of interest

N Zisapel is the founder of Neurin Pharmaceuticals. The authors have, however, limited this paper to the discussion of published literature from peer-reviewed journals and the official European Medicines Agency website. P Lemoine is the primary investigator in two Circadin clinical trials.

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Repeated Melatonin Supplementation Improves Sleep in Hypertensive Patients Treated with Beta-Blockers: A Randomized Controlled Trial

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Study Objectives: In the United States alone, approximately 22 million people take beta-blockers chronically. These medications suppress endogenous nighttime melatonin secretion, which may explain a reported side effect of insomnia. Therefore, we tested whether nightly melatonin supplementation improves sleep in hypertensive patients treated with beta-blockers.

Design: Randomized, double-blind, placebo-controlled, parallel-group design.

Setting: Clinical and Translational Research Center at Brigham and Women's Hospital, Boston.

Patients: Sixteen hypertensive patients (age 45-64 yr; 9 women) treated with the beta-blockers atenolol or metoprolol.

Interventions: Two 4-day in-laboratory admissions including polysomnographically recorded sleep. After the baseline assessment during the first admission, patients were randomized to 2.5 mg melatonin or placebo (nightly for 3 weeks), after which sleep was assessed again during the second 4-day admission. Baseline-adjusted values are reported. One patient was removed from analysis because of an unstable dose of prescription medication.

Measurements and Results: In comparison with placebo, 3 weeks of melatonin supplementation significantly increased total sleep time (+36 min; P = 0.046), increased sleep efficiency (+7.6%; P = 0.046), and decreased sleep onset latency to Stage 2 (-14 min; P = 0.001) as assessed by polysomnography. Compared with placebo, melatonin significantly increased Stage 2 sleep (+41 min; P = 0.037) but did not significantly change the durations of other sleep stages. The sleep onset latency remained significantly shortened on the night after discontinuation of melatonin administration (-25 min; P = 0.001), suggesting a carryover effect.

Conclusion: In hypertensive patients treated with beta-blockers, 3 weeks of nightly melatonin supplementation significantly improved sleep quality, without apparent tolerance and without rebound sleep disturbance during withdrawal of melatonin supplementation (in fact, a positive carryover effect was demonstrated). These findings may assist in developing countermeasures against sleep disturbances associated with beta-blocker therapy.

Clinical Trial Information: This study is registered with ClinicalTrials.gov, identifier: NCT00238108; trial name: Melatonin Supplements for Improving Sleep in Individuals with Hypertension; URL: http://www.clinicaltrials.gov/ct2/show/NCT00238108.

Keywords: Actigraphy, adrenergic beta-antagonists, atenolol, autonomic nervous system, hypertension, hypnotics, melatonin, metoprolol, polysomnography, sleep

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INTRODUCTION

Approximately 22 million Americans use beta-adrenergic receptor antagonists, or beta-blockers.[1] Despite their demonstrated benefit in various cardiovascular and non-cardiovascular diseases, beta-blockers have been associated with an incidence of adverse side effects including nighttime sleep disturbances and daytime fatigue.[2-6] These side effects have mainly been ascribed to central nervous system and cardiac effects. However, beta-1-blockers not only reduce sympathetic outflow to the cardiovascular system but also block sympathetic signaling to the pineal gland, resulting in suppression of nighttime levels of the soporific hormone melatonin,[7-10] which may help explain the insomnia associated with beta-blocker use.[2,11,12] Thus, melatonin supplementation may potentially counteract the sleep disturbances associated with beta-blocker use.[10,11] However, there has been no clinical trial to determine the efficacy of melatonin supplementation for improving sleep in patients treated chronically with a beta-blocker. Moreover, considerable data support adverse cardiometabolic effects of short sleep duration,[13-16] leading to speculation that such outcomes could be improved in patients using beta-blockers by maintaining sleep duration. Thus, in the current study we tested the hypothesis that repeated melatonin supplementation (3 weeks) improves sleep in hypertensive patients chronically treated with beta-blockers. Changes in sleep were assessed by polysomnography (PSG) under standardized laboratory conditions.

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METHODS

Patients

Sixteen patients completed the study (9 women; mean ± standard deviation [range]; age: 56.1 ± 6.1 yr [45–64 yr]; body mass index: 28.04 ± 3.86 kg/m^2 [21.5–34.7 kg/m^2]). These patients received a diagnosis of uncomplicated essential hypertension (blood pressure: 130/79 ± 15/9 mm Hg while on anti-hypertensive treatment) and were treated for at least 6 mo with beta-1-selective blockers (atenolol, 50 mg/day [n = 8], atenolol, 25 mg/

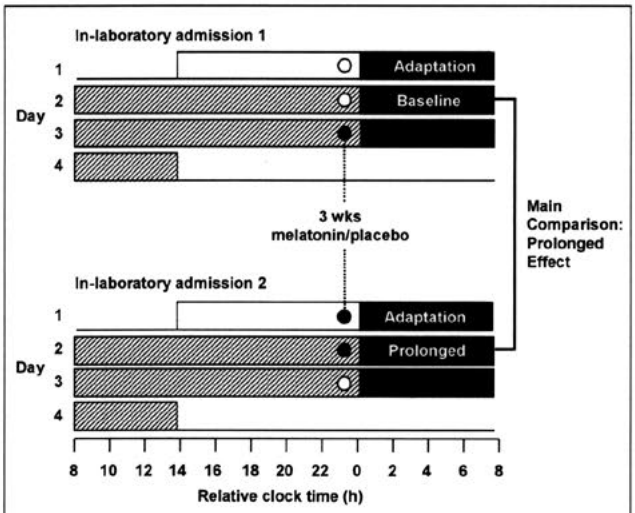

Figure 1—Example of in-laboratory protocol for patient with habitual bedtime of midnight. Polysomnography (PSG) collected on the baseline night (second sleep episode of first admission) was compared with PSG collected on the night after 3-week melatonin/placebo administration (second sleep episode of second admission) as the main comparison to assess the effect of 3-week melatonin supplementation. Patients were kept in the laboratory for an extra night after first administration and the first night after discontinuation as a safety precaution and to address exploratory questions (see Supplemental Material). Filled circles, double-blind study drug (melatonin/placebo); open circles, single-blind placebo; black bars, sleep episodes in complete darkness; hatched bars, wakefulness in dim light for assessment of endogenous melatonin profile (approximately 4 lux); white bars, wakefulness in room light (approximately 90 lux).

day [n = 5], atenolol, 100 mg/day [n = 1], metoprolol, 50 mg/day [n = 1], and metoprolol, 100 mg/day [n = 1]). In addition to a beta-blocker, 4 patients took an angiotensin-converting enzyme inhibitor and/or thiazide diuretic before and throughout the study. Patients were not on any medication other than antihypertensive medication and were otherwise healthy as confirmed by extensive history, physical, psychologic, and laboratory examination. Sleeping problems were not part of the inclusion criteria. Patients underwent an overnight polysomnographic diagnostic test according to recommended criteria[17] to exclude severe sleep apnea (apnea hypopnea index > 30/hr) and periodic limb movement during sleep (> 15/hr), as well as for patients to become accustomed to wearing the polysomnographic equipment before the start of the clinical trial. The average apnea-hypopnea index was 11/hr (range: 1-27/hr). One of the 16 patients was excluded from analysis because of a change in the dosing of prescription medication while enrolled in the study. Patients reported no shift work experience for at least 3 yr and not crossing more than two time zones in the 3 mo prior to the study. Toxicology screens at the time of both admissions confirmed that patients were free of any drugs (including caffeine, alcohol, and nicotine) apart from prescribed antihypertensive medications. Patients provided written informed consent, and the study was approved by the local Human Research Committee.

Study Design

The study had a randomized, double-blind, placebo-controlled, parallel design (Figure 1). Sequential randomization based on blocks of two was performed using dedicated software (http://www.randomization.com) by Brigham and Women's Hospital pharmacy department to ensure that the randomization list was not known to the patients, investigators, and staff involved in the study and sleep scoring. Melatonin 2.5 mg capsule or matching placebo was taken orally 1 hr before bedtime each night for approximately 3 weeks (once nightly for a mean of 23 days; range, 20-28 days, the exact timing depending on the availability of laboratory suites, staff, and patients). The melatonin/placebo capsules were prepared by the Brigham and Women's Hospital pharmacy department in compliance with Food and Drug Administration (FDA) Good Compounding Practice regulations and USP 795. Melatonin (2.5 mg; Regis Chemical Co. or Nature's Bounty, Inc.) was added to microcrystalline cellulose, National Formulary, and encapsulated in gelatin capsules. The placebo for melatonin was prepared by filling matching gelatin capsules with microcrystalline cellulose. The Certificates of Analysis confirmed the melatonin and microcrystalline met the United States Pharmacopeial Convention standards for identity, purity, dose, and stability. This protocol, including the sources of melatonin, was approved by the FDA. A dose of 2.5 mg of melatonin was selected to achieve melatonin values at or above physiologic concentrations for approximately 8 hr, for the full duration of the sleep episode.[18]

Study Protocol

The study protocol (Figure 1) consisted of the following five sequential parts: (1) 2-week baseline ambulatory monitoring (not depicted); (2) first 4-day in-laboratory monitoring; (3) 3-week ambulatory monitoring while taking the study drug; (4) second 4-day in-laboratory monitoring; and (5) 2-day poststudy drug ambulatory monitoring (not depicted). The drug or placebo was started during the in-laboratory segment (2) and stopped during the in-laboratory segment (4), as detailed below. To ensure stable rhythmicity of the circadian system immediately prior to both laboratory admissions, the patients maintained a self-selected and fixed sleep/wake cycle with 8 hr time in bed per night as verified by sleep/wake diaries, call-in times to a time-stamped voice recorder, and wrist actigraphy (Actiwatch; Minimitter, Bend, OR) during all ambutatory parts (for a minimum of 2 weeks prior to baseine and the full duration between both admissions).

During the 4-day in-laboratory admissions, patients stayed continuously in a private suite in the Clinical and Translational Research Center at Brigham and Women's Hospital. The schedule in the laboratory consisted of 16 hr of scheduled wakefulness per day and 8 hr of scheduled bed rest per night at the same times as patients had maintained during the ambulatory parts in the week prior to both admissions. The light level was 90 lux for the wake episode of the first day of each in-laboratory admission (adaptation day) and 4 lux for the remaining three wake episodes to allow assessment of dim light melatonin concentrations (see Supplement). Light levels were 0 lux for all sleep episodes. Throughout each of the sleep episodes, the patients maintained a supine posture and received a urinal or bedpan as needed. During the wake episodes patients refrained from exercise, and spent most of their time seated, reading, watching movies, or engaged in the study procedures. Patients were not permitted to lie on the bed during the wake

episodes. Meals were provided 1 hr 50 min (breakfast), 5 hr 25 min (lunch), 11 hr 25 min (dinner) and 13 hr 25 min (snack) after scheduled awakenings. A daily shower was scheduled at 2 hr 55 min after scheduled awakenings.

On the first in-laboratory admission, patients received a single-blind placebo capsule before sleep episodes 1 and 2, and a double-blind capsule (2.5 mg melatonin or placebo) before sleep episode 3 (Figure 1). After the first discharge from the laboratory, patients continued taking the double-blind capsule at home for 3 weeks until the second laboratory admission whereupon patients continued to take the double-blind capsule before sleep episodes 1 and 2, and received a single-blind placebo capsule before sleep episode 3. All melatonin or placebo capsules were taken 1 hr before the scheduled sleep episodes. Similarly, all patients took their beta-blocker 1 hr before bedtime for at least 2 weeks before and throughout the study. Study drug and prescribed beta-blocker use was verified by diaries and daily call-in times to a time-stamped voice recorder at home and by staff during the in-laboratory parts.

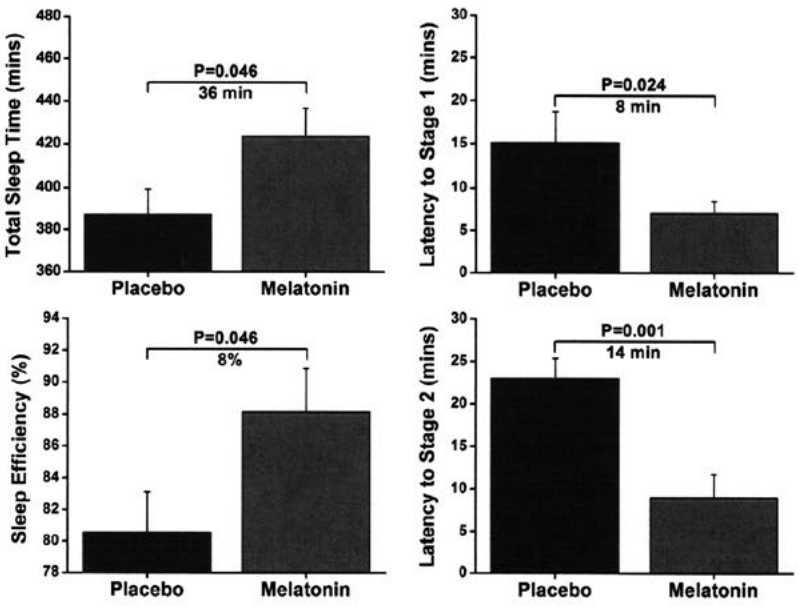

Figure 2—Melatonin improves PSG-assessed sleep quality. Three-week melatonin supplementation significantly increased total sleep time and sleep efficiency (left panels), and significantly decreased latency to Stage 1 and Stage 2 sleep (right panels). P values, significance of melatonin effect; numbers underneath P values, magnitude of melatonin effect.

Measurements

Polysomnography

Sleep was recorded on the second and third night of each laboratory admission by PSG (Vitaport-3, Temec Instruments, Kerkrade, B.V., The Netherlands), including electroencephalography (EEG), left and right electrooculography (EOG), bipolar submental electromyography (EMG), and bipolar electrocardiography (ECG). EEG was recorded from C3, C4, O1, O2 (referenced to A1 or A2), with C3-A2 and C4-A1 used for sleep scoring. Sleep stages were scored in 30-s epochs,[19] with scorer blinded to condition. Sleep efficiency was calculated as the total duration of sleep divided by the time in bed (8 hr).

Actigraphy

To estimate sleep quality at home, actigraphy data were collected throughout the ambulatory and in-laboratory portions of the study using an Actiwatch (Actiwatch-64 or the Actiwatch-L, Minimitter, Bend, OR). Patients wore this device on their nondominant wrist with integrated activity data recorded at 2-min intervals. Automatic sleep/wake scoring was performed with Actiwatch software (Actiware-Sleep 3.4; Minimitter Co. Inc.; sensitivity set at medium) between "bedtime" and "get-up time" derived from the sleep/wake diaries (at home) or between lights-off and lights-on (in-laboratory). Three sleep variables were objectively computed by this analysis: sleep onset latency, total sleep time, and sleep efficiency. Sleep onset latency was the calculated time between bedtime (from diary) and sleep onset (from actigraphy). Total sleep time was the calculated time asleep from actigraphy. Sleep efficiency was defined as the percentage of time asleep, as derived from actigraphy, while in bed, as derived from diary (at home), or while lights were off (in-laboratory).

Analysis

To determine the effect of 3-week melatonin supplementation on PSG, a generalized linear model was used with data collected at the end of 3 weeks of melatonin/placebo administration (second night of second laboratory admission) serving as outcome variables, treatment group as a fixed factor and baseline measurement (second night of first admission) as a covariate.[20] To determine the effect of repeated melatonin supplementation on sleep as estimated by actigraphy, a generalized linear mixed model was used with averaged actigraphy data collected throughout each of the 3 weeks of melatonin/placebo administration serving as repeated outcome variables, treatment group as a fixed factor, and the averaged ambulatory baseline data as a covariate.[20] All statistical procedures were performed via SPSS version 19 for Windows (IBM SPSS Statistics). Statistical significance was set at $P < 0.05$. Baseline-adjusted model estimated mean ± standard error data are presented.

RESULTS

Repeated melatonin supplementation (3 week) increased total sleep time by 37 min (placebo: 387 min vs. melatonin: 424 min; $P = 0.046$), increased sleep efficiency by 7.6% (80.5% vs. 88.1%; $P = 0.046$), and decreased latency to onset of Stage 1 sleep by 8 min (15 min vs. 7 min; $P = 0.024$) and latency to onset of Stage 2 sleep by 14 min (23 min vs. 9 min; $P = 0.001$) as assessed by PSG (Figure 2). Repeated melatonin supplementation significantly increased Stage 2 sleep by 41 min (230 vs. 272 min [note: numbers may not add up due to rounding];

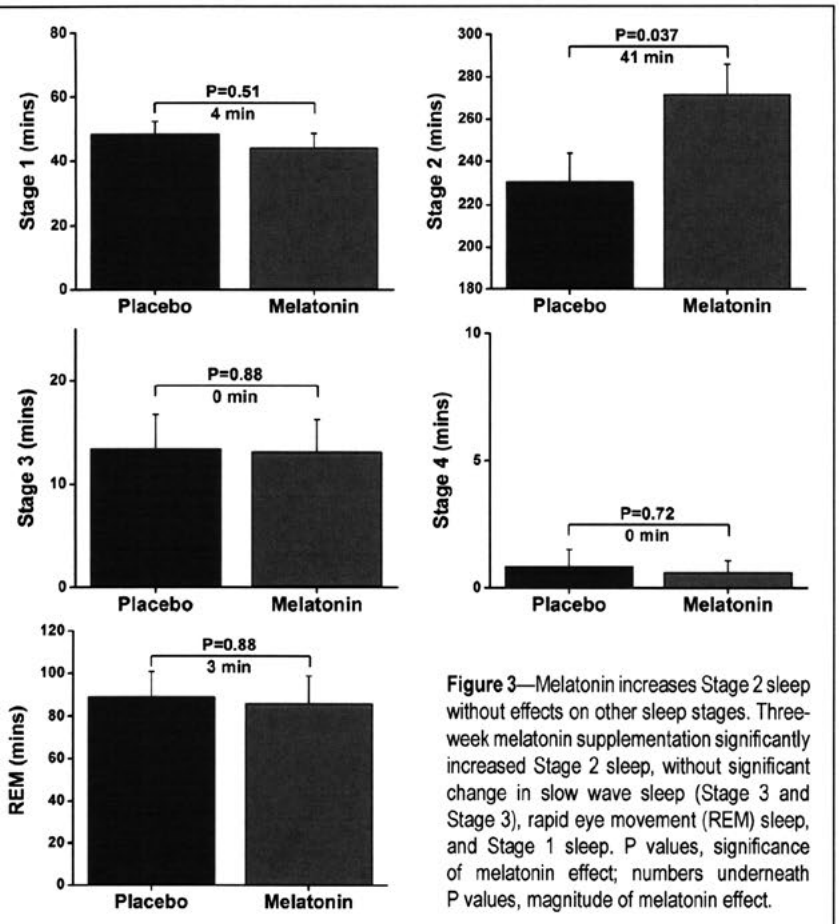

Figure 3—Melatonin increases Stage 2 sleep without effects on other sleep stages. Three-week melatonin supplementation significantly increased Stage 2 sleep, without significant change in slow wave sleep (Stage 3 and Stage 3), rapid eye movement (REM) sleep, and Stage 1 sleep. P values, significance of melatonin effect; numbers underneath P values, magnitude of melatonin effect.

P = 0.037). Melatonin did not significantly affect durations of Stage 1 sleep, slow wave sleep (Stages 3 and 4), rapid eye movement (REM) sleep (Figure 3), or the number and duration of awakenings.

Melatonin also significantly improved actigraphy-estimated total sleep time (377 min vs. 390 min; P = 0.011) and sleep efficiency (78% vs. 81%; P = 0.010), but not actigraphy-estimated sleep onset latency, at home throughout the 3 weeks in between in-laboratory stays. The improvement of total sleep time and sleep efficiency was similar between the three separate weeks (Figure 4), suggesting that melatonin was effective in improving sleep from the first week to the last.

This significant effect in the home was evident despite the fact that actigraphy underestimated the melatonin-induced changes in sleep as evident by comparing the actigraphy-estimates with PSG simultaneously in the laboratory, presumably due to misclassification of motion-free relaxed wakefulness as sleep when using actigraphy (see Figure S1).

In exploratory analysis, including the PSG assessments after single administration, 3-week administration, and after discontinuation of melatonin, there was a main effect of melatonin for the duration of Stage 2 sleep (lengthened), latency to Stage 1 sleep (shortened), and latency to Stage 2 sleep (shortened) (see Figure S2). In *post-hoc* analysis with Bonferroni correction, we found that latency to Stage 2 sleep remained significantly shortened after discontinuation of melatonin supplementation (P = 0.001), suggesting a carryover effect. In these *post-hoc* analyses we found no acute effect of melatonin on any PSG measure (i.e., no effect of a single dose of melatonin) and no sign of rebound sleep disturbance following discontinuation of melatonin (i.e., no worsening of any PSG measure compared with placebo).

Melatonin had no adverse effects on the patients' general health complaints as determined through a questionnaire inquiring about the presence or absence of headache, insomnia, hyperactivity, irritability, nausea, "sleeping limbs," dizziness, constipation, "shaky hands," stomach cramp, drowsiness, sweating, hunger, weakness, and sore eyes, at the first laboratory admission (baseline) and the second laboratory admission (repeated use).

Of the 10 patients in whom we could assess the plasma melatonin concentrations hourly for 24 hr under the dim light conditions during baseline conditions (see Supplemental Material), 6 patients had melatonin values in the typical range, between approximately 40-100 pg/ml, and 4 patients had very low melatonin values (around or below 10 pg/ml), presumably in part due to inhibition of endogenous melatonin secretion by use of beta-blocker (see Figure S3, top panel). Even though the dose distribution is limited and the sample size is relatively low given the large interindividual differences in nighttime melatonin plasma concentrations even in young healthy control patients, the relationship between the peak nighttime melatonin concentrations at baseline and beta-blocker dose is consistent with a higher beta-blocker dose being more likely to lead to lower melatonin secretion, i.e., around or below 10 pg/ml (see Figure S4).

In the patients randomized to placebo, the four 24-hr dim light plasma melatonin profiles (baseline, acute, prolonged, and carryover) were very similar (see Figure S3, middle panel). In the patients randomized to melatonin supplementation, melatonin intake resulted in either supraphysiologic or physiologic nighttime plasma melatonin concentrations for the full 8-hr sleep episodes, with the profiles very similar after acute (single dose) and 3-week administration (see Figure S3, bottom panel). After discontinuation of melatonin administration, the melatonin profile phase and amplitude returned to baseline levels (see Figure S3, bottom panel).

DISCUSSION

To our knowledge, this is the first study to show effectiveness of repeated melatonin supplementation on PSG-recorded sleep in people chronically treated with beta-blockers. Our results show that 3 weeks of melatonin supplementation improved sleep quality in hypertensive patients chronically treated with beta-blockers. Melatonin supplementation decreased sleep onset latency and increased sleep maintenance. There was no sign of the development of tolerance to melatonin supplementation,

and no sign of rebound sleep disturbance during withdrawal of melatonin supplementation.

Potential Clinical Relevance

These results of improved sleep with melatonin supplementation may have particular relevance to the millions of people around the world who chronically take beta-blockers.[1] Beta-blockers are widely prescribed for a variety of cardiovascular disorders including hypertension, congestive heart failure, cardiac arrhythmias, angina pectoris, for cardioprotection after myocardial infarction, and for some non-cardiovascular disorders such as migraine, posttraumatic stress disorder, and generalized anxiety disorder.[21] Beta-blockers are prescribed more than diuretics, angiotensin-converting enzyme inhibitors, or calcium channel blockers.[1] Moreover, in Americans 60 yr of age and older, beta-blockers are the second most frequently used prescription drugs (second to cholesterol-lowering medications).[1] In addition, the current findings may have relevance to thousands of patients with tetraplegia, because cervical spinal cord transection abolishes endogenous melatonin production, which may contribute to their reported sleeping problems.[22,23] Furthermore, there is evidence for decreased nighttime melatonin levels in patients with coronary artery disease and non-dipper hypertensive patients (in whom blood pressure does not decrease at night), either due to or independent of beta-blocker use,[24-26] in whom the effect of melatonin supplementation on sleep deserves further study. It has also been reported that melatonin supplementation can improve sleep in older individuals with insomnia and low endogenous melatonin concentrations.[27,28] Finally, self-reported short sleep duration has been linked to increased risk for diabetes, obesity, and cardiovascular disease in epidemiologic studies and experimental sleep curtailment leads to decreased glucose tolerance and leptin values, and increased blood pressure and ghrelin values.[13-16] Therefore, measures to improve sleep may not only have beneficial psychologic and cognitive effects, but also have potential for beneficial cardiovascular and metabolic health effects in some cases.

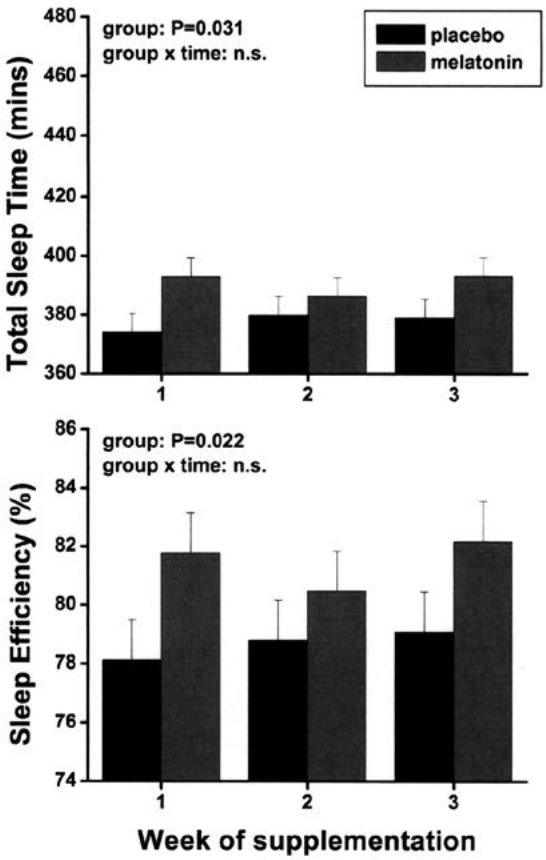

Figure 4—Time course of the effect of melatonin on actigraphy-estimated sleep. Melatonin increased total sleep time and sleep efficiency as estimated by actigraphy similarly during the 1st, 2nd, and 3rd week of supplementation at home, i.e., without significant interaction with time. P values, significance of melatonin effect (group; melatonin vs. placebo) and interaction of group with week of supplementation (group × time).

Mechanism

The suprachiasmatic nucleus (SCN) drives the circadian process of sleep regulation and contains high-affinity MT1 (Mel1b) and MT2 (Mel1b) receptors.[29-31] Nighttime melatonin administration has been proposed to be able to influence sleep by three mechanisms: (1) long-term effects on the SCN via high-affinity melatonin receptors, amplifying or synchronizing the SCN neural activity rhythm and its output, resulting in a stronger nighttime sleep drive;[32,33] (2) immediate/short-term effects on the SCN via high-affinity melatonin receptors, inhibiting SCN multiunit electrical activity to levels more typical of the biologic night and thereby increasing circadian sleep drive;[32] and (3) immediate vasodilatory effects on proximal and distal skin via high-affinity melatonin receptors, leading to increased skin temperature and heat dissipation, which may increase sleep propensity.[34] Our exploratory analysis showed that the latency to Stage 2 sleep was still significantly shortened after discontinuation of melatonin administration, i.e., when exogenous melatonin had cleared from the plasma, indicating a long-term effect of melatonin on sleep regulation. An amplification or phase advance of the output of the SCN that could outlast daily melatonin administration theoretically could explain such carryover effects of melatonin. However, the plasma melatonin profile was not amplified and not phase advanced, which does not support this hypothesis. The absence of a significant phase-shifting effect of 2.5 mg of melatonin given around the time of the dim light melatonin onset, as in our study, is consistent with the phase response curve to a similar dose of melatonin (3 mg) at that time.[31] The absence of a significant acute effect of melatonin on sleep in our exploratory analyses in this population does not support the hypothesis that the effects on sleep were mediated through acute vasodilatory and thermoregulatory effects.

Comparison With Hypnotic Agents

Sleep disturbances in patients treated with beta-blockers presumably could be negated by use of prescribed hypnotic drugs, such as benzodiazepines. However, there are several side effects and limitations associated with many of these sleep aids[35,36] that are not apparent with the use of melatonin supplementation. First, some hypnotic agents, including some benzodiazepines, have decreased effectiveness after chronic use (tolerance) and rebound insomnia after discontinuation, increasing the risk for dependence and abuse.[37,38] Thus, although

beta-blocker therapy for cardiovascular disorders is typically used for life, many sleep aids are not suitable for long-term use. In the current study, there was no sign of adverse side effects, tolerance (the effect after 3-week administration was not reduced as compared with single administration), or rebound insomnia with melatonin supplementation. Second, although benzodiazepines increase total sleep time, they often decrease slow wave and/or REM sleep,[39] whereas melatonin increased total sleep time without decreasing slow wave and REM sleep in the current study. Third, there is a dose-dependent effect of hypnotic agents, ranging from anxiolytic to soporific, anesthesia, coma, and death, and an associated risk of drug overdose,[40] benzodiazepine receptor agonists have been associated with memory and balance impairment,[41] and a recent correlational report found a threefold increased mortality associated with prescribed hyponotic (mostly benzodiazepine) use,[42] whereas melatonin appears relatively safe for the short-term use (weeks) for which there are good data, even at relatively high doses.[43] Fourth, traditional hypnotic agents generally do not tackle the cause of sleeping problems if these are due to suppressed melatonin. Thus, based on these apparent potential benefits of melatonin over traditional hypnotic agents, larger-scale clinical trials are likely warranted to determine whether melatonin or melatonin agonists can be used as an alternative or add-on to hypnotic agents in treating sleeping problems in people treated with beta-blockers

Previous Studies Linking Beta-blockers, Melatonin, and Sleep

All beta-1-selective blockers investigated, including hydrophilic atenolol and moderately lipophilic metoprolol (both used in this study) and bisoprolol, suppress nighttime melatonin.[2,7-11,44] Because the pineal gland lies outside of the blood-brain barrier, both lipophilic and hydrophilic beta-blockers suppress melatonin production. Also, the nonselective beta-blocker propranolol (beta-1- plus beta-2-blocker) suppresses melatonin.[2,10] The lack of a suppression of melatonin by the nonselective beta-blockers carvedilol (includes alpha-1-blocking activity) and nebivolol (has nitric oxide-mediated vasodilatory effects) has been hypothesized to be due to a compensatory increase in sympathetic activity following vasodilatation, and requires further investigation.[45]

Although it is clear that beta-1-selective blockers suppress melatonin levels and that melatonin administration during the daytime (when endogenous melatonin levels are low) improves sleep,[46] there are few studies in the literature regarding the effects of beta-blockers on sleep at night and the role of melatonin suppression.

In studies based on self-reported measures of sleep in hypertensive patients, it was found that both 6 and 10 weeks of treatment with propranolol or ridazolol suppressed urinary 6-sulphatoxymelatonin levels by approximately 40-50% but had no effect on self-reported sleep complaints;[47] that with 4 weeks of metoprolol there was a correlation between the magnitude of suppression of nighttime urinary melatonin excretion and the percentage of disturbed nights[2]; and in a randomized study of 149 patients over 6-12 mo revealed that replacement of beta-blockers (mostly atenolol) with angiotensin-converting enzyme inhibitors resulted in a decrease in sleep complaints.[48] In one of the few studies investigating the effect of beta-blockers on PSG-assessed sleep in hypertensive patients, Danchin et al. reported a (nonsignificant) tendency for total sleep time to decrease by 34 min after a single dose of atenolol (100 mg) in a double-blind randomized crossover trial in 8 patients.[5]

To our knowledge, only one study has investigated the use of melatonin supplementation after beta-blocker use on PSG-assessed sleep. In that (acute) study, 100 mg atenolol in healthy volunteers led to decreases in urinary 6-sulphatoxymelatonin and total sleep time, whereas 5 mg of melatonin supplementation while on atenolol restored sleep to baseline levels without atenolol.[11] The current study extends these observations to include a relevant patient population and demonstrates that repeated melatonin supplementation improves PSG-assessed sleep in hypertensive patients chronically treated with beta-blockers.

Interestingly, the anxiolytic property of beta-blockers has led people to test the effect of beta-blockers in the treatment of insomnia, with surprisingly disappointing results, including worsened sleep disturbances.[49] This raises the question whether any anxiolytic effect of beta-blockers that might improve sleep is counteracted by the adverse melatonin-suppressing effects of beta-blockers. The potential clinical benefit of a combination of beta-blockers plus nighttime melatonin in the treatment of sleep disturbances, and in particular anxiety-related insomnia, warrants further investigation.

Limitations and Future Directions

The main limitations of the current study include the small sample size, and the fact that we could not determine the effect of the chronic beta-blocker use on melatonin production and sleep quality because we had no recordings before the chronic beta-blocker therapy. Furthermore, we cannot determine the extent to which the observed beneficial effect on sleep quality of melatonin supplementation is specific to beta-blocker-induced melatonin suppression. Indeed, our previous work indicates that even in hypertensive patients without any beta-blocker therapy, repeated nighttime melatonin supplementation improves actigraphy-estimated sleep quality.[50] Larger studies are needed to determine which patient populations treated with beta-blockers may benefit most from melatonin supplementation. Such studies should assess both hard cardiovascular endpoints as well as patient-reported outcomes to determine the full effect of melatonin supplementation to beta-blocker treatment.

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Contributors

Conceived and designed the experiments: Drs. Scheer and Shea. Performed the experiments: Drs. Scheer, Morris, Garcia, Smales, Kelly, Marks, and Malhotra. Analyzed the data: Drs. Scheer and Morris. Wrote the paper: Drs. Scheer, Morris, Malhotra, and Shea.

DISCLOSURE STATEMENT

This was not an industry supported study. Dr. Shea serves on the Board of Directors of the American Academy of Sleep Medicine. The other authors have indicated no financial conflicts of interest.

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Actigraphy Underestimated the Effect of Melatonin on Sleep: A Comparison With Polysomnographically Assessed Sleep

The magnitude of effect of melatonin on sleep at home as estimated by actigraphy was smaller than the magnitude of effect of melatonin on sleep in the laboratory as determined by polysomnography (PSG). This difference could be due to 3 factors: (1) difference in measurement technique (PSG vs. actigraphy); (2) difference in measurement location (laboratory [PSG] vs. home [actigraphy]); and/or (3) difference in duration of supplementation (throughout the 3 wk of taking the study drug [actigraphy] or after 3 wk of taking the study drug [PSG]). To test whether it was caused by a difference in measurement technique (1), while keeping the other factors (2) and (3) constant, we assessed actigraphy-estimated sleep on the same nights in the laboratory as when PSG was recorded.

These analyses indicate that the smaller effect of melatonin on sleep as estimated by actigraphy as compared to that measured by PSG was primarily due to the measurement technique (3.3% difference in sleep efficiency; comparing middle gray bar with right red bar; Figure S1) and less by location (factor 2) or duration (factor 3) of supplementation (combined 1.4% difference; comparing left gray bar with middle gray bar; Figure S1). Thus, it is likely that the actigraphy-estimated effect of melatonin on sleep also underestimated the true effect of melatonin while patients were sleeping at home. Actigraphy is considered less reliable for detecting disturbed sleep and especially less useful for assessing sleep latency which may explain this underestimation.[1]

No Development of Tolerance After Repeated Melatonin Use and No Rebound Insomnia After Discontinuation of Melatonin

For the exploratory analysis, to determine the acute effect of melatonin and the carry-over effect of melatonin, a generalized linear mixed model was used with data collected after single administration (acute; the third sleep episode during the first admission starting 1 hr after the first administration of the study drug), after repeated administration, and after discontinuation (carryover; the third sleep episode on the second admission starting 25 hr after discontinuation of the 3-wk administration of the study drug) of melatonin/placebo serving as the outcome variables, treatment group, and measurement time as fixed factors, and baseline measurement as a covariate.[2]

These analyses showed a significant main effect of melatonin, shortening the latency to onset of Stage 2 sleep ($P = 0.030$). *Post-hoc* analysis with Bonferroni correction showed that melatonin significantly shortened the latency to onset of Stage 2 sleep after 3-wk use (24 vs. 10 min; $P = 0.044$) and also after discontinuation of melatonin (carryover effect; 37 vs. 13 min; $P = 0.001$), without significant effect after acute melatonin administration (after one dose) (Figure S2). In addition, there was a significant main effect of melatonin shortening latency to onset of Stage 1 sleep ($P = 0.027$), without significant effect in *post-hoc* analysis. Finally, there was a significant main effect of melatonin increasing Stage 2 sleep ($P = 0.044$), without significant effect in *post-hoc* analysis. None of the other PSG sleep variables showed a significant main effect of melatonin.

Melatonin Had No Effect on Subjective Sleep Quality

While in the laboratory, patients completed a post-sleep questionnaire within the first 15 min after each scheduled awakening, including the questions "how sound did you sleep last night" (5 categories from "very sound" to "very restless") and "how good would you rate your sleep to have been last night" (5 categories from "very good" to "very bad"). Melatonin had no significant effect on either measure.

Melatonin Supplementation Resulted in Plasma Melatonin Levels At or Above Endogenous Nighttime Levels Throughout Sleeping Episodes

An intravenous catheter (20 g 1.25 inch in forearm) was inserted in the patients' dominant arm on study day 2 of both laboratory admissions and blood was sampled via 12-ft tubing every 60 min beginning 8 hr after scheduled awakening on the second study day and continued until discharge on the fourth day of each laboratory stay. Plasma melatonin levels were assayed via radioimmunoassay 1125 (Pharmasan Laboratories, Osceola, WI, USA). The sensitivity was 0.7 pg/ml and the interassay coefficient of variation was 13.2% and 8.4% at a mean

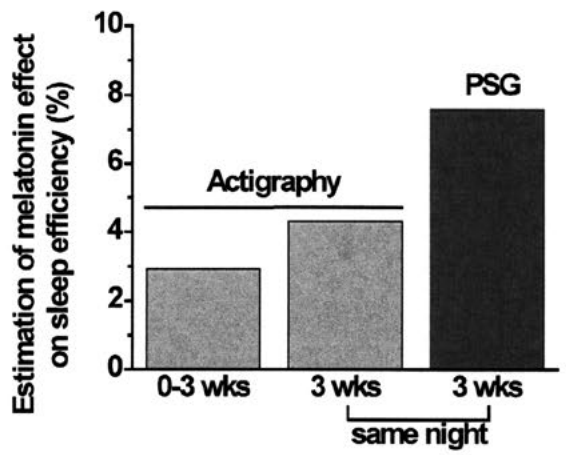

Figure S1—Actigraphy underestimates the effect of melatonin on sleep efficiency. The magnitude of effect of melatonin on sleep efficiency as estimated by actigraphy at home (left gray bar) was smaller than that as determined by polysomnography (PSG) in the laboratory (right red bar). This difference was mainly due to an underestimation of the magnitude of effect by actigraphy as compared with the assessment by the golden standard, PSG. First, as measured under the same laboratory conditions and on the same recording night, the effect of melatonin on sleep efficiency as estimated by actigraphy was 3.3 % less than as measured by PSG (comparing middle gray bar and right red bar). Second, the difference in magnitude of effect of melatonin on sleep efficiency due to a combined difference in duration (as an average throughout 3 wk of supplementation vs. one night at the end of 3 wk of supplementation) and location (home vs. laboratory) was much smaller, i.e., 1.4 % (comparing left and middle gray bars).

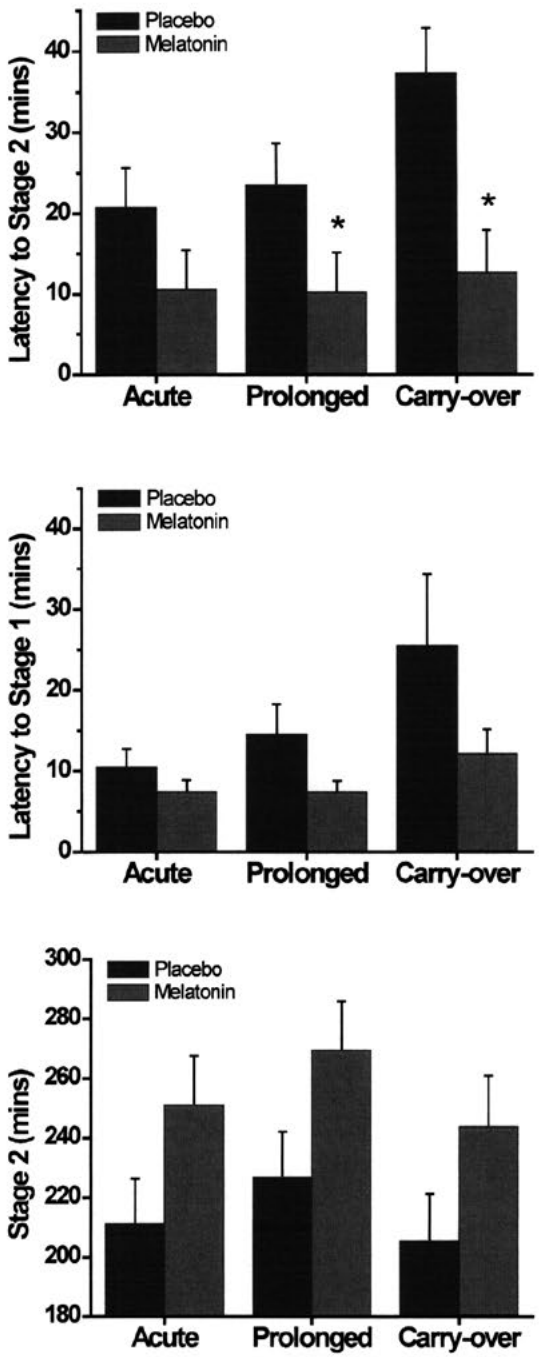

Figure S2—Acute, prolonged, and carryover effects of melatonin on PSG-assessed sleep quality. In exploratory analysis, including the PSG assessments after single administration, 3-wk administration, and after discontinuation of melatonin, there was a main group effect for Stage 2 sleep (P = 0.030), latency to Stage 1 sleep (P = 0.027), and latency to Stage 2 sleep (P = 0.044). In *post-hoc* analysis, acute melatonin supplementation (after 1 dose) had no significant effect on any PSG measure. On the night after discontinuation of melatonin (i.e., the sleep opportunity starting 25 hr after the last dose), there was no worsening of any PSG measure (no sign of rebound insomnia). In fact, the latency to Stage 2 sleep remained significantly shortened for at least one night after discontinuation of melatonin (P = 0.001; top panel; Carry-over). Asterisk indicates significance of melatonin effect for *post- hoc* analysis.

concentration of 17.3 and 69 pg/ml, respectively. Due to subject characteristics (e.g., artificial body parts increasing risk of infection or intolerance precluding intravenous insertion) and technical difficulties, we could only analyze 24-hr melatonin profiles of 10 patients (4 of whom were randomized to the melatonin group).

On the baseline day while on placebo (second day of first admission to the laboratory) there were large interindividual differences in nighttime plasma melatonin values: 6 patients had melatonin values in the typical range, between approximately 40-100 pg/ml, and 4 patients had very low melatonin values (≤ 10 pg/ml) (Figure S3, top panel). Because we had no assessments of melatonin profiles before the patients were prescribed their chronic beta-blocker therapy and because there is large interindividual variability even among healthy and unmedicated people, we could not determine the extent to which beta-blockers suppressed melatonin concentrations. Indeed, it has been proposed that the individual decline in melatonin production capacity, e.g., with aging, disease or medication use, may better correspond to sleep disruption than absolute melatonin concentrations.[3,4] In those patients randomized to melatonin supplementation, melatonin plasma concentrations rose to a level more than an order of magnitude higher than during baseline conditions, stayed at or above nighttime levels for the full 8-hr sleep episode, and declined to levels below 20 pg/ml approximately 4 hr after scheduled awakening. This pattern was similar for both the acute administration and with repeated administration (Figure S3, bottom panel). The endogenous melatonin profile observed the day after discontinuation of melatonin supplementation, after exogenous melatonin had completely disappeared from the circulation and no longer obscured the endogenous melatonin profile, showed that repeated melatonin administration had not resulted in an amplification of the endogenous melatonin profile amplitude, and had not resulted in an advance of the dim light melatonin onset or dim light melatonin offset.

Relationship Between Beta-blocker Dose and Peak Nighttime Melatonin Concentration

Although the dose distribution was limited and the sample size was relatively low, the individual peak nighttime melatonin concentrations were consistent with a higher beta-blocker dose being more likely to lead to lower melatonin secretion (Figure S4).

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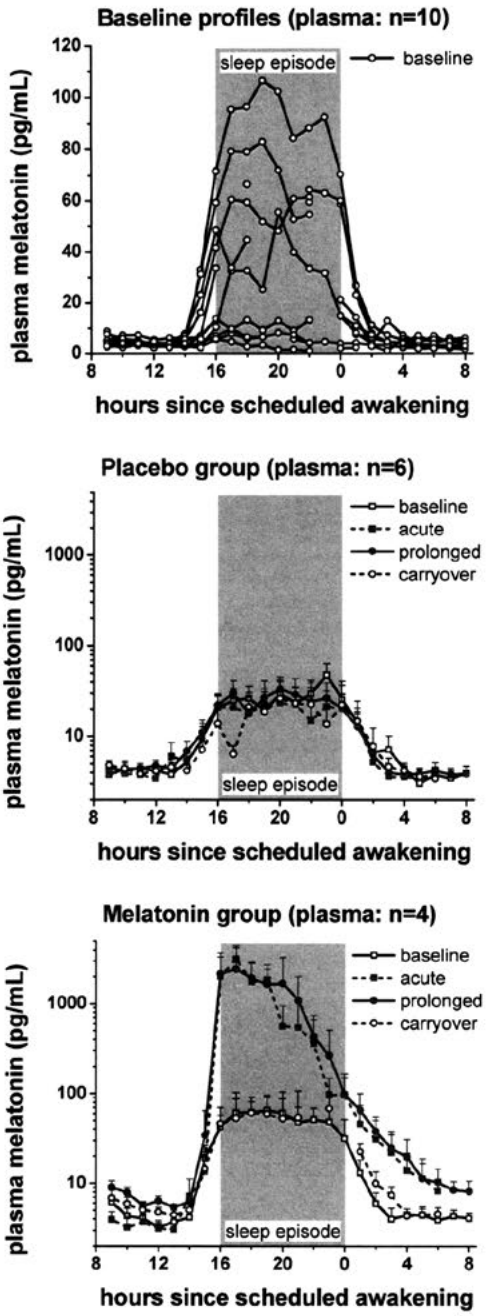

Figure S3—Plasma melatonin profiles. In the 10 volunteers in whom we could collect blood, 24-hr baseline melatonin profiles are depicted (top panel). In the placebo group, the melatonin profiles were very consistent across the four separate 24-hr windows (middle panel; log scale). In the melatonin group, plasma melatonin concentrations were increased by more than 20-fold in the first few hours after melatonin administration, and remained at superphysiologic or physiologic nighttime concentrations for the full 8-hr sleep episodes (bottom panel; log scale). Interestingly, melatonin profiles after a single melatonin dose (acute) and after 3 wk of daily melatonin dosing (prolonged) were comparable, and melatonin profiles at baseline and on the night following the last dose (carryover) were comparable. All melatonin samples were collected in dim light conditions (≤ 4 lux). Gray bar, 8-hr scheduled sleep episode in complete darkness in a supine posture at rest.

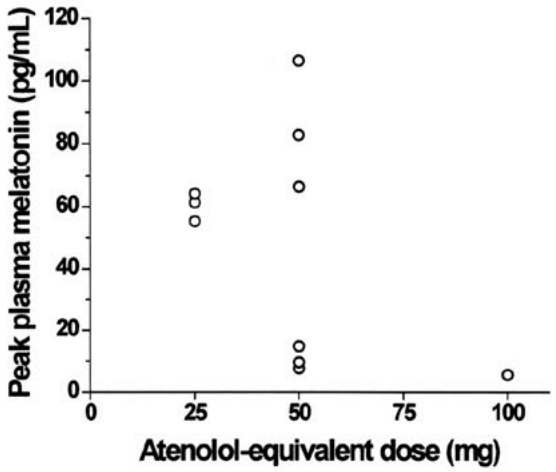

Figure S4—Relationship between beta-blocker dose and peak nighttime melatonin concentrations. For the 10 volunteers in whom we could collect blood, the relationship between their daily beta-blocker dose and the peak nighttime melatonin concentration at baseline is shown. Atenolol-equivalent beta-blocker dose was determined based on the approximation that twice the dose of metoprolol is required to achieve the same effectiveness as with atenolol (e.g., 100 mg metoprolol has an atenolol-equivalent dose of 50 mg).[5]

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Contribution of prolonged-release melatonin and anti-benzodiazepine campaigns to the reduction of benzodiazepine and z-drugs consumption in nine European countries

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Abstract
Background Benzodiazepines (BZD) and benzodiazepine receptor agonists (zolpidem, zaleplon, zopiclone, altogether Z-drugs) are most commonly prescribed for the treatment of insomnia. However, long-term use of BZD/Z-drugs is associated with major adverse events including, but not limited to, falls and fractures, domestic and traffic accidents, confusion, cognitive impairment, Alzheimer's disease and cancer. The prolonged use of these drugs is thought to be related to severe withdrawal symptoms and potential dependency. The chronic and extensive use of BZD/Z drugs has become a public health issue and has led to multiple campaigns to reduce both prescription and consumption of BZD/Z-drugs. Prolonged-release (PR) melatonin is the first of a new class of melatonin receptor agonist drugs that has demonstrated clinically relevant efficacy on improving quality of sleep and morning alertness, with a good safety profile.
Objective This study aimed to analyze and evaluate the impact of anti-BZD/Z-drugs campaigns and the availability of alternative pharmacotherapy (PR-melatonin) on the consumption of BZD and Z-drugs in several European countries.
Methods Annual sales data from nine European countries were extracted from the IMS sales database and analyzed to determine whether trends in use of these treatment options were attributed to campaigns and/or availability and affordability of safer alternatives on the market.
Results Campaigns aiming to reduce the use of BZD/Z-drugs failed when they were not associated with the availability and market uptake of PR-melatonin. The reimbursement of PR-melatonin supports better penetration rates and a higher reduction in sales for BZD/Z-drugs.

Keywords Insomnia · Benzodiazepines (BZD) · Benzodiazepine receptor agonists · Z-drugs · Prolonged-release (PR) melatonin · Addiction

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Introduction

Insomnia is a disorder characterized by difficulties in initiating and/or maintaining sleep, nighttime or early awakenings, and nonrestorative or poor quality sleep for at least 1 month [1, 2]. Its diagnosis is further subdivided into primary insomnia with an absence of comorbid conditions, and secondary insomnia if it is associated with other conditions (physical, mental, environmental causes). Insomnia is associated with clinically significant daytime distress resulting in a reduced quality of life. Mental health problems, such as a reduction of cognitive abilities, memory and attention, as well as cardiovascular, respiratory and metabolic disorders are associated with insomnia [3]. Direct and indirect costs of insomnia represent a substantial societal economic burden [4]. The prevalence of primary insomnia ranges from 1 % to 10 % in the general

population and up to 25–30 % in the elderly, [5–9] for whom treatment of insomnia is a clear medical need.

Benzodiazepines (BZD) and benzodiazepine receptor agonists (zolpidem, zaleplon, zopiclone, altogether Z-drugs) are most commonly prescribed for the treatment of insomnia [10, 11]. A meta-analysis of the risks and benefits of these therapeutic options in elderly patients reported statistically significant improvements in sleep, but also reported a statistically significant risk of adverse events [12, 13], including life-threatening ones [14]. Indeed these drugs are only approved by regulatory authorities for 2–4 weeks because of safety concerns. The Z-drugs, which unlike BZD are used exclusively for the treatment of insomnia, were thought to have a lesser tendency to induce physical dependence and addiction than BZDs [15], and are therefore widely prescribed for the treatment of insomnia, particularly in elderly patients [16–18]. Nevertheless, safety issues are still a matter of concern [19–25]. Long-term BZD and Z-drug use is not recommended, as tolerance and addiction can occur [26]. A population-based survey of patients using Z-drugs and BZD hypnotics found that Z-drug users were more likely to report that they had tried to stop using their hypnotic drug and were more likely to want to stop taking Z-drugs than BZD users. Adverse effects were reported in over 41 % of users with no difference between these two classes. Efficacy also did not differ between Z-drugs and BZD users [13].

In patients over 60 years of age, chronic BZD or Z-drug use carries the risk of exacerbations of pre-existing psychomotor or cognitive impairment, which may result in an increased risk of falls, motor vehicle collisions, household accidents or confusion and memory problems [27]. Recent studies have also pinpointed the potential increased risk of Alzheimer's disease [28], cancer, and mortality [29] after chronic consumption of hypnotic drugs.

These safety concerns relating to the treatment of insomnia with hypnotic drugs, as well as the possibility of dependence, are a significant public health issue.

It has also been demonstrated that in some countries such as France, BZD and Z-drugs are overused and prescribed for a much longer time than the indicated 4 weeks [26, 30]. As a result, more and more health authorities in Europe are initiating policies and recommendations in order to decrease the consumption of BZD and Z-drugs [30–35]. However, the anti-BZD and Z-drug campaigns initiated in most countries have been unsuccessful, and despite the guidelines and national recommendations, the use of BZD and especially Z-drugs has continued to increase.

Prolonged-release (PR) melatonin is the first of a new class of drugs known as "melatonin receptors agonists," and is a non-sedative hypnotic which has demonstrated clinically relevant efficacy on quality of sleep and morning alertness, with a good safety profile [36–39]. No evidence of dependence, withdrawal effects, rebound insomnia or negative influence on daytime alertness has been observed with its use [40, 41]. Several clinical trials demonstrated that PR-melatonin could help reduce BZD and Z-drugs consumption [42, 43].

PR-melatonin is only available as trade name Circadin, manufactured by Neurim Pharmaceuticals, Tel-Aviv, Israel. PR-melatonin 2 mg is the only alternative to BZD and Z-drugs, approved by the European Medicines Agency (EMA) in 2007 for patients aged 55 or over, as monotherapy for the short-term treatment (up to 13 weeks) of primary insomnia.

As PR-melatonin was launched in many European markets in 2008, it was interesting to evaluate how campaigns to decrease BZD and Z-drugs prescriptions affected consumption of these drugs in real life, with or without market uptake of PR-melatonin.

Objectives

The objective of this study was to analyze and evaluate the impact of anti-BZD/Z-drug use campaigns and the availability of alternative pharmacotherapy (PR-melatonin) on the consumption of BZD and Z-drugs in several European countries.

Methodology

The selection of European countries considered in the scope of this study was based on two criteria: countries having national or regional (in Spain) anti-BZD campaigns, and/or countries where PR-melatonin was launched and reached at least 4 % volume market share of the total insomnia market for N5B1 (NON-BARBITURATE PLAIN). The countries with anti-BZD campaigns were selected after reviewing their respective Ministry of Health, national public health, HTA agency, and regional health authorities' websites. To determine countries with significant PR-melatonin market share, the IMS sales database was used. IMS Health is a global company that provides information, services and technology for the healthcare industry. The market volume is defined as the ratio of the number of PR-melatonin standard units sold to the total number of standard units sold for the treatment of insomnia. The Standard Unit (SU) is the smallest drug dose available on the market. The level of 4 % was considered to be a significant penetration rate—when the other hypnotics are generics with a price that is around 8 times lower, and the indication for PR-melatonin is only for insomnia patients aged over 55 while for the competitors are for all ages, 4 % in volume can reasonably be considered a significant penetration rate. It corresponds to approximately 20 % in value.

The European countries in the scope of this study were: Finland, Norway, Denmark, Sweden, Greece, France, the Netherlands, Spain and the United Kingdom.

This study was completed using the annual sale volumes of BZD/Z-drugs and PR-melatonin for each country in the scope, extracted from the IMS sales database. Data is expressed in SU. For each country, we studied the evolution of BZD/Z-drug sales volumes (together and separately) 3 years prior to the launch of PR-melatonin (at the end of 2007) and then 4 years after the launch of PR-melatonin (2011), as well as the evolution of PR-melatonin sales volumes. Additional parameters considered in the interpretation of the data were: the launch strategy of PR-melatonin (actively promoted/not promoted), product positioning and key messages, national or regional anti-BZD/Z-drugs campaigns (the type of campaign, their target and the recommendations), the penetration rate of PR-melatonin in 2011 and its reimbursement status compared to BZD/Z-drugs. As only the volume of sales is available in these databases, the assumption was made that the volume sold was equal to the prescribed and consumed volumes.

Results

Table 1 presents market status of BZD/Z hypnotics and PR-melatonin for the countries within the scope of the study. The market trends for each country are depicted in Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9 and are detailed below, by country.

Finland

In Finland, health authorities have been carrying out an anti-BZD campaign since 2005 [33]. This government-driven campaign was supported by publications and guidelines with negative recommendations on BZD and Z-drug use. PR-melatonin was launched in January 2008 and marketed actively, but was not reimbursed, while BZDs and Z-drugs are partially reimbursed. The price of PR-melatonin in Finland is about eight times higher (0.55€ ex-factory/day) than the mean price of BZD/Z-drugs (0.07€ ex-factory/day). Nevertheless, patients are used to paying for medications and the impact of price on patient decisions is limited.

As depicted in Fig. 1, BZD/Z-drug consumption remained stable between 2005 and 2007. More precisely, BZD sales decreased in proportion to the increase in Z-drug sales. Since the launch of PR-melatonin in 2008, BZD and Z-drugs sales have decreased substantially, with a reduction of up to 20.2 % between 2008 and 2011. PR-melatonin sales increased gradually between 2008 and 2011, to reach 5.1 % in volume (SU) market share and 27.1 % in value market share in 2011. In 2011, 5 million SUs of PR-melatonin were sold whereas the annual sales of BZD/Z-drugs reduced by 16.6 million SUs compared to 2005.

Norway

In Norway, an important anti-BZD government driven campaign was initiated in 2005 [44]. This campaign was supported by guidelines and especially focused on the issue of driving and BZD/Z-drug consumption [44]. PR-melatonin was launched in Norway in January 2008, but was not reimbursed. BZD and Z-drugs were not reimbursed as well.

Despite the anti-BZD campaign prior to the launch of PR-melatonin, BZD/Z-drug net consumption increased by 7.3 % between 2005 and 2007. BZD volume sales decreased (9.3 %) whereas Z-drug sales increased (11.7 %) during this period. After its launch in January 2008, PR-melatonin reached a volume market share of 4.5 % and a value market share of 21 % in 2011. BZD consumption decreased by 13.4 % between 2008 and 2011, whereas Z-drugs sales stabilized (+1.0 %).

Denmark

The Danish Institute of Rational Pharmacotherapy (within the Danish Medicines Agency) started a campaign against the use of BZD and Z-drugs in 2008, with three brochures provided to physicians, citizens and pharmacies [45]. They pinpoint the fact that long-term consumption of BZD and Z-drugs is associated with health risks. Approximately 100,000 people in Denmark permanently consume BZDs and are addicted to them [45].

PR-melatonin was launched in October 2007, without reimbursement status, similar to BZD/Z-drugs that are also not reimbursed. PR-melatonin was not recommended as first-line therapy in the treatment of primary insomnia [46]. Since Denmark citizens are used to paying for insomnia drugs, the non-reimbursement status did not differentiate PR-melatonin from BZD/Z-drugs.

Between 2005 and 2007, Z-drug sales remained at the same level whereas BZD sales started to decrease slightly, despite the absence of an anti-BZD campaign. In 2008, a campaign was launched alongside the introduction of PR-melatonin. PR-melatonin's market share reached 3.7 % of volume in 2011 and 21 % in value market share in 2011. From 2008, the sale of BZD/Z-drugs decreased quite substantially (by 24.7 %).

Sweden

In Sweden, all BZD and Z-drugs are reimbursed. An anti-BZD campaign was launched in 2001 [47], resulting in a stagnation of the sales of BZD, with an increase of Z-drugs sales. PR-melatonin was launched but was the only non-reimbursed hypnotic. It represented only 1 % of volume

Table 1 Market status of BZD/Z hypnotics and PR-melatonin for each country in the scope

Anti-BZD campaigns					PR-melatonin			BZD/Z-drugs		
Countries	Yes/No	year	Launch date	Promotion	Reimbursement	Volume market share in 2011 (SU%)	Price (€/tab)	Reimbursement	Volume market size in 2005 (million SU)	Zolpidem price (€/tab)
Finland	Yes	2005	January 2008	Actively promoted	No reimbursement	5.10 %	0.55	Partial reimbursement	105,962	0.07
Norway	Yes	2005	January 2008	Actively promoted	No reimbursement	4.50 %	0.53	No reimbursement	75,711	0.10
Denmark	Yes	2008	October 2007	Actively promoted	No reimbursement	3.70 %	0.55	No reimbursement	71,688	0.63
Sweden	Yes	2001	2008	Actively promoted	No reimbursement	1 %	0.62	Reimbursed	195,048	0.05
Greece	No	–	2008	Actively promoted	Reimbursed (automatic, at 75 %)	5.50 %	0.53	Reimbursed (automatic, at 75 %)	50,836	0.09
France	Yes	2008	June 2008	Not promoted	No reimbursement	<1 %	0.76	Reimbursed	766,207	0.16
Netherlands	Yes	2009	2009	Initially promoted	No reimbursement	<1 %	0.57	No reimbursement (since Jan 2009)	148,042	Not found
UK	Yes	2004	2008	Initially promoted	Reimbursed (automatic) not recommended by NICE[a]	<1 %	0.53	Reimbursed (automatic) Equal recommendation BZD and Z drugs by NICE	474,775	0.08
Spain	Regional small campaigns	–	Not launched	NA	NA	NA	NA	Reimbursed	390,005	0.06

[a] In the UK, drug prices are freely chosen by pharmaceutical firms and reimbursed at 100 %. The control of practices is made through the recommendations of the National Health Service (NHS) after advice from the National Institute for Health and Clinical Excellence (NICE). In a general way, a product negatively recommended is not prescribed.

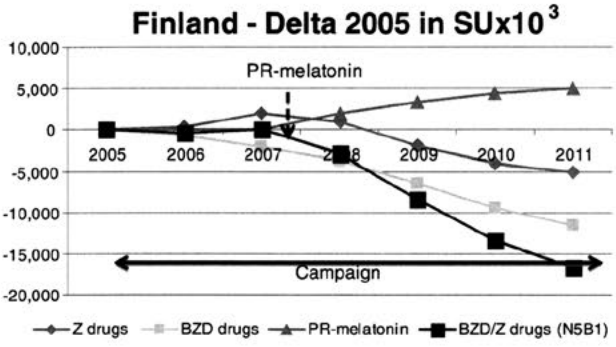

Fig. 1 Finland. Despite the anti-BZD campaign, BZD/Z-drugs consumption remained stable until the introduction of PR-melatonin. At PR-melatonin's launch in 2008, BZD and Z-drugs sales decreased substantially between 2008 and 2011

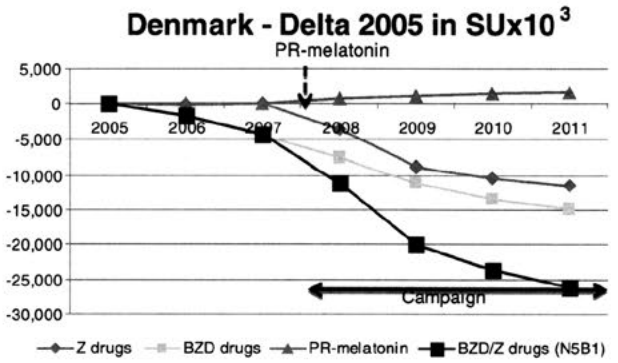

Fig. 3 Denmark. Despite the absence of an anti-BZD campaign between 2005 and 2007, BZD sales started to decrease slightly whereas Z-drug sales remained at the same level. From 2008, when both PR-melatonin and a BZD campaign was launched, the BZD/Z-drugs sales decreased quite substantially

market share and 11 % of value market share in 2011, as shown in Fig. 4. The BZD consumption rate was quite stable (−3.2 %) whereas sales of Z-drugs rose steadily (+20.5 %) from 2005 to 2011.

France

In France, health authorities are concerned about the overuse of BZD and Z-drugs. An initial report warning about the use of BZD/Z-drugs was issued in the early 90s [48], followed by a series of reports issued upthrough 2010 and many campaigns, the most prominent being in 2008 [26]. The risks of these products are well known, and the Haute Autorité de Santé (HAS) is trying to reduce their consumption. The HAS has published detailed recommendations on how to help patients withdraw from the use of BZD: "Psycho SA - Plaintes du sommeil-Insomnie 2010" [26]. Also, the Agence Française de Sécurité Sanitaire Produits de Santé (AFSSaPS) addressed the high levels of BZD consumption in France through a review of the last 10 years [30], and tried to reduce consumption by controlling use and strengthening the measures already initiated to promote the appropriate consumption of BZD and Z-drugs.

PR-melatonin was introduced to the French market in June 2008. The product was not actively promoted in France, since it was not reimbursed while all other hypnotics were reimbursed. The price was eight times higher than the mean BZD price. As French patients are not used to paying for their medication "out of pocket," there was a substantial disincentive for choosing PR-melatonin prescriptions.

As observed in Fig. 5, the sales of BZD/Z-drugs did not change significantly following the recommendations of the HAS, with the global variation of +1.8 %. More precisely, BZD sales decreased by −6.0 % whereas Z-drug sales increased by +4.7 % during the campaign. From 2007, both BZD and Z-drug sales remained stable despite the various reports and campaigns issued by the health authorities. PR-melatonin sales were negligible.

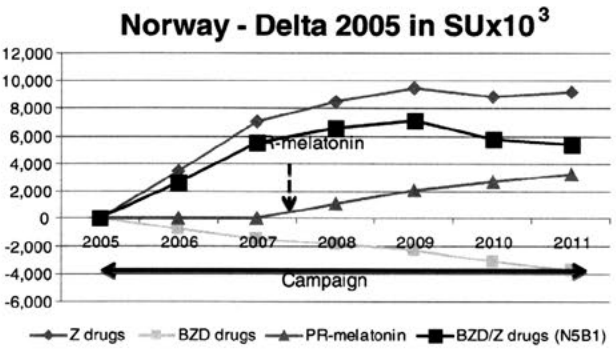

Fig. 2 Norway. Despite the anti-BZD campaign prior to the launch of PR-melatonin, BZD/Z-drug net consumption increased between 2005 and 2007. BZD volume sales decreased whereas Z-drug sales increased. After PR-melatonin launch, BZD consumption decreased by 13.4 % between 2008 and 2011, whereas Z-drugs sales have stabilized. PR-melatonin reached a volume market share of 4.5 % in 2011

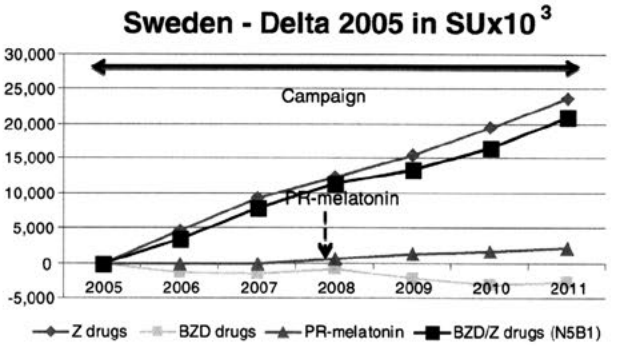

Fig. 4 Sweden. The BZD consumption rate is quite stable whereas Z-drugs sales rose steadily from 2005 to 2011 despite the anti-BZD campaign. PR-melatonin sales were negligible

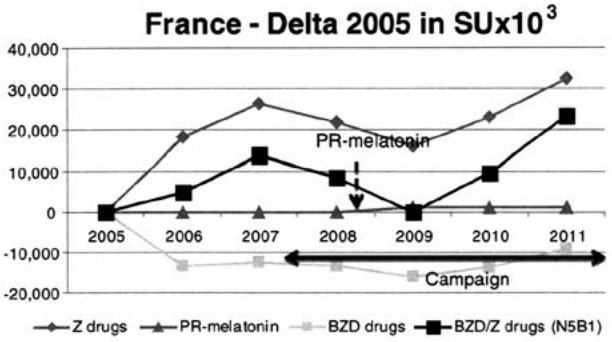

Fig. 5 France. BZD sales decreased whereas Z-drug sales increased during the anti-BZD campaign. From 2007, both BZD and Z-drug sales remained stable despite the various reports and campaigns. PR-melatonin sales were negligible

Greece

No anti-BZD campaign has been initiated in Greece. PR-melatonin was launched in January 2008 and reimbursed like all other hypnotics.

Before PR-melatonin's launch, consumption of BZD/Z-drugs decreased by 5.9 % between 2005 and 2007 (Fig. 6). After the launch, this phenomenon accelerated, with a decrease of 14.5 % in BZD/Z-drug consumption between 2008 and 2011. PR-melatonin penetration was progressive, reaching 5.5 % of volume market share and 28 % of value market share in 2011. Compared to sales in 2005, the annual sales of BZD/Z-drugs decreased by more than 11 million SUs. Approximately 2.4 million SUs of PR-melatonin were sold during 2011.

The Netherlands

The Netherlands College of General Practitioners recommended that BZD and Z-drugs should be prescribed only for short courses, and should generally be avoided in elderly patients. Indeed, around 30 % of Dutch patients using these compounds

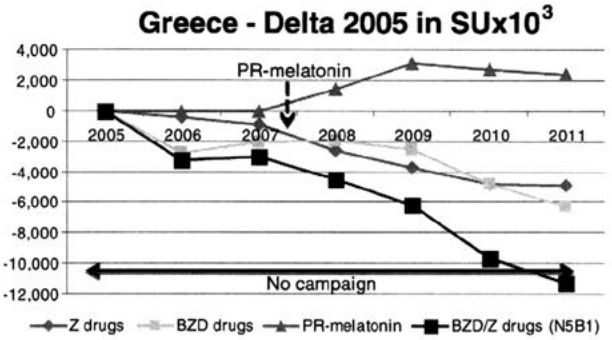

Fig. 6 Greece. Between 2005 and 2007, there was a decrease of BZD/Z-drug consumption. After the PR-melatonin launch in 2008, this phenomenon accelerated, with a bigger decrease of the BZD/Z consumption between 2008 and 2011. The PR-melatonin's penetration was progressive, finally reaching 5.5 % of volume market share

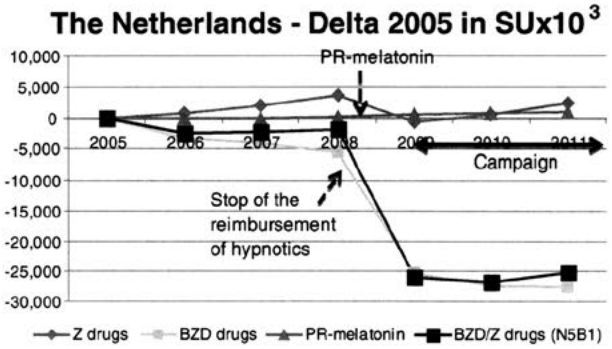

Fig. 7 The Netherlands. The sales of PR-melatonin were negligible. Looking separately at BZDs and Z-drugs, BZD sales decreased sharply whereas Z-drug sales were stable between 2008 and 2011

are chronic users [49]. In January 2009, the reimbursement status of BZD and Z-drugs changed, and became excluded from the Dutch reimbursement schemes. The aim of this change was to reduce the use of these medications for chronic use, and to limit the health care expenditures (high level of costs due to the volume of BZD use). After ending reimbursement, the Dutch Foundation for Pharmaceutical Statistics reported a 16 % reduction in the overall use of BZDs and Z-drugs [50].

In the Netherlands, PR-melatonin was launched without a reimbursement status. As shown in Fig. 7, the sales of PR-melatonin were negligible. Looking separately at BZDs and Z-drugs (Fig. 7), BZD sales decreased by −19.4 % whereas Z-drug sales were stable (−3.6 %) between 2008 and 2011 and since the ending of reimbursement in 2009, both BZD and Z-drugs sales are stable.

United Kingdom

In the United Kingdom, recommendations to restrict BZD and Z-drug usage were published on 2004, by the Department of Health (DoH) [51]. In this recommendation, doctors are warned that benzodiazepines should only be prescribed for short-term treatment, in light of evidence of problems associated with long-term use.

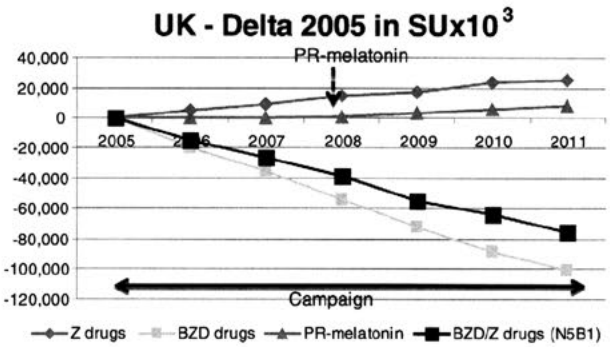

Fig. 8 The UK. The decrease in BZD SUs between 2005 and 2010 was steady. Similarly, the sales of Z-drugs increased during this period. PR-melatonin sales were negligible in the UK

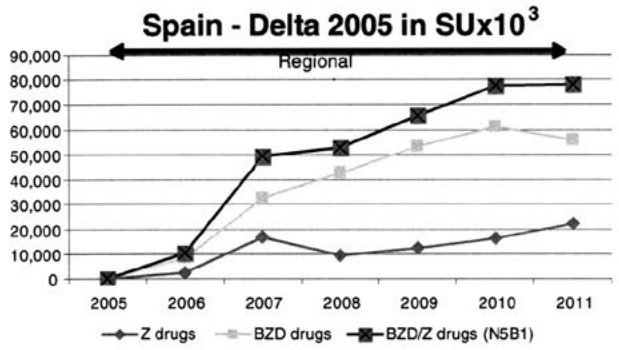

Fig. 9 Spain. There was a substantial increase in BZD and Z-drug consumption between 2005 and 2011 and PR-melatonin was not on the market

PR-melatonin was launched in 2008 and automatically reimbursed in the UK, but was not recommended by the National Institute of Clinical Excellence (NICE) and the Scottish Medicines Consortium (SMC).

The decrease in BZD SUs between 2005 and 2010 was steady, up to 31.7 % less, as shown in Fig. 8. Concurrently, the sales of Z-drugs increased during this period (+7.4 %). PR-melatonin sales were negligible in UK.

Spain

Campaigns were conducted at regional levels; in addition, PR-melatonin was approved, but not reimbursed and thus was not put on the market.

As shown in Fig. 9, there was a substantial 20 % increase in BZD and Z-drug consumption between 2005 and 2011.

Discussion

The results of this analysis suggest there are three common groups among the studied countries, with different BZD/Z-drug consumption trends:

- Countries where the sales of BZD and Z-drugs decreased since 2007: Greece, Finland and Denmark.

  In Greece there was no anti-BZD campaign before the launch of PR-melatonin, and the consumption of the BZD and Z-drugs was stable. BZD and Z-drug consumption decreased by 14.5 % over 3 years after the introduction of PR-melatonin in the market. The decrease in BZD/Z drug consumption since 2008 can thus be attributed to the launch of PR-melatonin and its considerable market penetration. On average, an increase in 1 SU of PR-melatonin was associated with a decrease of about 4 SUs of BZD/Z-drugs.

  The combined launch of PR-melatonin and anti-BZD campaigns in Finland and Denmark seems to be associated with a reduction of BZD/Z-drugs usage. This decrease is concomitant with the penetration of PR-melatonin on the market and the campaign implementation. Again, uptake of 1 SU PR-melatonin in Finland was associated with a decrease of 3 SUs of BZD/Z drugs consumption in this country.
- Countries where the sales of BZD decrease while Z-drugs increase: Norway, the Netherlands and the UK. In these countries the anti-BZD campaigns seem effective for BZDs, but essentially resulted in a shift in prescription patterns towards Z-drugs.

In Norway, there was an overall increase in BZD/Z drugs consumption since 2005 but the BZD sales decreased in favor of Z-drugs. Since PR-melatonin was launched, the increase in Z-drug sales stopped and the consumption was stabilized, as if the switch from BZDs gradually shifted from Z-drugs to PR-melatonin.

The same evolution of BZD and Z-drug sales was observed in the Netherlands, but the decrease in BZD sales was mostly related to the change in the reimbursement status, suggesting that BZD/Z drug consumption in this country is price sensitive and reimbursement itself has some encouraging effect on hypnotic drug consumption. Nevertheless, Z-drug sales remained stable between 2009 and 2011. PR-melatonin sales did not rise considerably in the Netherlands perhaps because it is more expensive than the other drugs and is not actively promoted in this country.

In the UK, a decrease was seen only in BZD. There was a steady increase in Z-drug use of up to 7.3 % in 2011, although NICE has issued the following recommendation: "It is recommended that, because of the lack of compelling evidence to distinguish between zaleplon, zolpidem, zopiclone or the shorter-acting benzodiazepine hypnotics, the drug with the lowest purchase cost (taking into account daily required dose and product price per dose) should be prescribed" [52]. Possibly, higher market acceptance of PR-melatonin might gradually change this situation as seen in Norway.

- Countries where the sales of BZD were stable and Z-drug use increased, resulting in overall increases in BZD and Z-drug sales despite anti-BZD campaigns: France, Sweden and Spain.
- In these countries the anti-BZD/Z-drug campaigns that were sometimes quite intense and long lasting (like in France) had no or very limited impact on prescription levels. As BZDs and Z-drugs are reimbursed while PR-melatonin is not, and these markets are reimbursement-sensitive, PR-melatonin was not commercially launched in France and was not put on the market in Spain.

Although real-life outcomes are difficult to interpret, as many factors could contribute to the occurrence of the

outcome, some conclusions may be drawn with a reasonable level of certainty in the light of this research.

In countries where BZD/Z-drug campaigns were launched and PR-melatonin was not promoted nor prescribed, all campaigns failed to reach the desired outcome. This was the case for France, Spain, and Sweden.

In Greece, where no campaign was initiated, the sales reduction of BZD/Z-drugs was concomitant with PR-melatonin uptake. The case of Greece is a robust argument in favor of the role of PR-melatonin in the reduction of BZD/Z-drug sales.

In Finland and Denmark, the concomitant launch of BZD/Z-drug campaigns and PR-melatonin made it difficult to weight the impact of each factor on the reduction of BZD/Z-drug sales. However, in Finland the 3-year campaign from 2005 to 2008 ended with no appreciable effect at the launch of PR-melatonin. The drop of BZD/Z-drug sales clearly followed the uptake of PR-melatonin.

It should be noted that a standard unit is the smallest available drug dose. For PR-melatonin, an SU is the defined daily dose (DDD), as there is only one dosage available on the market. For BZD/Z-drugs there are often several dosages and therefore more than one SU may account for a DDD. Thus, the volume of BZD/Z-drugs in SUs replaced by the sales of PR-melatonin is higher than the raw number of SUs of PR-melatonin sold. In addition, the lower SU volumes of sold PR-melatonin as compared to unsold BZD/Z-drug SU volumes may in part reflect the fact that PR-melatonin can be discontinued without difficulty while BZD/Z-drugs cause withdrawal, tolerance and dependency, making discontinuation very difficult and causing abuse.

In Norway, the prescription shift of BZD toward Z-drugs stopped suddenly when PR-melatonin was launched. PR-melatonin appears to be a successful alternative option to Z-drugs.

In the UK and the Netherlands, in the absence of PR-melatonin uptake the reduction of BZD sales was associated with an increase of Z-drug use. The objective of total reduction was not achieved. The shift cannot be considered a success of the anti BZD/Z drug campaign, as the risk associated with Z-drugs is not considered significantly different from BZD in most studies [27]. Some studies found Z-drugs to be even worse [53].

The marketing strategy toward positioning and promotion of pharmaceutical products is a critical element of the medical practice [54]. In this case in Greece, Finland, Denmark and later Norway, unlike Sweden, PR-melatonin was perceived as an option to help chronic users withdraw from BZD/Z-drugs. Although this was not the only positioning, it was an important element of the marketing strategy also leading to volume market shares of 4–5.5 %. In those countries, the sales of PR-melatonin were associated with a decrease of BZD/Z-drug sales.

The lack of success of anti-BZD/Z-drug campaigns in the absence of an alternative pharmacological treatment option (France, Sweden) raises the question of the utility of such campaigns. Even if BZD drugs were actually reduced in countries like the UK and Norway, they were always associated with a shift in prescription toward another pharmacological agent, namely Z-drugs alone (UK) or Z-drugs followed by PR-melatonin when it became available (Norway). When both PR-melatonin and Z-drugs were available the prescriptions were consistently channeled toward PR-melatonin, resulting in a net decrease of the whole sedative hypnotics class including BZDs and Z-drugs (Finland and Denmark). The availability on the market of pharmacological alternative options to replace BZD/Z-drugs appears to be a critical factor for success of such campaigns. In the Netherlands, despite the fact that reimbursement was ended for both BZD and Z-drugs, there was a shift toward Z-drug prescription for some of the patients. It is unclear how the other patients were managed. Additionally, no information is available on alternative pharmacological or nonpharmacological prescriptions. Therefore, it is not possible to appreciate potential harm associated with this shift in practice.

The adoption of PR-melatonin as a treatment option is also important for BZD/Z drug use in countries where the product is reimbursed (e.g. the UK and Greece). In the UK the PR-melatonin launch was not associated with an HTA recommendation and sales didn't take off, as the recommendation is a strong driver of general physician (GP) prescriptions. However in Greece, where the adoption of PR-melatonin was high, so was the decrease of sales of BZD/Z-drugs despite the lack of campaigns.

In Spain, where PR-melatonin was not available and the campaign was mild and geographically limited to regions, and in France were the campaign was intense and national, the sales of BZD/Z-drugs still tended to increase.

The findings of this study are consistent with a small size, double-blind randomized clinical trial that has shown the role of PR-melatonin in helping patients to withdraw from BZD/Z-drug use [43]. It is also supported by an observational study showing a low rate of reinitiation of BZD/Z-drug after a course of PR-melatonin when patients were previously treated by BZD/Z-drugs [42].

The study has also some limitations.

No country without any campaigns and without a significant PR-melatonin existence was selected as control country. However, it is established that prescription habits of doctors are deeply anchored and without any intervention, no change occurs [55]. Moreover, in order to compare trends with and without campaigns, some countries can be their own control by comparing the period before with the period after the launch of the campaign, for example France.

We used a database that is solely based on sales data and not prescriptions. We assumed that sales figures are a good proxy of what is consumed even if it is clearly higher. Indeed, some sold drugs are not then consumed by the

patients. However, we assumed that the proportion of drugs sold and actually consumed by patients is the same, whatever the product. There are no reasons to believe that the proportion is different between products.

Unlike Z-drugs, BZD could be used for other indications such as epilepsy or anxiety [56, 57]. However, we only considered N5B1 IMS classification in this study, which is for non-barbiturate drugs and is mostly used for insomnia, while BZDs used for other indications are more likely to be reported under other IMS classes, such as N5C (Antidepressants and Anxiolytics).

We didn't collect and analyze whole promotional materials, but we relied on interviews of the company's marketing leader that provided a clear picture of the positioning and promotion of PR-melatonin. The dichotomy of positioning (or not) of PR-melatonin to help patients discontinue BZD/Z-drugs was quite clear. Moreover, the people interviewed were not aware of the ultimate research goal and therefore were unlikely to be biased.

There were no prescriber interviews to appreciate the drivers of their prescriptions, and the role of campaign and PR-melatonin promotion. This could limit the interpretation of the reasons for prescribing PR-melatonin as a means of discontinuing BZD/Z-drug use. In this research we were not assessing the causal relationship but just the existence or not of a relationship.

Finally, we didn't perform a thorough review of campaigns to appreciate the reasons for failure, as this wasn't the objective of our research.

Conclusion

Long-term prescription of BZD/Z-drugs is associated with major adverse events including, but not limited to, falls and fractures, domestic and traffic accidents, confusion, cognitive impairment, Alzheimer's disease and cancer. The prolonged use of these drugs is thought to be related to severe withdrawal symptoms and potential dependency. The chronic and extensive use of BZD/Z-drugs has become a public health issue and led to multiple campaigns to both reduce the prescription of BZD/Z-drugs and achieve discontinuation of long-term treatment. In our research, we observed the failure of those campaigns when they were not associated with the availability and uptake of sales of PR-melatonin. The reimbursement of PR-melatonin may support a better market penetration and a higher reduction of sales of BZD/Z-drugs. The non-reimbursement of BZD/Z-drugs appeared to have no effect on Z-drug prescription, and even showed an increase in prescription during 2011. When considering campaigns aiming to limit the usage of BZD/Z-drugs, policy makers should carefully consider the availability of reimbursed effective and safe pharmacological alternatives.

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Efficacy of prolonged release melatonin in insomnia patients aged 55–80 years: quality of sleep and next-day alertness outcomes

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ABSTRACT

Objective: Melatonin, the hormone produced nocturnally by the pineal gland, serves as a circadian time cue and sleep-anticipating signal in humans. With age, melatonin production declines and the prevalence of sleep disorders, particularly insomnia, increases. The efficacy and safety of a prolonged release melatonin formulation (PR-melatonin; Circadin* 2 mg) were examined in insomnia patients aged 55 years and older.

Design: Randomised, double blind, placebo-controlled.

Setting: Primary care.

Methodology: From 1248 patients pre-screened and 523 attending visit 1, 354 males and females aged 55–80 years were admitted to the study, 177 to active medication and 177 to placebo. The study was conducted by primary care physicians in the West of Scotland and consisted of a 2-week, single blind, placebo run-in period followed by a 3-week double blind treatment period with PR-melatonin or placebo, one tablet per day at 2 hours before bedtime.

Main outcome measures: Responder rate (concomitant improvement in sleep quality and morning alertness on Leeds Sleep Evaluation Questionnaire [LSEQ]), other LSEQ assessments, Pittsburgh Sleep Quality Index (PSQI) global score, other PSQI assessments, Quality of Night and Quality of Day derived from a diary, Clinical Global Improvement scale (CGI) score and quality of life (WHO-5 well being index).

Results: Of the 354 patients entering the active phase of the study, 20 failed to complete visit 3 (eight PR-melatonin; 12 Placebo). The principal reasons for drop-out were patient decision and lost to follow-up. Significant differences in favour of PR-melatonin vs. placebo treatment were found in concomitant and clinically relevant improvements in quality of sleep and morning alertness, demonstrated by responder analysis (26% vs. 15%; $p = 0.014$) as well as on each of these parameters separately. A significant and clinically relevant shortening of sleep latency to the same extent as most frequently used sleep medications was also found (–24.3 vs.–12.9 minutes; $p = 0.028$). Quality of life also improved significantly ($p = 0.034$).

Conclusions: PR-melatonin results in significant and clinically meaningful improvements in sleep quality, morning alertness, sleep onset latency and quality of life in primary insomnia patients aged 55 years and over.

Trial registration: The trial was conducted prior to registration being introduced.

* Circadin is a registered trade name of Neurim Pharmaceuticals Ltd, Tel Aviv, Israel

Introduction

Insomnia is a subjective complaint of sleep described as delayed, insufficient in duration and/or poor in quality (non-restorative sleep). The sleep disturbance (or associated daytime fatigue) causes clinically significant distress or impairment in social, occupational, or other important areas of functioning (Diagnostic and Statistical Manual for Mental Disorders [DSM-IV]). While inadequate quantity of sleep (sleep duration, sleep latency, number of arousals) are reliably measured in the sleep laboratory, the term 'sleep quality' represents a complex phenomenon that is difficult to define and measure objectively as it contains purely subjective aspects such as 'depth' or 'restful' sleep. Nevertheless 'sleep quality' better reflects the concept of insomnia defined in DSM-IV in the sense that it is more closely related to daytime functioning and wellbeing than the objective, sleep laboratory measurements where there is a significant overlap in the distribution of sleep recordings for subjectively defined insomniacs and good sleepers. The elderly are particularly liable to suffer from insomnia[1]. Even in healthy subjects age is negatively correlated with subjective sleep quality and daytime dysfunction[2]. Non-restorative sleep (perceived poor quality of sleep) and subsequently poor daytime functioning are increasingly recognized as a leading syndrome in the diagnostic and therapeutic process of insomnia complaints[3-5]. Average sleep quality rather than quantity appears to be better related to health and affects balance and satisfaction with life[6,7]. Thus, non-restorative sleep and poor quality of sleep constitute a major component of the problem of insomnia, which in itself is a common complaint and is highly associated with impaired daytime functioning[8]. Although much of the outcome of insomnia derives from the extent to which it impairs daytime functioning, insomnia drugs have been approved on the basis of improvements in sleep induction and/or maintenance but not in sleep quality and next day performance[9,10].

Among the wide variety of available treatments for sleep disturbances, the most commonly prescribed hypnotics are benzodiazepines and non-benzodiazepines ('Z-drugs'), both classes of which are gamma-aminobutyric acid (GABA)-A receptor modulators. Hypnotics primarily address insomnia related to quantitative sleep problems (increased sleep latency, shorter sleep duration) but not necessarily sleep quality, and furthermore fail to improve and even adversely affect daytime vigilance[11,12]. Newer treatments of insomnia with favourable daytime consequences are therefore sought.

Melatonin (N-acetyl-5-methoxytryptamine), the major hormone nocturnally produced by the pineal gland, is a sleep regulator and signal of darkness in humans[13]. Thus, the circadian rhythm in synthesis and secretion of melatonin is closely associated with the sleep rhythm in both sighted and blind subjects[14,15]. Daytime administration of exogenous melatonin (when it is not present endogenously) promotes sleep in humans[16,17], presumably by inhibiting circadian wakefulness mechanisms[18,19] and results in modified brain activity compatible with sleep anticipation[20,21]. It is also known that endogenous melatonin levels decrease with age[22]. Decline in melatonin may contribute to the common complaint of poor sleep quality seen among elderly people[23-25]. This raises the possibility of improving sleep in elderly patients by treatment with melatonin substitution. Melatonin itself has a very short half-life and is quickly cleared from the circulation with physiological levels being maintained during the night by continuing output from the pineal gland. PR-melatonin (Circadin* 2 mg) is a prolonged-release formulation of melatonin which when administered orally produces levels of melatonin over the subsequent 8–10 hours thus mimicking the physiological profile. Many previous studies of melatonin in insomnia have been hampered by the wide variety of formulations and doses of melatonin studied, the range of ages of patients studied and the incosistency of outcomes evaluated and insufficient statistical power[26]. A number of studies have demonstrated the objective effectiveness of PR-melatonin in various sleep parameters: sleep latency, efficiency and wake after sleep onset in patients aged 55 years and older. This study was conducted to investigate whether or not treatment with PR-melatonin 2 mg would improve quality of sleep and next-day alertness of older patients suffering from primary insomnia.

We hypothesized that PR-melatonin would significantly improve both quality of sleep and morning alertness compared to placebo in these patients. We selected the Leeds Sleep Evaluation Questionnaire (LSEQ) as the primary tool for these measurements[27]. The LSEQ comprises 10 horizontal 100 mm visual analog scales relating to the following aspects of sleep and daytime behaviour: getting to sleep (GTS; questions 1, 2 and 3); quality of sleep (QOS) (questions 4 and 5); awakening from sleep (AFS; questions 6 and 7); and behaviour following wakening (BFW; questions 8, 9 and 10). The LSEQ is a valid and reliable measure of the effects of drugs on sleep and daytime effects[28] and has been validated in a number of studies, including some involving the PR-melatonin target population (insomnia patients 55 years and older)[29,30]. Furthermore, impaired quality of sleep as assessed by LSEQ was strongly associated with impaired quality of life[31].

* Circadin is a registered trade name of Neurim Pharmaceuticals Ltd, Tel Aviv, Israel

Responder rate analysis for establishing clinical relevance of observed effects in clinical trials is well recognized and is recommended in the European regulatory guidelines for clinical trials[32]. To establish clinical relevance of the observed effects, the minimal clinically significant difference in QOS, which is the difference that is clinically meaningful to the patient, was determined by an anchor-based method using ratings on a five-point severity scale and found to equal 10 mm[33]. As a clinically significant improvement in quality of sleep should lead to improved morning alertness, a responder was defined as a patient who showed improvement in both parameters, that is concomitant improvement from baseline by 10 mm or more on both the QOS and BFW variables of the LSEQ.

Patients and methods

Study design

This was a randomised, double-blind, parallel group clinical trial comprising a 2 week, single-blind, placebo run-in period followed by a 3 weeks double-blind treatment period in which patients were randomised to receive PR-melatonin (Circadin 2 mg, Neurim Pharmaceuticals Ltd, Tel Aviv, Israel), or placebo, given orally as one tablet per day 2 hours before bedtime.

Study subjects

General practitioners in Glasgow and the surrounding areas (West of Scotland) recruited patients into the study. Study visits were conducted by specially selected and trained general practitioners (GPs) along with full-time professional trained research nurses assigned to the practices especially for this purpose.

Patients expressing interest in participating in the study were pre-screened for suitability by the nurses using a telephone interview. A four-step process was used for screening out patients with secondary insomnia and other sleep disorders. The initial diagnosis of primary insomnia was performed using a sleep history questionnaire (SHQ) adopted from The Management of Insomnia Guidelines for Clinical Practice[34]. A similar SHQ has recently been recommended by Clinical Practice Guideline-Adult Insomnia[35]. The SHQ characterises the primary sleep complaint according to the different diagnostic criteria (DSM-IV and International Classification of Diseases [ICD]-10). The questionnaire also helps in differentiating primary insomnia from secondary insomnia due to medical and psychiatric disorders (including depression and anxiety) and specific insomnia disorders like circadian rhythm disorders, movement disorders, parasomnias and breathing related sleep disorders.

Then, a physical examination, an important element in the evaluation of insomnia patients with medical symptoms[35], was performed at the screening visit by a qualified clinician. In order to rule out psychiatric disorders, including depression, anxiety and dementia, the patients went through a detailed psychological assessment that included the Raskin Depression scale, Covi anxiety scale and the Mini Mental State (MMS) on the first visit. Patients who scored 6 or more on the Raskin depression scale and Covi anxiety scale and patients with a score ≤ 24 or ≤ 26 on the MMS (depending on the socio-educational level of the patient) were non-eligible for inclusion in the study. History of severe psychiatric disorders, especially psychosis, anxiety and depression were major exclusion criteria. Finally, patients that were using psychotropic treatments (neuroleptics, antiepileptics, barbiturates, antidepressants, anxiolytics or lithium) in the 3 months before the study were excluded. A positive drug screen on visit 2 for benzodiazepines or morphine derivatives led to immediate exclusion. Suitable patients were invited to visit 1 during which they were consented and assessed for inclusion. Major exclusion criteria for the study included use of benzodiazepine or non-benzodiazepine hypnotics within the previous 2 weeks or any psychoactive treatment within the previous 3 months, sleep disorders associated with a psychiatric disorder (e.g., depression, anxiety, dementia), sleep disorders secondary to another medical condition (e.g., sleep apnoea, circadian rhythm sleep disorder), use of prohibited concomitant medication or excessive alcohol consumption, any chronic medical condition that was likely to be the cause of the sleep problem (e.g., chronic pain, benign prostatic hypertrophy) or might interfere with the conduct of the study or a lifestyle likely to interfere with sleep patterns (e.g., shift work, jet-lag). Patients considered for entry into a 2 week placebo run-in phase were males and females aged between 55 and 80 years who were suffering from primary insomnia according to the DSM-IV criteria.

After the 2 week placebo run-in period patients returned for visit 2 and their eligibility was re-evaluated. Patients completed the Leeds Sleep Evaluation Questionnaire (LSEQ) for the three consecutive nights by the end of the baseline period. Patients were eligible to be included in the analysis of the pre-defined study primary and secondary endpoints and were randomised, provided they continued to meet major entry criteria and demonstrated persistent sleep quality complaints (QOS) rating of 40 mm and over on the LSEQ at the end of the single-blind placebo run-in period.

Randomisation was achieved by making a call to an Interactive Voice Response System and receiving

an assigned treatment pack number. The random sequences were in the form of randomly permuted blocks of four nested within study site.

The final study assessments at visit 3 were made 3 weeks after randomisation. Samples were taken at each visit for haematology, biochemistry and urinalysis and adverse events were recorded.

The study protocol and relevant documents were approved by Huntingdon Multi-centre Research Ethics Committee, Cambridge, UK. Participants provided written informed consent.

Endpoints

The primary objective of the study was to compare the relative frequencies of occurrence of patients showing concomitant improvements of 10 mm or more on QOS and BFW in the two treatment groups.

Patients completed the LSEQ for the three consecutive nights at the end of each period. A score on the left of each scale of the LSEQ represents deterioration from usual and the right of each scale represents improvement from usual. A mark in the middle of the scale indicates that no change from usual has been reported. A 3 nights' mean score was calculated for each of the two variables (QOS, BFW) recorded on the last 3 nights of the baseline and treatment periods.

The secondary objectives were to compare the effects of PR-melatonin versus placebo at the end of the 3-week treatment period on the following variables:

(1) The 3 nights' mean of individual parameters derived from the LSEQ (namely GTS, QOS, AFS and BFW); recorded on the last 3 nights of the baseline and treatment periods.

(2) The global score from the Pittsburgh Sleep Quality Index (PSQI)[36,37]. The PSQI comprises nine questions relating to the patient's usual sleep habits during the previous 2 weeks; the second and third weeks of active treatment. It addresses possible reasons for trouble in sleeping as well as daytime behaviour. The patient is asked to give the most accurate reply for the majority of days and nights during this period. An algorithm is used to calculate seven component scores and these are added to give a global PSQI score. The PSQI has been recommended as an essential measure for global sleep and insomnia symptoms in recent expert consensus recommendations for a standard set of research assessments in insomnia[36].

(3) The PSQI component scores, Question 2 (sleep latency) and Question 4 (total sleep time) after 3 weeks' double-blind treatment and the change from baseline levels of these parameters. It has been shown that each of the PSQI individual component scores measures a particular aspect of the overall construct. Furthermore, control subjects differ from insomnia patients in all individual components[2]. However, the correlation between individual items and global score ranged from 0.83 (subjective sleep quality) to 0.07 (cough or snore during sleep)[2]. In the evaluation of the drug effects it was therefore interesting to look at each component.

(4) The quality of night (QON) and quality of day (QOD) mean daily scores derived from a sleep diary. Patients were instructed to rate each morning the quality of their sleep (QON) over the previous night; and each evening the overall quality of their day (QOD) on a five-point severity rating scale: 1, very bad; 2, bad; 3, fair; 4, good; 5, very good. The results of the 3 last nights of each period were averaged and the changes in each parameter from run-in placebo to treatment were calculated for each patient.

(5) The Clinical Global Improvement (CGI) score[38] was assessed by the clinician at visit 3 following 3 weeks double-blind treatment, the comparison being to baseline, visit 2.

(6) Quality of life derived from the WHO-5 Wellbeing index[39]. This covers positive mood, vitality and general interests.

Statistical issues

Baseline characteristics are summarised as means and standard deviations for continuous variables and ordinal scores, and counts and percentages for categorical variables.

The results presented in this paper are based on patients who met all major entry criteria, had persistent sleep quality complaints at the end of the placebo run-in period and were randomised and provided outcome data at visit 3. This is referred to as the 'full analysis set'. The primary endpoint was analysed using a chi-square test for association, with the odds ratio and 95% confidence interval for PR-melatonin versus placebo calculated from a logistic regression model with randomised treatment group as the only independent variable. For the primary outcome an additional 'intention to treat' analysis was carried out based on all randomised patients, with those without follow-up at visit 3 assumed not to have achieved a primary outcome. It was estimated that 166 patients per treatment group would be required to detect a difference in response rates in the primary efficacy variable between PR-melatonin groups and the placebo group at a 5% significance level with 80% power, assuming the true response rates were 46% for PR-melatonin and 31% for placebo.

For the secondary endpoints (1), (2), (3) (4) and (6) above, the outcomes at visit 3 were compared between the treatment groups, adjusting for the visit 2 measurement using analysis of covariance. Analysis of covariance was also used for the CGI at visit 3 adjusted for the score at visit 2.

Adverse event data were summarised for all subjects randomised to study medication.

Results

Patient disposition and demographics

The passage of the participants through the study is depicted in the CONSORT diagram in Figure 1. A total of 523 patients attended visit 1 and provided informed consent. Of these, 99 failed to demonstrate persistent sleep quality complaints and 70 did not meet other inclusion/exclusion criteria. The remaining 354 patients were eligible for inclusion in the analysis of the primary and secondary outcomes and were randomised: 177 to receive PR-melatonin and 177 to receive placebo. Eight patients (3.5%) in the PR-melatonin group and 12 patients (5.3%) in the placebo group were withdrawn during the double-blind phase and had no outcome data at visit 3. The full analysis set therefore comprised 334 patients – 169 in the PR-melatonin group and 165 in the placebo group. Patients' baseline characteristics were similar in the two treatment groups (Table 1).

Efficacy evaluation

For the full analysis set, 44 (26.0%) patients in the PR-melatonin group showed an improvement of 10 mm or more on both the QOS and BFW scales of the LSEQ, while 25 (15.2%) of the placebo group demonstrated this improvement ($p = 0.014$), odds ratio (95% CI) for

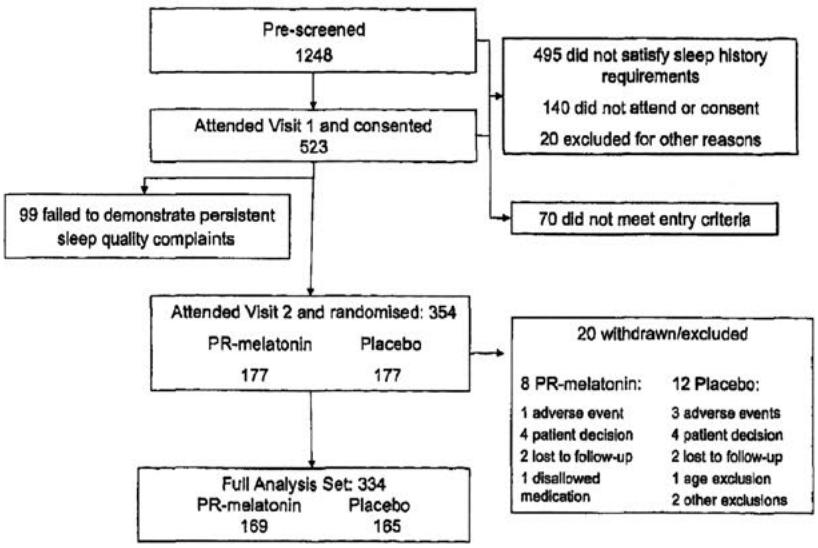

Figure 1. Consort diagram

Table 1. Baseline characteristics of the full analysis set of patients. Numbers are mean (standard deviation) unless stated otherwise indicated

	PR-melatonin ($n = 169$)	Placebo ($n = 165$)	Total ($n = 334$)
Age, years	66.1 (6.4)	65.3 (6.3)	65.7 (6.4)
BMI, kg/m^2	26.5 (4.0)	26.6 (3.4)	26.6 (3.7)
Seated SBP, mmHg	140 (15)	136 (15)	138 (15)
Seated DBP, mmHg	80 (11)	79 (9)	80 (10)
Heart rate, bpm	71 (9)	71 (8)	71 (9)
Sex, *n* (%) male	68 (40)	65 (39)	133 (40)
Current smoker, *n* (%)	20 (12)	21 (13)	41 (12)

BMI = body mass index; bpm = beats per minute; DBP = diastolic blood pressure; SBP = systolic blood pressure

PR-melatonin versus placebo 1.97 (1.14, 3.41). The corresponding result for the intention to treat analysis yielded a 25% improvement rate for PR-melatonin compared to 14% for placebo ($p = 0.011$), odds ratio (95% CI) 2.01 (1.17, 3.46) (Table 2).

The results for the secondary outcomes are given in Table 3. These demonstrate statistically significant improvement in the PR-melatonin group compared to placebo for the individual components of the LSEQ (QOS and BFW) of the primary endpoint when assessed on a continuous scale ($p = 0.014$ and $p = 0.038$, respectively) and for GTS ($p = 0.013$), with a trend to improvement for AFS. There was a trend to improvement for the PSQI total score ($p = 0.081$). There was a significant improvement for sleep quality (Component 1 of the PSQI) ($p = 0.036$). PR-melatonin improved mean sleep latency (Q2 of the PSQI) by 24.3 minutes compared to 12.9 minutes for the placebo. With PR-melatonin, baseline adjusted sleep latency was shorter by 8.8 minutes ($p = 0.028$, 95% CI (1.0, 16.7)mins) over that with placebo. Total sleep time (Q3 of the PSQI) was not significantly improved (0.8 hour improvement on PR-melatonin vs. 0.6 on placebo) on PR-melatonin ($p = 0.14$, 95% CI (–0.2, 0.5) hours).

Similarly, there were trends to improvement for QON and QOD, as measured from the patient diary cards, that just failed to reach statistical significance for QON ($p = 0.054$). These findings were supported by a trend to improvement for the CGI.

A statistically significant better outcome for the PR-melatonin group on the WHO-5 well being index ($p = 0.034$) was demonstrated and 70% of patients who responded to PR-melatonin (i.e. demonstrated concomitant improvements in QOS and BFW)

Table 2. *Primary endpoint: responder rate analysis of PR-melatonin versus placebo in two components of the Leeds Sleep Evaluation Questionnaire: quality of sleep (QOS) and behaviour following wakefulness (BFW)*

	PR-melatonin		Placebo	
	n	%	*n*	%
Improvement of ≥ 10 mm on the Leeds QOS and BFW scales				
Yes	44	(26)	25	(15)
No	124	(73)	139	(84)
Missing	1		1	

Odds-ratio for PR-melatonin versus placebo = 1.97 (95% CI 1.14, 3.41)
Chi-square test = 6.04, $p = 0.014$

Table 3. *Secondary endpoint data: Results presented are mean (standard deviation) of results at visit 2 (V2) and visit 3 (V3) and of the change (V3 – V2) for each outcome and for each treatment group*

	PR-melatonin			Placebo				
	V2	V3	(V3 – V2)	V2	V3	(V3 – V2)	ETE	*p*-value
LSEQ, mm								
QOS	54.5 (9.3)	45.9 (16.0)	–8.6 (16.3)	53.7 (9.7)	49.5 (14.8)	–4.2 (14.7)	–4.0 (–7.2, –0.8)	0.014
BFW	51.6 (10.6)	44.7 (15.3)	–7.0 (14.1)	52.2 (12.1)	48.0 (14.4)	–4.1 (13.9)	–3.0 (–5.9, –0.2)	0.038
GTS	53.0 (7.6)	45.7 (13.8)	–7.3 (13.3)	52.0 (7.5)	48.4 (11.4)	–3.6 (11.3)	–3.3 (–5.8, –0.7)	0.013
AFS	52.0 (8.4)	47.5 (14.2)	–4.5 (13.4)	52.7 (9.6)	49.8 (13.0)	–2.9 (14.3)	–2.0 (–4.8, 0.8)	0.16
PSQI								
Total	10.6 (2.6)	8.1(3.7)	–2.5 (3.3)	10.4 (2.7)	8.6 (3.7)	–1.8 (3.3)	–0.6 (–1.3, 0.1)	0.081
C1 (sleep quality)	2.0 (0.7)	1.4 (0.8)	–0.6 (0.9)	2.0 (0.7)	1.6 (0.8)	–0.4 (0.8)	–0.2 (–0.3, –0.0)	0.036
Q2 (sleep latency, minutes)	65.1 (70.7)	40.8 (54.5)	–24.3 (47.6)	57.9 (65.4)	45.0 (59.0)	–12.9 (39.7)	–8.8 (–16.7, –1.0)	0.028
Diary								
QON	2.6 (0.7)	3.0 (0.8)	0.4 (0.8)	2.6 (0.7)	2.9 (0.9)	0.3 (0.8)	0.2 (–0.0, 0.3)	0.054
QOD	3.1 (0.7)	3.4 (0.6)	0.2 (0.7)	3.3 (0.6)	3.4 (0.7)	0.1 (0.7)	0.1 (–0.0.0.2)	0.21
CGI	N/A	3.0 (1.1)	N/A	N/A	3.2 (1.1)	N/A	–0.2 (–0.4, 0.1)	0.14
WHO-5 index	16.0 (3.4)	17.7 (3.9)	1.7 (3.3)	15.5 (4.5)	16.6 (4.5)	1.1 (4.0)	0.8 (0.1, 1.5)	0.034

The estimated treatment effect (ETE) [PR-melatonin – placebo] (95% confidence interval) and associated *p*-value is also given as estimated from the ANCOVA. The exception is for the Clinical Global Improvement scale (CGI) where there was no equivalent baseline score and adjustment is for the Global Clinical Impression at baseline

AFS = awakening from sleep; BFW = behaviour following wakening; C1 = component 1; CGI = Clinical Global Improvement scale; GTS = getting to sleep; LSEQ = Leeds Sleep Evaluation Questionnaire; PSQI = Pittsburgh Sleep Quality Index; Q2 = question 2; QOD = quality of day; QON = quality of night; QOS = quality of sleep

experienced a clinically relevant improvement in quality of life (equivalent to 3 units or more on the WHO-5 scale) compared to only 24% in non-responders.

Safety evaluation

Adverse events were ascertained for all patients during the study and up to 30 days following completion of the double-blind therapy. In the PR-melatonin group 43 (24%) patients reported 50 events. In the placebo group 37 patients (21%) reported 49 events. The most commonly reported adverse events were 'Nasopharyngitis' and 'Headache or migraine'. 'Nasopharyngitis' was reported by five patients in the PR-melatonin group and by four patients in the placebo group. 'Headache or migraine' accounted for four events in the PR-melatonin patients and 11 in placebo patients. Only one adverse event was reported as severe. This was a case of 'emotional distress due to a bereavement' in a patient in the PR-melatonin group.

Pulse and temperature measurements were similar for the two treatment groups at each visit (data not shown) and there were no differences between the two groups in laboratory measurements.

Discussion

The primary endpoint for the study was the rate of patients responding to the dual outcome of improvement in quality of sleep and morning alertness. The results show that PR-melatonin was superior to placebo and this is supported by improvement in the individual components of the LSEQ. Further, all variables studied were either significantly improved in the PR-melatonin group or tended to benefit. In particular, there were significant improvements in sleep latency as measured by the PSQI and the LSEQ and in quality of life as measured by the WHO-5 index. The difference in the percentage of responders between the PR-melatonin and placebo groups is 11% in the full analysis set, corresponding to a number needed to treat (NNT) value of 9. For comparison, the results of a recent meta-analysis which evaluated the efficacy of hypnotic drugs in the elderly population show that these drugs, which are acknowledged effective hypnotics, have a NNT value of 13[11] in improving sleep quality. No improvement of morning alertness or daytime vigilance has ever been claimed or demonstrated for any of these drugs. The chance of being a responder showing a concomitant improvement in quality of sleep and morning alertness in the PR-melatonin group was almost twice that of the placebo group (odds ratio 1.97). The odds ratio for response in the single outcome of sleep quality with zaleplon 10 mg was reported to be 1.12 after 1 week and 0.86 after 2 weeks of treatment[40]. Indeed, zaleplon is not claimed or demonstrated to have a beneficial effect on quality of sleep. An odds ratio of 2 provides clear evidence that PR-melatonin's effect on the subjective quality of sleep and morning alertness is clinically relevant.

Sleep performs a restorative function for the brain and body, improving the sense of energy and 'wellbeing'[41]. Improvement in sleep should thus improve the patient's wellbeing the following day. This has proven difficult to demonstrate for most hypnotics. We have demonstrated significant improvements in morning alertness as measured by LSEQ and quality of life as measured by WHO-5. This effect on quality of life further demonstrates the clinical relevance of the positive effect on morning alertness. Thus, not only is the percentage of subjects likely to respond to PR-melatonin twice that of placebo, but also that the improvement with PR-melatonin is more likely to result in improved quality of life. In contrast, according to a recent meta-analysis[11] of sedative hypnotics adverse cognitive events were 4.78 times more common ($p < 0.01$) and reports of daytime fatigue were 3.82 times more common ($p < 0.001$) in individuals using a hypnotic compared with placebo.

Significant differences in favour of PR-melatonin were also found in sleep latency as measured by the PSQI. The improvement in sleep latency (8.8 minutes over placebo) is of a magnitude similar to that of zaleplon and ramelteon (8 minutes over placebo)[12,40,42].

PR-melatonin demonstrated a good safety profile with no obvious differences in safety parameters between the active treatment and placebo groups. It is also important to note that unlike benzodiazepine and non-benzodiazepine ('z-drugs') hypnotics, PR-melatonin use is not associated with impairment of psychomotor functions, memory recall and driving skills in this population[43].

Future studies should assess the implications of the improvement in morning alertness on social and occupational functioning and maintenance of these effects.

Conclusions

The results of this study demonstrate that in older patients suffering from non-restorative sleep

the use of PR-melatonin can produce clinically relevant improvements in sleep quality and morning alertness resulting in an improvement in a sense of wellbeing. Improvements in sleep latency were also observed.

The safety and efficacy profile of PR-melatonin, as used in this study, and lack of detrimental effects on memory and vehicle driving shown in other studies, supports its use in the treatment of primary insomnia in patients over the age of 55 years.

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The effect of raising and lowering tryptophan levels on human mood and social behaviour

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Review

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The effect of raising and lowering tryptophan levels on human mood and social behaviour

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Acute tryptophan depletion (ATD) studies indicate that low serotonin can lower mood and also increase aggression, although results vary somewhat between studies with similar participants. Lowering of mood after ATD is related to the susceptibility of the study participants to clinical depression, and some participants show no effect on mood. This indicates that low serotonin can contribute to lowered mood, but cannot—by itself—cause lowered mood, unless other unknown systems interact with serotonin to lower mood. Studies using tryptophan supplementation demonstrate that increased serotonin can decrease quarrelsomeness and increase agreeableness in everyday life. Social interactions that are more agreeable and less quarrelsome are associated with better mood. Thus, serotonin may have direct effects on mood, but may also be able to influence mood through changes in social behaviour. The increased agreeableness and decreased quarrelsomeness resulting from increases in serotonin will help foster congenial relations with others and should help to increase social support. As social support and social isolation have an important relationship with both physical and mental health, more research is needed on the implications of the ability of serotonin to modulate social behaviour for the regulation of mood, and for future physical and mental health.

1. Introduction

The demonstration, shortly after the discovery of antidepressants, that levels of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) are low in the cerebrospinal fluid (CSF) of depressed patients [1] was one of the factors that resulted in the hypothesis that depression might be associated with a deficit in serotonin function, and that antidepressants act by increasing serotonin function [2]. However, subsequent studies found that the difference between CSF 5-HIAA in depressed patients and controls was small, with a large overlap between groups, and that low CSF 5-HIAA was associated with suicide, and in particular violent suicide rather than depression [3]. Suicide tends to be associated with aggression, and a large body of animal research suggests that lowering serotonin causes aggression. Furthermore, low CSF 5-HIAA is associated with aggression in humans [4]. Also, much of the evidence relating low serotonin to mood or aggression in humans was correlational, with no direct evidence on causation. Therefore, in the early 1980s the extent to which serotonin might have a direct control on mood or aggression or both was not clear. The idea behind the acute tryptophan depletion technique was simplistic—temporarily lower serotonin in human brain by lowering tryptophan levels, and study the effects on mood and aggressive responding in a laboratory setting.

2. The acute tryptophan depletion technique

In humans, as in experimental animals, tryptophan hydroxylase is usually only about half saturated with tryptophan [5], so giving tryptophan can increase the rate of serotonin synthesis up to two-fold, whereas decreasing brain tryptophan will lower serotonin synthesis. Research on rats showed that feeding the

animals a meal containing all the essential amino acids except for tryptophan resulted in a decline in tryptophan levels in blood and brain, and, therefore, in brain serotonin [6]. The meal induces protein synthesis and as tryptophan is incorporated into protein its level in blood and tissues declines [7]. Concu *et al.* [8] demonstrated that a 18.7 g amino acid mixture containing no tryptophan caused a 42 per cent decline in human plasma tryptophan. Subsequently, Young *et al.* [9] used a 100 g amino acid mixture and found a much greater lowering of tryptophan levels, which was associated with a modest lowering of mood in males without overt psychopathology. The 100 g mixture lowered human brain serotonin synthesis by more than 85 per cent, according to a method using positron emission tomography and α-[^{11}C]methyl-L-tryptophan as a tracer [10].

3. The effect of acute tryptophan depletion on mood

Numerous studies have looked at the effect of acute tryptophan depletion (ATD) on mood in healthy participants, in healthy participants with a family history of depression, in patients with depression, in newly recovered patients on antidepressants and in recovered depressed patients off antidepressants. A number of reviews have summarized these studies [11–14]. Results are somewhat variable, but some important patterns emerge. In healthy individuals, there is little or no lowering of mood, although results can be quite variable between studies, with some lowering of mood seen more often in women than in men. I suggest a possible cause for this variability later in this review. In healthy participants with a family history of depression, there is a lowering of mood although mood remains within the normal range of mood. In newly recovered depressed patients on antidepressants that act on the serotonergic system, 50 per cent or more of the patients show a temporary reappearance of the depressed mood they experienced before recovery. In recovered depressed patients off antidepressants, only a small percentage of the patients show a marked lowering of mood. In recovered depressed on noradrenergic antidepressants, there is no lowering of mood.

The results of the ATD studies suggest that lowering serotonin synthesis can lower mood in some circumstances, and that the magnitude of the effect tends to be greater in people with a greater susceptibility for depression. Low serotonin by itself is not enough to cause depressed mood, but of course it would be surprising if it did, given that the number of serotonin neurons in the brain is small. Several possible mechanisms might explain the different response in those who exhibit a lowering of mood after ADT and those who do not. First, in those who do not show a lowering of mood, other neuronal systems that modulate mood may be able to buffer the effects of low serotonin, whereas in those who show lowered mood, there may be suboptimal function of those other systems. Second, the lack of effect on mood in some people may be due to adaptive changes. One suggestion is the downregulation of cortical serotonin$_2$ receptors that has been shown in healthy volunteers undergoing ATD [15], and possibly this does not occur in those showing a lowering of mood. Third, lowered mood may be associated with decreased release of serotonin, but serotonin release may not be changed by ADT in those who show no lowering of mood. Decreased synthesis of serotonin over a period of a few hours may not always be enough to deplete serotonin stores sufficiently to lower serotonin release from neurons. Decreased serotonin release presumably implies a decrease in the amount of serotonin stored in each vesicle in the pool of vesicles that is preferentially released on neuronal firing. How this might be influenced by decreased serotonin synthesis is not clear, but it will presumably depend in part on the rate of firing of serotonin neurons, and the extent to which the serotonin that is released is recycled into vesicles. ATD is likely to have a greater effect when there is a greater release of serotonin, which should exhaust stores of serotonin more quickly. In experimental animals, serotonin release increases with increasing arousal [16]. Therefore, a plausible hypothesis is that greater arousal is more likely to lead to a greater effect of ATD on mood. However, arousal has not yet been studied as a factor that might explain some of the variability in response to ATD.

Presumably, if serotonin neurons are firing at a very low rate, any effect of ATD is likely to be small. In experimental animals, serotonin neurons virtually cease activity during rapid-eye-movement (REM) sleep [17]. If the same is true in humans, then people who have a marked lowering of mood after ATD when awake should not show any lowering of mood during dreams, if they undergo ATD before sleep.

A review combing the results from a number of studies investigated variables that might explain why some recovered depressed patients show a marked lowering of mood after ATD, whereas others do not [18]. The reduction in plasma tryptophan levels was 77 ± 15% (mean ± s.d.), and, at this level of depletion, there was no relationship between the degree of plasma tryptophan depletion and the degree of lowering of mood. This suggests that, above a certain threshold in depletion of tryptophan, serotonin synthesis is small enough that its variation is no longer functionally significant in the control of mood. Among the clinical variables, chronicity of depression was the most powerful predictor of relapse in mood, but female gender, exposure to SSRI (selective serotonin reuptake inhibitor) antidepressant and previous serious suicidal thoughts or attempts were also significant predictors. Women may be more susceptible to the effect of ATD, because the procedure lowers serotonin synthesis more in women than in men [10]. The lowering of mood in patients on SSRIs may be related more to the mechanism of action of SSRIs than to the role of serotonin in the aetiology of depression. The other variables are all consistent with the idea that a susceptibility to depression is an important factor in the response to ATD.

4. The effects of acute tryptophan depletion on aggression and irritability

A review published in 2002 included 11 studies looking at the effect of ATD on laboratory measures of aggression or irritable mood [13]. Most of the studies were on men, but two were on women. The majority of studies found increased aggressive responding or irritability, but some did not. An increase in aggression did not seem to be related always to the susceptibility of the participants to aggression. For example, those with intermittent explosive disorder showed no increase in irritability or aggressive outbursts after ATD [19]. An additional four more recent studies all found increased aggression after ATD. This was so for women

performing a laboratory task of aggression during the premenstrual phase [20], and for healthy women whose menstrual cycle was not controlled for, but only in those with higher plasma tryptophan [21]. Males were also tested using laboratory tests of aggression, and ATD increased aggressive responding in children with attention deficit hyperactivity disorder [22], and in healthy controls and patients with intermittent explosive disorder [23].

While the results suggest that ATD can increase aggressive responding under some circumstances, the results are variable. Two factors might explain this. The first is biological. Twelve of the men with high trait aggression, who participated in an ATD study looking at changes on an aggression rating scale [24], also participated in a study investigating 5-HT_{1A} receptor sensitivity using temperature change in response to ipsapirone [25]. Combining the results from the two studies revealed that the six participants who had a marked increase in ratings of aggression after ATD, compared with the six who showed no marked increase in aggression, had a blunted hypothermic response to ipsapirone [26]. This suggests that those who showed increased aggression after ATD have decreased 5-HT_{1A} receptor sensitivity. While differences in the serotonin system may account for some of the differences between studies, the second possible cause is methodological. While there are well-established scales for measuring mood and irritability, aggression is more difficult to study over a short time period in a controlled setting. Two behavioural measures have been used to study aggression after ATD in a laboratory setting: the Taylor aggression task [27] and the point subtraction task [28]. In both tasks the participants are told that they are competing against another person, but in fact they are playing against a computer program. In the Taylor task, participants are told that they are participating in a reaction time task. Each time they win they deliver an electric shock to their 'partner', using any of eight buttons that deliver varying shock intensities from mild to just below the painful threshold. When they lose, they receive a shock of varying intensity in each trial. The measure of aggression is the average intensity of shocks delivered to the 'partner'. In the point subtraction task, players can press one button to obtain points that are later exchanged for cash, or another button that subtracts points from their 'partner'. The measure of aggression is the number of points subtracted from the 'partner', in response to different levels of point subtraction from the participant by the 'partner'. Both tasks depend on deception, and the extent to which the deception might have been successful is not always assessed. The tasks are also artificial, and response may depend on such factors as the exact instructions given, the way in which they are delivered, the fact that participants may want to be seen acting in a positive manner and so on. All these are factors that may differ between studies and may explain part of the variability between ATD aggression studies.

5. Tryptophan supplementation and social behaviour

Aggression is an extreme but important form of social behaviour, and regulation of aggression is a phylogenetically old function of serotonin. Serotonin plays a role in the regulation of aggression in many invertebrates [29], although the effects are not always consistent between species and may depend on context. For example, serotonin modulates aggression in lobsters, but the exact effect depends on factors such as the relative dominance of the animals involved [30]. However, in many situations, low serotonin promotes aggression, whereas increased serotonin promotes prosocial behaviours. For example, increased serotonin levels are involved in the transformation in locusts from being solitary to collecting together in swarms [31].

A wealth of data support the idea that low serotonin promotes aggression in mammals [32]. The idea that low serotonin may contribute to aggression resulted in two small clinical trials comparing tryptophan supplementation with placebo for the treatment of aggression. In the first, 12 aggressive patients with schizophrenia, whose aggression was not treated adequately with antipsychotic drugs, were given tryptophan and placebo for 4 weeks in a cross-over study [33]. Tryptophan, relative to placebo, decreased incidents on the ward requiring intervention. In the second study on 20 aggressive psychiatric inpatients, tryptophan, relative to placebo, decreased the need for antipsychotics and sedatives [34]. Subsequently a number of trials have shown the efficacy of serotonergic antidepressants in the treatment of aggression [35].

Overt aggression is at one end of an axis that is described, among other names, as affiliative–agonistic. The traditional view of serotonin is that it inhibits response to a number of different stimuli, of which provocation, that might lead to aggression, is just one [36,37]. However, increased serotonin function may also promote affiliative behaviours. As mentioned above, serotonin is involved in the swarming of locusts [31], and in vervet monkeys increasing serotonin through a variety of pharmacological interventions will increase the extent to which an animal will approach, sit next to and groom another animal [38,39]. This raises the question of whether serotonin regulates more normal aspects of human social behaviour, along the affiliative–agonistic axis, in addition to its effect on overt aggression.

Research in social psychology over the past few decades has resulted in the development of a method for studying human social behaviour in everyday life along two independent axes, agreeable–quarrelsome and dominant–submissive [40]. A recent review discusses this method, and how it can be applied studying the biological aspects of behaviour [41]. The method uses a technique referred to as ecological momentary assessment (EMA). Participants fill in a one-page form after each social interaction lasting more than 5 min, throughout the day. The form contains a number of statements concerning the participant's behaviour during the interaction. There are groups of statements for each of four behaviours, agreeable, quarrelsome, dominant and submissive. Example of behaviours for each of these categories are: 'I complimented or praised the other person' (agreeable); 'I discredited what someone said' (quarrelsome); 'I assigned someone to a task' (dominant); 'I did not state my own views' (submissive) [40]. The participant checks off each behaviour on the list that she or he exhibited during the interaction. The setting (home, work, other setting) and the interaction partner (romantic partner, friend, acquaintance, supervisor, supervisee, co-worker, parent, other) are also marked on the form. Obviously, the behaviours vary greatly from one interaction to another. However, with increasing measurements, mean values become increasingly stable up

to about 70 measurements. As most participants fill in about 6 forms per day, 12 days of measurement are usually enough. Mean values obtained this way are stable across time for any individual [42].

To test whether increasing serotonin might alter behaviour along the agreeable–quarrelsome axis, Moskowitz *et al.* [43] studied 98 healthy men and women in a double-blind, placebo-controlled study. The treatments, 3 g of tryptophan per day and placebo, both given with meals, were given for 12 days each in counterbalanced order, with a 2 day washout period between treatments. Tryptophan caused a significant decrease in quarrelsome behaviours, but only when placebo was given first. This result was seen in both men and women, in different settings and with different types of interaction partners. There was no effect of tryptophan on agreeableness or on mood. At the end of study, the participants were asked to guess which treatment they were taking during which period. The guesses of the women were slightly better than that expected by chance, but the men did no better than chance. Therefore, the effect of tryptophan was not likely due to unblinding. Nor was the decreased quarrelsomeness due to better mood.

The results of this study raise two main questions. Why was there no effect of tryptophan when it was given first, and why was there no increase in agreeableness with increased serotonin? The original report of the study suggests that the lack of effect of tryptophan when given first could be due to a carryover effect. If the participants were more agreeable to those they frequently conversed with, the more agreeable tone may have been reciprocated, causing a change in tone of the interactions that may have persisted beyond the time tryptophan was given. If this is true, then increasing the washout period should result in a decrease in quarrelsomeness whatever the order of treatments. The lack of an increase in agreeableness after tryptophan may have been due to a ceiling effect. The participants in the study described above were typical of healthy people, in that they exhibited agreeable behaviours much more frequently than quarrelsome behaviours. Therefore, there may have been little scope for an increase in agreeableness. If the lack of an effect on agreeableness was due to a ceiling effect, tryptophan should increase agreeableness in less agreeable people.

The two ideas put forward in the paragraph above were tested in a study by aan het Rot *et al.* [44]. Participants were recruited for this second study from those who responded to an advertisement that included statements such as: Do you have problems with irritability? Do you repeatedly lose control of your temper? Do you get easily agitated? Those who answered the advertisement were screened to rule out the presence of current depression or alcoholism, and had to score at least one standard deviation above the mean on one scale of irritability, and at least half a standard deviation above the mean on another scale of irritability. The scales were the Buss–Durkee Hostility Inventory [45], and an adapted version of the NEO Five-Factor Inventory that included the entire Angry Hostility subscale of the Revised NEO Personality Inventory [46]. A total of 39 men and women were recruited. The design, as in the previous study, was double-blind placebo-controlled crossover using 3 g tryptophan or placebo. The wash out period was increased from 2 to 6 days, and to ensure that the participants started each arm of the study on the same day of the week, the treatment period was increased from 12 to 15 days. Tryptophan decreased quarrelsome behaviours with a medium effect size, and this effect was seen irrespective of which treatment was given first. This supports the idea that, in the first study, quarrelsomeness was not reduced by tryptophan when tryptophan was given first because of the short washout period and a carryover effect from the tryptophan treatment to the placebo treatment. In the study on quarrelsome people, tryptophan increased agreeable behaviours, supporting the idea that the lack of effect on agreeableness in the first study was due to a ceiling effect.

In a more recent study carried out in a laboratory setting, boys with a history of physical aggression, average age 10, were given tryptophan (500 mg; $n = 12$) or placebo ($n = 11$) under double-blind conditions [47]. In one of the tests tryptophan, relative to placebo, resulted in a trend ($p = 0.07$) towards increased prosocial behaviour. The study demonstrated the feasibility of using tryptophan in children, and suggested that in children, as in adults, increasing serotonin may increase prosocial behaviours.

Overall, the results discussed above suggest that altered serotonin can influence behaviour along the entire spectrum of behaviour from agreeable to quarrelsome to overt aggression.

6. Potential implications of the effect of serotonin on social behaviour

(a) Social behaviour and health

The effect of serotonin on social behaviour has implications for both mental and physical health. Hostility, which is the mental state associated with quarrelsomeness, is associated with decreased social support and social isolation [48]. Furthermore, research confirms that positive emotions and agreeableness foster congenial relationships with others [49,50]. This in turn will create the conditions for an increase in social support. The implications of social support or lack of social support for human health are great. As stated in a recent review, 'Social interactions have long-term physiological, psychological, and behavioural consequences. Social isolation is a well recognized, but little understood risk factor and prognostic marker of disease; it can have profoundly detrimental effects on both mental and physical well being, particularly during states of compromised health. In contrast, the health benefits associated with social support (both reduced risk and improved recovery) are evident in a variety of illnesses and injury states' [51, p. 67]. A meta-analysis of the effects of social relationships and mortality concluded that poor social integration is as big a risk factor for mortality as well-established factors such as smoking [52]. Research has also shown associations between health and behaviour along the agreeable–quarrelsome axis. For example, hostility is a risk factor for many disorders such as coronary heart disease (CHD) [53], whereas agreeableness was a significant protective factor against mortality in a sample of older, frail patients [54]. So far, the amount of evidence suggesting that serotonin is an important factor in the relationship between social behaviour and health is very limited. However, a recent study looked at serotonin-related measures, including measuring the levels of the serotonin metabolite 5-hydroxyindoleacetic acid in CSF, and concluded that their results were 'consistent with the hypothesis that increased CNS serotonin is associated with a more favourable

psychosocial/metabolic/cardiovascular profile, whereas decreased CNS serotonin function is associated with a less favourable profile' [55, p. 601]. A review concluded that the serotonin system, as indexed by polymorphisms of the serotonin transporter, 'is an important link between the social environment and health' [56, p. 107].

The extent to which behaviours mediated by serotonin or social networks act to influence health is not known. Social factors may influence factors such as inflammation and stress responses that can impact on health [51]. Alternately, neurotransmitter systems may have separate effects on social behaviour and aspect of metabolism associated with health. For example, in mice, 5-HT_{2C} receptors expressed by pro-opiomelanocortin neurons are physiologically relevant regulators of insulin sensitivity and glucose homeostasis in the liver [57]. Whatever the mediating mechanisms, there is great scope for future studies looking at the effects of tryptophan administration on social behaviour and health-related measures in humans. For example, in irritable people, would supplemental tryptophan help them to expand their social network? Would tryptophan be a useful adjunct in couple counselling with quarrelsome couples? Would tryptophan decrease hostility, alter metabolic profiles and decrease CHD in irritable people at risk for CHD? Would it alter metabolic profiles and decrease CHD in people who are not irritable but are at risk for CHD, thereby suggesting that irritability is not a causative factor in CHD? While the long-term risks of tryptophan administration would have to be balanced carefully against potential benefits, in general, side effects and adverse effects of tryptophan are small [58].

(b) Possible role of social behaviour as a factor mediating the effects of acute tryptophan depletion on mood

As discussed above, there is a degree of variability in the effect on mood of ATD that is not explained. Part of this may be due to a lack of control of social behaviour during ATD studies. A decrease in quarrelsome behaviours is associated with improved mood [44], and presumably an increase in quarrelsomeness would contribute to a lowering of mood. Therefore, the lowering of mood after ATD in healthy people may depend on the extent to which they have the opportunity to have quarrelsome social exchanges with others. One method to test this would be to give tryptophan-deficient amino acid mixtures to groups of participants. If a group of people all had a deficient amino acid mixture together, this would increase the opportunity for quarrelsome interactions. Given that the perceptions that others are less agreeable and more quarrelsome is also associated with lowered mood [59], lowering serotonin in a group of individuals could potentiate any lowering of mood (or create a lowering of mood) both because of the individuals' quarrelsome behaviours and because of their interaction partners' quarrelsomeness. This hypothesis could be tested easily. Of course, the hypothesis does not imply that all changes of mood are mediated by altered social interaction. The dramatic lowering of mood seen in newly recovered depressed patients on antidepressants that act on the serotonergic system could not be due just to changes in social behaviour. Presumably, while serotonin has separate actions on mood and social behaviour, there is a two-way interaction between mood and social behaviour in the same way that there is a two-way interaction between mood and cognition.

(c) Serotonin's effect on social behaviour as a possible explanation for serotonin's effect on more complex behaviours

As discussed above, the regulation of social behaviour is a phylogenetically old function of serotonin. Based on the evidence already reviewed, the role of serotonin in social behaviour is to regulate the tone of interactions along the axis that is often described as agonistic–affiliative in animals, and, in humans, runs from agreeable to quarrelsome to overt aggression. In this way serotonin differs from oxytocin, which seems to be involved in the formation of bonds [60]. The evidence described above suggests that serotonin may alter the tone of the interactions between individuals once some sort of bond has formed. High serotonin resulting in more agreeable behaviours might help strengthen bonds once formed, but there is currently no evidence that serotonin is involved in the initial formation of social bonds. Another interesting difference between serotonin and oxytocin is that, while serotonin exists in species with a very primitive nervous system, oxytocin itself exists only in mammals, although there are related peptides in lower species [61].

Tryptophan can increase agreeable behaviours without the individual being aware of this change, given that the adult participants in the two studies described above could not always guess, better than by chance, when they were on tryptophan and when they were on placebo. This suggests that the drive to be agreeable or quarrelsome that serotonin modulates is not in areas of the brain accessed by consciousness. This conclusion is not surprising given that serotonin modulates social behaviour in species with a very primitive nervous system. Thus, serotonin may be modulating a basic drive to be social. As discussed below, serotonin has also been implicated in more complex aspects of social behaviour and cognition in humans. However, sometimes these more complex changes may be a secondary effect of changes along the agreeable–quarrelsome axis.

The effect of ATD was studied on behaviour in the Prisoner's Dilemma (PD) game [62], which measures social cooperation based on reciprocal altruism. In the iterated PD game used in this study the participant plays against a series of 'partners' (actually a computer program). In the game, both players decide separately whether they want to cooperate or defect. In this study, if both cooperated they received a monetary prize, 20 pence. If one player cooperated and the other defected, the one who defected received 30 pence and the other nothing. If both defected, they received 10 pence. The participants played two games of 20 rounds, with the 'partner' always choosing first in each round whether to cooperate or defect. In the first game, the partner chose to cooperate, and in the second game to defect. In subsequent trials, the partner followed a tit-for-tat strategy. ATD, relative to the control amino acid mixture, resulted in a significant reduction in cooperation. A plausible interpretation of this result is that the increased quarrelsomeness resulting from ATD could have been the basic mechanism that resulted in the decrease in cooperation.

A second study involved ATD and a different game using monetary gain, the ultimatum game [63]. Once again, the

participant played with a 'partner', and once again the 'partner' was really a computer program. However, each trial was with a different partner, and a picture of each partner's face was shown on a monitor. In the ultimatum game, the partner proposed to split a sum of money with the participant. The partner specified the percentage that would go to the participant, and the percentage the partner would keep. The participant could accept the offer, in which case both were paid according to the proposed split of the money, or reject the offer, in which case neither received anything. ATD resulted in the participants rejecting more unfair offers, defined as those in which the participant was offered only 20 per cent of the money. This was interpreted as serotonin modulating behavioural reactions to unfairness. However, an ATD-induced increase in quarrelsomeness is a possible primary factor in mediating these results.

The participant stood to gain the most money by accepting all offers, as each trial was with a new 'partner', thus preventing retaliation and learning. Again, a plausible explanation for the results is that, after ATD, the participant had a more quarrelsome disposition towards the partners, so that punishing the partner became more of a priority than monetary gain.

A recent review suggests that the link between serotonin and prosocial behaviour has been demonstrated convincingly, but considers that the mechanism is not known, given that 'constructs such as cooperation, affiliation, and aggression are complex and are likely composed of several smaller elements' [64, p. 78]. For example, the effect of altered serotonin on cooperation in games such as those described above 'could be motivated by a desire for fairness, a fear of retaliation, or long-term strategic goals, any of which could be modulated by 5-HT' [64, p. 78]. However, while this may be true in humans, such complex cognitions are less likely to play a role in non-human primates, and are certainly not mediators of the effects of serotonin on social behaviour in more primitive species. The hypothesis I am proposing is that the effect of ATD on behaviour in games such as those described above is mediated by a non-conscious drive that alters how people react to others along the agreeable–quarrelsome axis. This is certainly not incompatible with such a drive altering different aspects of cognition, and those cognitions being mediating factors in the resulting behaviour. However, it places the phylogenetically old function of serotonin as the primary factor with altered cognitions as possible secondary mediating factors.

(d) Serotonin, mood and cognition

One open question is the extent to which serotonin influences mood directly or though cognitive effects. The first study demonstrating a lowering of mood after ATD measured one aspect of cognitive performance in addition to measuring mood [9]. The participants performed a proofreading task while listening to low, high and dysphoric distractors. After tryptophan depletion, but not after the control amino acid mixture, the participants performed worse with the dysphoric distractor than with the low or high distractor. The suggestion that ATD may result in enhanced attention to dysphoric stimuli has been confirmed in many other studies. These studies were reviewed by Harmer *et al.* [65] and Harmer [66], who suggest that the lowering of mood after ATD may be secondary to cognitive changes. Harmer [66, p. 1026] also suggests that raising serotonin by giving antidepressants causes 'positive re-biasing of automatic processing' and that this may explain how antidepressants work. The idea that the lowering of mood after ATD is secondary to cognitive changes is supported by two studies on recovered depressed patients [67,68]. In both studies, a low-dose tryptophan-depleting amino acid mixture had no effect on mood, but altered cognitive processing of emotional stimuli in the direction seen in depressed patients. However, in my opinion, the direction of causality remains an open question. The lack of change in mood with changes in cognition in the two studies mentioned above may have been due to the greater sensitivity of the cognitive measures to detect change. Furthermore, the interaction between mood and cognition may be in both directions, with altered mood influencing cognitive processing of emotional stimuli and cognitive processing of emotional stimuli influencing emotion.

(e) Serotonin, social behaviour and social cognition

If the effect of serotonin on the cognitive appraisal of emotional stimuli can contribute to serotonin's role in regulating mood, can alterations in social cognition influence serotonin's effect on social behaviour? A number of studies have looked at the effect of ATD on the response to faces with different expressions. Results have been rather variable. In healthy participants, ATD impaired recognition of fearful faces only in women [69], but in another study impaired recognition occurred only in carriers of the short allele of the serotonin transporter, irrespective of gender [70]. A different specificity has also been seen in healthy participants, with ATD decreasing recognition of facial expressions depicting anger, disgust and surprise, but not fear and sadness [71]. Furthermore, in two other studies on healthy participants ATD failed to alter recognition of emotion in faces [72,73]. One study found different results in healthy women and those with a history of depression. There was no effect on recognition of fearful faces, but ATD increased recognition of happiness in women who had never been depressed, and decreased it in recovered depressed women [67]. Raising tryptophan with alpha-lactalbumin, a protein with high tryptophan levels, enhanced the recognition of both fearful and happy faces in healthy participants [74]. However, in another study, in which healthy people received tryptophan supplements for two weeks, tryptophan increased the recognition of happy facial expressions but decreased the recognition of disgusted facial expressions in women, without altering the responses of men [75].

Investigations on how altered tryptophan levels influence the recognition of different facial expressions are relatively easy to perform, but have not produced particularly consistent or interesting results. This may not be a fruitful topic to pursue until there is some understanding of the sources of variability. Furthermore, the implications that serotonin-induced alterations in the recognition of facial expressions have for actual social functioning in everyday life are not clear.

Two recent studies looked at different aspects of social cognition after ATD in healthy participants. In the first, the women rated photographs of happy faces as less attractive, and were less aroused by angry faces, after ATD [76]. In the second study men and women looked at photographs of heterosexual couples with neutral expressions. Half of the

couples were touching and half were standing slightly apart. ATD reduced the participant's assessment of the degree of intimacy and romance of the couples [77]. After ATD, in only the women, the touching couples were rated as more able to resolve conflicts.

The results of the last two studies mentioned, on ATD and social cognition, are in part consistent with the results of the studies on the effect of altered tryptophan levels on the behavioural axis from agreeableness to quarrelsomeness to overt aggression. As discussed in §§4 and 5, lowered serotonin makes people less agreeable and more aggressive. Rating happy faces as less attractive, when tryptophan and serotonin levels are low [76], could be considered a secondary effect of a less agreeable and more irritable attitude towards others owing to low serotonin. Similarly, feelings of lower agreeableness and greater irritability towards others might well lead to similar attributions to others, and results in ratings of couples as less intimate and romantic [77].

The fact, discussed in §5, that serotonin influences behaviour along the agonistic–affiliative axis even in animals with a very primitive nervous system suggests that serotonin-mediated changes in social behaviour are not mediated by changes in social cognition. Nonetheless, the possibility remains that there is a two-way interaction between each of the following pairs: mood and social behaviour, mood and cognition, social behaviour and cognition. Thus, the exact mechanisms whereby serotonin influences mood, social behaviour and cognition should remain a topic of research for many decades to come.

7. Conclusion

Serotonin neurons project from the brainstem, innervating all areas of the brain diffusely. In keeping with what might be expected from the neuroanatomy, serotonin modulates many aspects of brain function. A recent review on the biology of serotonin lists some of those functions, but while aggression is included in the list, regulation of prosocial behaviours is not [78]. The discovery that increasing serotonin can increase prosocial behaviours is relatively recent and so far not much attention has been paid to this topic. This is in spite of the large body of data relating aspects of social behaviour to both physical and mental health, and the fact that most psychiatric disorders are associated in some way with disruptions in the normal patterns of social interactions. Much of the work on social neuroscience related to serotonin has focused more on social cognition and neuroanatomical considerations than actual human social behaviour. When behaviour has been studied it is usually in a laboratory rather than in everyday life [64,79,80]. While research on social cognition and the neuroanatomical basis of social cognition are certainly important, a more balanced approach, with more studies looking at everyday social behaviour, would probably help the field to advance more rapidly.

While the measurement of social behaviour in everyday life using EMA has become as established technique in social psychology its use in more biologically oriented studies is still rare. An important take-home message of this review is that EMA methodology is simple to perform and can produce interesting results when combined with well-established strategies that influence neurotransmitter function.

The studies described in this review suggest that while alterations in serotonin do not always alter mood in humans, effects on social behaviour along the agreeable–quarrelsome axis can occur even in the absence of any change in mood. The effects of serotonin on mood and social behaviour are presumably mediated by different neuroanatomical systems. However, the tone of social interactions can have effects on mood, and mood can have effects on social behaviour. For example, irritability is a common symptom in clinical depression. The two-way interaction between mood and social behaviour could be a fruitful area of research that may reveal more about how both are regulated.

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Review

Effects of tryptophan loading on human cognition, mood, and sleep

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ABSTRACT

Modulating central serotonergic function by acute tryptophan depletion (ATD) has provided the fundamental insights into which cognitive functions are influenced by serotonin. It may be expected that serotonergic stimulation by tryptophan (Trp) loading could evoke beneficial behavioural changes that mirror those of ATD. The current review examines the evidence for such effects, notably those on cognition, mood and sleep. Reports vary considerably across different cognitive domains, study designs, and populations. It is hypothesised that the effects of Trp loading on performance may be dependent on the initial state of the serotonergic system of the subject. Memory improvements following Trp loading have generally been shown in clinical and sub-clinical populations where initial serotonergic disturbances are known. Similarly, Trp loading appears to be most effective for improving mood in vulnerable subjects, and improves sleep in adults with some sleep disturbances. Research has consistently shown Trp loading impairs psychomotor and reaction time performance, however, this is likely to be attributed to its mild sedative effects.

Contents

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1. Introduction

1.1. Serotonin

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter, responsible for neurochemical signal transduction between neurons. The neurons of the raphe nuclei are the principal source of 5-HT release in the brain. Although there are relatively few serotonergic neurons in the brain, these neurons innervate widespread areas of the brain, such as the forebrain, hippocampus, cerebellum and spinal cord (Haider et al., 2006), and are considered important in the modulation of several essential behavioural and physiological functions, such as mood, sleep and wakefulness, cognition, sexual behaviour, appetite, aggression, impulsivity, neurodevelopment, circadian rhythms, body temperature, and neuroendocrine function (Jacob and Fornal, 1995).

Reduced 5-HT function is recognised as a contributing factor in affective disorders, such as depression, bipolar disorder, anxiety disorders, and obsessive compulsive disorder (Davis et al., 2002). Medicinal drugs that stimulate 5-HT activity throughout the brain, predominantly selective serotonin reuptake inhibitors (SSRIs) and various tricyclic antidepressants (TCAs), are effective in ameliorating the symptoms of these disorders. Furthermore, cognitive deficits that frequently accompany these disorders have also been shown to improve with pro-serotonergic pharmacological therapies (Schmitt et al., 2006).

1.2. Modulation of serotonergic function by dietary tryptophan

For a limited number of neurotransmitters, such as serotonin, dietary precursors can influence the rate of synthesis and function of the neurotransmitters. The synthesis of 5-HT is dependent on the brain availability of its precursor, the amino acid L-tryptophan (Trp). The amino acid is converted via a short metabolic pathway consisting of the two enzymes tryptophan hydroxylase and amino acid decarboxylase to serotonin. Tryptophan hydroxylase, the rate-limiting enzyme on the pathway from Trp to 5-HT, is not normally saturated with Trp. Thus, increasing Trp levels can increase 5-HT synthesis as much as twofold following 3 g pure Trp load (Young, 1996; Young and Gauthier, 1981), which is significant to modulate mood, cognition and behaviour (Attenburrow et al., 2003; Cunliffe et al., 1998; Marsh et al., 2002; Markus et al., 2008; Yuwiler et al., 1981). While decreasing Trp availability can cause a considerable decline in 5-HT synthesis and turnover (Nishizawa et al., 1997).

Trp is transported across the blood–brain barrier by a specific active transport system, which also transports a number of other large neutral amino acids (LNAAs: leucine, isoleucine, tyrosine, phenylalanine, and valine) into the brain. As a result, Trp competes with these other LNAAs for active transport sites. Therefore, the uptake of Trp does not depend on total concentration of plasma Trp alone, but primarily on the plasma ratio of Trp to the sum of other LNAAs (Trp–LNAA ratio) (Fernstrom and Wurtman, 1972). An increase in the plasma Trp–LNAA ratio can result in an increased uptake of Trp in the brain. Thus, the relative amount of LNAAs in the diet has a major impact on the levels of Trp in the brain. A diet high in Trp, but with a large amount of LNAAs, will not result in higher brain Trp levels, and may even decrease Trp uptake into the brain. An intervention rich in Trp relative to other LNAAs is needed in order to boost uptake of Trp, and consequently serotonin production, in the brain.

1.3. Increasing brain tryptophan

Increases in plasma Trp–LNAA ratio can be achieved by giving Trp the advantage in competition for access to the brain (Fernstrom and Wurtman, 1971), either through the intake of pure Trp (Markus et al., 2008; Sobczak et al., 2002, 2003), increasing carbohydrate intake (Fernstrom and Wurtman, 1971, 1972; Markus et al., 1998), or through consumption of tryptophan-rich α-lactalbumin protein (Markus et al., 2000, 2002). Throughout the review these methods of increasing brain Trp will be referred to as Trp loading.

α-Lactalbumin is a whey-derived protein with the highest Trp content and highest Trp–LNAA ratio of all food protein sources (Heine et al., 1996). α-Lactalbumin has been shown to increase plasma Trp–LNAA ratio up to 130% (Booij et al., 2006; Markus et al., 2000, 2005; Merens et al., 2005; Scrutton et al., 2007). Ingestion of normal protein, which also contains Trp, decreases brain Trp. This is because Trp is the least abundant amino acid in protein, and therefore the increase in plasma Trp is less than the increase in plasma LNAAs that compete with Trp for transport into the brain. Carbohydrates, on the other hand, which contain no Trp, increase brain Trp and 5-HT, due to a carbohydrate-induced rise in glucose, which triggers insulin secretion. Insulin stimulates the uptake of LNAA in skeletal muscles, with the exception of Trp (Fernstrom and Wurtman, 1971). Consequently, LNAA plasma levels fall, competition for the transport of Trp decreases, and brain levels of Trp and 5-HT increase. However, a carbohydrate rich/protein poor (CR-PP) diet increases plasma Trp–LNAA ratio (20–25%; Markus et al., 1998, 1999) considerably less than α-lactalbumin.

For the purpose of the present review, the effects of Trp loading in humans (clinical populations, vulnerable volunteers, and healthy volunteers) on cognitive function, mood, and sleep are considered, to explore the potential benefits of serotonergic stimulation through Trp loading. As previously mentioned, brain Trp can be increased through intake of either pure Trp, a carbohydrate rich/protein poor diet, or α-lactalbumin. Therefore, studies employing these Trp loading manipulations are discussed.

2. Methods

2.1. Selection procedures

An extensive medline search was performed from 1966 to January 2009 using the search terms: "tryptophan", "α-lactalbumin", "cognition", "memory", "attention", "vigilance", "executive function", "emotional processing", "mood", and "sleep". The search was limited to human studies only. The bibliographies of the references identified were searched for additional papers that met the following inclusion criteria: (1) original papers written in English appearing in a peer-reviewed journal, (2) include a comparison condition (Trp loading versus placebo or ATD), (3) specify sample characteristics for the participants, (4) include cognitive, mood and/or sleep assessments. All studies that assessed Trp loading on cognition, mood and/or sleep meeting the above criteria were included in the review.

2.2. Methodological remarks

Forty-three studies were identified for inclusion in the review. Sixteen studies assessed the effects of Trp loading on cognitive functioning. Thirteen articles assessed the effects of Trp loading on mood, and 21 studies assessed the effects of Trp loading on sleep measures. Twenty-three studies included only healthy subjects, 8 studies assessed healthy volunteers and vulnerable populations (i.e. mental illness, participants with family history of mental illness, stress-prone, premenstrual women, individuals with sleep disturbances), and 12 studies included only vulnerable populations. Thirty-one studies increased brain Trp with pure Trp. Nine studies increased brain Trp with α-lactalbumin, and three studies increased brain Trp with a carbohydrate rich/protein poor diet. The Trp loading studies included in the review are summarized in Tables 1–3.

Table 1
Summary of cognitive findings.

Study	Subjects	Dose	Increase from baseline in plasma Trp–LNAA ratio	Intervention type	Measures	Results
Luciana et al. (2001)	19 healthy adults	10.3 g L-Trp	Not available; total plasma Trp levels increased by tenfold from 53.22 to 551.4 μmol/L	Acute, repeated-measures, double-blind design (no placebo – Trp loading or depletion)	Spatial working memory; affective working memory; verbal fluency; sustained attention and short-term memory span; motor speed and accuracy	Decrements in working memory for verbal and affective stimuli relative to Trp depletion; decrements in motor performance; improved sustained attention; no effect on mood
Morgan et al. (2007)	8 healthy adults	30 mg/kg body weight L-Trp	Not available	Acute, repeated-measures, double-blind, placebo-controlled design	Executive function	No effects found
Attenburrow et al. (2003)	24 healthy females	Nutritionally sourced pure Trp (1.8 g Trp)	Not available	Acute, double-blind, parallel group, placebo-controlled design	Emotional processing	Trp enhanced perception of fearful and happy facial expressions relative to placebo
Murphy et al. (2006)	38 healthy adults	14 days Trp intervention of 1 g three times a day	Not available	Sub-chronic, double-blind, parallel group, placebo-controlled design	Emotional processing (facial expression recognition, emotion-potentiated startle, attentional probe, emotional categorisation and memory); mood	Trp increased the recognition of happiness and decreased recognition of disgust in females; Trp decreased attentional vigilance towards negative stimuli and reduced baseline emotional startle response in females; no effects on mood
Scrutton et al. (2007) (10)	28 healthy females	40 g α-lactalbumin-rich drink (total Trp level 1.8 g)	80%	Acute, double-blind, parallel group, placebo-controlled design	Emotional processing; mood	No effects found; increase in subjective rating of nausea 150 min after α-lactalbumin ingestion
Winokur et al. (1986)	11 healthy males	5, 7.5 and 10 g L-tryptophan; saline administered intravenously		Acute, repeated-measures, double-blind, placebo-controlled design	Psychomotor performance; subjective ratings of fatigue	L-Trp produced a dose-dependent impairment in motor performance; L-Trp increased mental and physical sedation, but did not alter subjective ratings of tranquilization
Cunliffe et al. (1998) (11)	6 healthy adults	30 mg/kg body weight L-Trp	41% (peak)	Acute, repeated-measures, double-blind, placebo-controlled design	Subjective measure of fatigue (VAS), objective measure of central fatigue (Flicker Fusion Frequency task), simple reaction time, peripheral fatigue (grip strength and wrist ergometry)	Trp decreased performance on the Flicker Fusion Frequency task (measure central fatigue); Trp slowed reaction time performance; Trp increased subjective ratings of fatigue
Dougherty et al. (2007)	18 healthy adults	5.15 g Trp	127%	Acute, repeated-measures, double-blind, placebo-controlled design	Sustained attention, impulsivity	Trp loading produced fewer errors of omission during a vigilance task in the Trp loading condition relative to the Trp depletion condition
Markus et al. (2005) (7)	Healthy subjects with (n = 14) or without (n = 14) mild sleep complaints	40 g (2 × 20 g) tryptophan-enriched α-lactalbumin protein (4.8 g/100 g Trp)	130% increase from placebo	Repeated-measures, double-blind, placebo-controlled design	Subjective sleepiness; vigilance; EEG (ERPs)	α-Lactalbumin reduced sleepiness in the morning, improved morning alertness and attention (P300 ERP) in both groups; α-lactalbumin improved next morning vigilance performance in subjects with mild sleep complaints
Markus et al. (2002) (4)	23 high stress-vulnerable and 29 low stress-vulnerable subjects	40 g α-lactalbumin-rich drink (2 × 20 g containing 12.32 g/kg Trp; Trp/ΣLNAA ratio of 8.7%)	Not available; Trp–LNAA ratio was 43% greater after α-lactalbumin diet than after control diet	Acute, repeated-measures (diet), between-subject, double-blind design	Memory scanning	Improved memory scanning in high stress-vulnerable subjects compared to placebo; no effect of Trp in low stress-vulnerable subjects
Markus et al. (1999)	22 high stress-vulnerable and 21 low stress-vulnerable subjects (aged 19–26 yrs)	Carbohydrate rich/protein poor diet versus protein rich/carbohydrate poor diet	Not available	Acute, repeated-measures (diet), between-subject, double-blind design	Memory scanning	Improved memory scanning after experimental stress only in high-stress volunteers with carbohydrate rich/protein poor diet

Table 1 (*Continued*)

Study	Subjects	Dose	Increase from baseline in plasma Trp–LNAA ratio	Intervention type	Measures	Results
Markus et al. (1998) (5)	24 high stress-Vulnerable and 24 low stress-vulnerable subjects (aged 18–25 yrs)	Carbohydrate rich/protein poor diet versus protein rich/carbohydrate poor diet	Not available; Trp–LNAA ratio increased 48% in the carbohydrate rich/protein poor diet from the protein rich/carbohydrate poor diet	Acute, repeated-measures (diet), between-subject, double-blind design	Memory scanning; mood	Failed to demonstrate memory scanning improvements following a carbohydrate rich/protein poor diet in stress-prone subjects following experimental stress although basic reaction speed was increased in pooled groups; in high stress subjects a carbohydrate rich/protein poor diet prevented deterioration of feelings of depression and vigour during stress manifested after protein rich/carbohydrate poor diet
Schmitt et al. (2005) (1)	16 Females with premenstrual symptoms	40 g α-lactalbumin-rich drink (2 × 20 g containing 12.32 g/kg Trp; Trp/ΣLNAA ratio of 8.7%)	6–25%	Acute, repeated-measures, double-blind, placebo-controlled design	Short- and long-term memory; executive function	Improved long-term memory for abstract figures, but not for words; no effect on executive function
Sayegh et al. (1995) (2)	24 Premenstrual females with PMS	Carbohydrate rich drink	29%	Acute, repeated-measures, double-blind, placebo-controlled design	Verbal recognition memory; verbal retrieval; mood	Improved verbal recognition memory; decreased self-report measures of depression, anger, confusion
Sobczak et al. (2003, 2002) (9)	30 healthy first-degree relatives of bipolar patients and 15 matched controls	7 g Tryptophan intravenous	Trp–LNAA ratio increased 1500% as baseline ratio was 0.11 and 105 min after Trp ratio was 1.835	Acute, between-group, repeated-measures, double-blind, placebo-controlled design	Planning; sustained attention; focused attention; divided attention; response inhibition; psychomotor performance; short- and long-term memory; verbal fluency; mood	Trp impaired long-term memory retrieval and storage and decreased movement time on a psychomotor task in both groups; Trp impaired focused attention and planning in subjects with first-degree relative with bipolar disorder; Trp increased feelings of anger, depression, fatigue, tension, and decreased feelings of vigour and feelings of alertness in both groups relative to placebo
Booij et al. (2006) (3)	23 recovered depressed patients (21 F and 2 M) and 20 controls (17 F and 3 M)	40 g α-lactalbumin-rich drink (2 × 20 g containing12.32 g/kg Trp; Trp/ΣLNAA ratio of 8.7%)	21%	Acute, repeated-measures, double-blind, placebo-controlled design	Short- and long-term memory; focused attention and response inhibition; motor speed; executive function; mood	Improved abstract visual memory, in both recovered depressed patients and healthy controls; slowed motor response in both groups; no effect on mood or other cognitive functions

Table 2
Summary of mood findings.

Study	Subjects	Dose	Increase from baseline in plasma Trp–LNAA ratio	Intervention type	Measures	Results
Luciana et al. (2001)	19 healthy adults	10.3 g L-Trp	Not available; total plasma Trp levels increased by tenfold from 53.22 to 551.4 μmol/L	Acute, repeated-measures, double-blind design (no placebo – Trp loading or depletion)	Mood; spatial working memory; affective working memory; verbal fluency; short-term attention and memory span; motor speed and accuracy	No effect on mood; decrements in working memory for verbal and affective stimuli relative to rp depletion; decrements in motor performance; improved sustained attention
Markus et al. (2008) (8)	18 healthy subjects	15 g α-lactalbumin whey-protein with 0.8 g Trp and 9.4 g LNAA (Trp–LNAA ratio 0.1); hydrolysed protein (Pep2Balance) with 0.8 g Trp and 4 g LNAA (Trp–LNAA ratio 1.1); 0.8 g pure Trp; and 1.2 g synthetic peptide containing 0.8 g Trp; 20 g casein protein with 0.4 g Trp and 10 g LNAA (Trp–LNAA ratio 0.04)	α-Lactalbumin–67% Pep2balance–255% pure Trp–191% synthetic peptide–263%	Acute, repeated-measures, double-blind, placebo-controlled design	Mood and plasma amino acids	Hydrolysed protein (Pep2Balance™) produced faster and greater increases in plasma Trp–LNAA ratio compared to α-lactalbumin and pure Trp; Mood improved 60 min after consumption hydrolysed protein and pure Trp. Most profound and durable mood enhancing effects observed 210 min after intake of hydrolysed protein. No mood effects observed with α-lactalbumin or synthetic Trp peptide
Murphy et al. (2006)	38 healthy adults	14 days Trp intervention of 1 g 3 times a day	Not available	Sub-chronic, double-blind, parallel group, placebo-controlled design	Mood; emotional processing (facial expression recognition, emotion-potentiated startle, attentional probe, emotional categorisation and memory)	No effects on mood; Trp increased the recognition of happiness and decreased recognition of disgust in females; Trp decreased attentional vigilance towards negative stimuli and reduced baseline emotional startle response in females
Scrutton et al. (2007) (10)	28 healthy females	40 g α-lactalbumin-rich drink (total Trp level 1.8 g)	80%	Acute, double-blind, parallel group, placebo-controlled design	Mood; emotional processing;	No effects found; increase in subjective rating of nausea 150 min after α-lactalbumin ingestion
Beulens et al. (2004) (12)	18 healthy males	12 g α-lactalbumin-enriched whey-protein (Trp–LNAA ratio of 0.16) with carbohydrates versus carbohydrates only	16%	Acute, repeated-measures, double-blind, placebo-controlled design	Mood	No effects
Yuwiler et al. (1981)	5 healthy males	50 mg/kg L-Trp acute; 100 mg/kg L-Trp acute; 50 mg/kg L-Trp sub-chronic for 14 days	Not available	Repeated-measures, double-blind design	Mood and alertness	No effect of Trp on valence of mood; Trp increased lethargy and drowsiness within 30 min after 50 mg/kg L-Trp ingestion
Markus et al. (1998) (5)	24 high stress-Vulnerable and 24 low stress-vulnerable subjects (aged 18–25 yrs)	Carbohydrate rich/protein poor diet versus protein rich/carbohydrate poor diet	Not available; Trp–LNAA ratio increased 48% in the carbohydrate rich/protein poor diet from the protein rich/carbohydrate poor diet	Acute, repeated-measures (diet), between-subject, double-blind design	Mood; memory scanning	In high stress subjects a carbohydrate rich/protein poor diet prevented deterioration of feelings of depression and vigour during stress manifested after protein rich/carbohydrate poor diet; failed to demonstrate memory scanning improvements following a carbohydrate rich/protein poor diet in stress-prone subjects following experimental stress

Table 2 (*Continued*)

Study	Subjects	Dose	Increase from baseline in plasma Trp–LNAA ratio	Intervention type	Measures	Results
Markus et al. (2000) (6)	29 high stress-Vulnerable and 29 low stress-vulnerable subjects	40 g α-lactalbumin-rich drink (2 × 20 g containing12.32 g/kg Trp; Trp/ΣLNAA ratio of 8.7%)	Not available; Trp–LNAA ratio increased 48% after α-lactalbumin diet from the control diet	Acute, repeated-measures (diet), between-subject, double-blind, placebo-controlled design	Mood	α-Lactalbumin-rich diet reduced depressive symptoms in stress-vulnerable subjects after experimental stress
Sayegh et al. (1995) (2)	24 Premenstrual females with PMS	Carbohydrate rich drink	29%	Acute, repeated-measures, double-blind, placebo-controlled design	Mood; verbal recognition; memory; verbal retrieval	Decreased self-report measures of depression, anger, confusion; Improved verbal recognition memory
Steinberg et al. (1999)	80 females with premenstrual dysphoric disorder	6 g L-Trp (given as 2 g three times a day) for 17 days	Not available	Sub-chronic, between-subject, randomised, double-blind, placebo-controlled design	Mood	L-Trp more effective than placebo in controlling extreme mood swings, dysphoria, irritability, and tension
Sobczak et al. (2003, 2002) (9)	30 healthy first-degree relatives of bipolar patients and 15 matched controls	7 g Tryptophan intravenous	Trp–LNAA ratio increased 1500% as baseline ratio was 0.11 and 105 min after Trp ratio was 1.835	Acute, between-group, repeated-measures, double-blind, placebo-controlled design	Mood; planning; sustained attention; focused attention; divided attention; response inhibition; psychomotor performance; short- and long-term memory; verbal fluency	Trp increased feelings of anger, depression, fatigue, tension, and decreased feelings of vigour and feelings of alertness in both groups relative to placebo; Trp impaired long-term memory retrieval and storage and decreased movement time on a psychomotor task in both groups; Trp impaired focused attention and planning in subjects with first-degree relative with bipolar disorder
Merens et al. (2005) (13)	23 recovered depressed adults and 20 healthy adults	40 g α-lactalbumin-rich drink (2 × 20 g containing12.32 g/kg Trp; Trp/ΣLNAA ratio of 8.7%)	21%	Acute, repeated-measures, between-subject, double-blind, placebo-controlled design	Mood	α-Lactalbumin had no effect in improving mood after experimental stress
Booij et al. (2006) (3)	23 recovered depressed patients (21 F and 2 M) and 20 controls (17 F and 3 M)	40 g α-lactalbumin-rich drink (2 × 20 g containing 12.32 g/kg Trp; Trp/ΣLNAA ratio of 8.7%)	21%	Acute, repeated-measures, double-blind, placebo-controlled design	Mood; short- and long-term memory; focused attention and response inhibition; motor speed; executive function	No effect on mood; improved abstract visual memory in both recovered depressed patients and healthy controls; slowed motor response in both groups

Table 3
Summary of sleep findings.

Study	Subjects	Dose	Increase from baseline in plasma Trp–LNAA ratio	Intervention type	Measures	Results
Wyatt et al. (1970) (1)	Healthy adults	7.5 g Trp	Not available		Sleep parameters	Trp decreased REM sleep and increased non-REM sleep
Nicholson and Stone (1979) (2)	6 healthy males	2, 4, and 6 g L-Trp	Not available		Sleep parameters	4 g L-Trp increased percentage of REM sleep and duration of stage 3 daytime sleep; no modulations to sleep found during nighttime sleep following 2, 4, and 6 g L-Trp
Hartmann et al. (1974) (3)	Healthy adults	1–15 g Trp (dose–response study)	Not available		Sleep parameters	1–15 g Trp decreased sleep latency, but only in doses above 5 g modulations to sleep stages observed, specifically, decreases in desynchronised sleep % increases in slow-wave sleep
Leatherwood and Pollet (1984) (19)	Healthy adults	500 mg Trp (5 nights)		Sub-chronic, double-blind, repeated-measures, placebo-controlled design	Sleep parameters	Trp decreased sleep latency, sleep depth increased, increased sleepiness, and calming effects were reported; younger females more sensitive to the sedating effects of Trp than any other group
George et al. (1989) (6)	10 healthy adults	1.2 or 2.4 g L-Trp	Not available	Acute, repeated-measures, double-blind, placebo-controlled design	Objective (sleep latency) and subjective measures of sleepiness and their relationship to blood L-Trp levels	Both L-Trp doses reduced sleep latency at 1 h, with reduction persisting at 2 h for 2.4 L-Trp only; positive correlation between subjective and objective sleepiness measures for 2.4 g dose only; correlation between blood Trp and sleep latency found at 0, 60 min and 120 min for both doses
Spinweber et al. (1983) (8)	20 healthy adults	4 g L-Trp	Not available; however plasma total Trp levels increased 260% and free Trp 343% relative to placebo	Acute, repeated-measures, double-blind, placebo-controlled design	Waking EEG and daytime sleep	L-Trp reduced sleep latency without altering nap sleep stages; during waking EEG L-Trp increased alpha latency, theta latency, theta amplitude, and decrease alpha frequency; conclusion L-Trp effective sleep hypnotic
Thorleifsdottir et al. (1989) (15)	20 healthy adults	2 g L-Trp	Not available	Acute, repeated-measures, double-blind, placebo-controlled design	Daytime arousal measured with EEG	Trp increased drowsiness reflected by increases in theta amplitude and decreases in alpha amplitude; subjective ratings of sleepiness increased with Trp; psychomotor performance not affected with Trp
Chauffard-Alboucq et al. (1991) (20)	9 healthy females	500 mg and 1 g L-Trp combined with a carbohydrate load	200% (500 mg) 300% (1 g)	Acute, double-blind, repeated-measures, placebo-controlled design	Perceived sleepiness; sedative effects	Both Trp doses increased sleepiness and sedative effects relative to placebo. Effect was observed when plasma Trp–LNAA ratio increased 200% (500 mg) and 300% (1 g) from baseline, peaking 90 minu after Trp administration. Peak in perceived sleepiness found 90 min after Trp consumption
Körner et al. (1986) (7)	10 adults with sleep disturbances	5 g L-Trp	Not available	Acute, repeated-measures, double-blind, placebo-controlled design	Sleep parameters	Trp decreased sleep latency, improved sleep period time and total sleep time; no effect on slow-wave sleep
Brown et al. (1979) (5)	18 females with laboratory sleep-onset latency greater than 20 min	1 g or 3 g L-Trp for 10 nights	Not available	Sub-chronic, repeated-measures, double-blind, placebo-controlled design	Sleep parameters	Trp had no effect on amount of REM, slow-wave sleep and wakefulness relative to placebo; reductions in sleep-onset latency with 3 g Trp
Hartman and Spinweber (1979) (9)	15 mild insomniacs	250 mg, 500 mg and 1 g L-Trp	Not available	Acute, repeated-measures, double-blind, placebo-controlled design	Sleep parameters	1 g Trp reduced sleep latency, whereas the lower Trp doses produced trends in same direction; Stage IV sleep increased with 250 mg Trp
Hudson et al. (2005) (10)	57 chronic insomniacs	25 mg deoiled butternut squash seed meal (contains 22 mg Trp/1 g protein) mixed with 25 mg dextrose; 250 mg pharmaceutical Trp mixed with 25 mg dextrose and 25 g rolled oats; rolled oats (placebo)	Not available	Acute, between-subjects, double-blind, placebo-controlled design	Objective (total sleep time, sleep efficiency, total wake time, time awake-middle of the night) and subjective (overall perceived quality) measures of sleep	Protein source Trp and pharmaceutical grade Trp improved subjective and objective sleep measures
Hartman et al. (1983) (11)	96 chronic insomniacs	1 g L-Trp; 100 mg secobarbital; 30 mg flurazepam; placebo for 7 days	Not available	Sub-chronic, between-subjects, double-blind, placebo-controlled design	Sleep parameters	Trp did not improve sleep during treatment phase (7 days), however post-treatment Trp improved sleep latency

Table 3 (*Continued*)

Study	Subjects	Dose	Increase from baseline in plasma Trp–LNAA ratio	Intervention type	Measures	Results
Demisch et al. (1987a) (13)	39 chronic insomniacs	2 g L-Trp and 0.04 g L-Trp (instead of placebo)	Not available	Acute, repeated-measures, double-blind design	Sleep parameters	Full L-Trp (2 g) dose administered first improved sleep relative to low Trp dose. However, when low Trp dose administered first, no difference found between two treatment conditions. Authors argue that L-Trp seems to be effective in promoting sleep in subjects with chronic insomnia
Demisch et al. (1987b) (14)	25 chronic insomniacs	2 g L-Trp for 4 weeks and 4 weeks no treatment	Not available	Sub-chronic, repeated-measures, double-blind design	Sleep parameters	Trp improved sleep patterns; subjective sleep ratings improved with Trp; sleep deteriorated in only half of the patients during the control period (no treatment)
Spinweber (1986) (4)	20 male chronic sleep-onset insomniacs	3 g L-Trp (6 nights)	Not available	Sub-chronic, between-subject, double-blind, placebo-controlled design	Sleep; performance; arousal; brain electrical activity	No effect of L-Trp on sleep latency during first three nights of administration; nights 4–6 sleep latency reduced; no effect on sleep stages; Trp did not impair performance; Trp elevated arousal threshold; Trp did not alter brain electrical activity
Schneider-Helmert (1981) (12)	8 severe insomniacs	2 g L-Trp (3 nights) followed by 4 night placebo period	Not available	Sub-chronic, repeated-measures, double-blind, placebo-controlled design	Sleep parameters	Improvements to sleep found to continue during a four night placebo period compared to the pre-Trp baseline, suggesting interval therapy to be useful method in cases of severe insomnia
Aparicio et al. (2007) (18)	18 healthy infants	Standard infant commercial milk (1.5% Trp) administered during daytime and nighttime for 1 week; second condition Trp enriched milk (3.4% Trp) given during light-time (06:00–18:00) and standard commercial milk given during nighttime (18:00–06:00) for 1 week; experimental condition infants received the standard commercial milk during daytime and Trp enriched milk during nighttime for 1 week	Not available	Sub-chronic, double-blind, repeated-measures design	Sleep patterns	Infants receiving low Trp formula during the day and high Trp formula during the night, slept more, manifested better sleep efficiency, increased immobility time, had fewer night movements and waking episodes. No statistical differences found between two control groups despite the fact that quite different amounts of Trp were administered (1.5% and 2.72%). Conclusion: milk formulas with varying Trp contents that are appropriate to light–dark variations improve the sleep/wake cycles of infants who are not breast fed
Yogman and Zeisal (1983) (16)	20 healthy newborn infants (2–3 days old)	Trp in 10% glucose or valine in 5% glucose	Not available	Acute, between-subjects design	Sleep patterns	Newborns fed Trp had shorter sleep latencies, and entered rapid eye movement and quiet sleep sooner than when fed commercial formula
Steinberg et al. (1992) (17)	57 healthy infants	Formula containing 0, 294, 588, 882 μmol/L of added Trp; for comparative purposes standard human milk and commercial formula included	Plasma Trp–LNAA ratio greatest for infants fed human milk (0.132) and formula containing highest level of added Trp (0.129)	Sub-chronic, randomised, between-subjects design	Sleep patterns	Trp–LNAA ratio, not plasma Trp concentrations, predicted differences in sleep latency across the different treatment conditions. Sleep latency was shorter for infants with the highest Trp–LNAA ratios. Infants consuming formulas with lower Trp loading had sleep latencies similar to those of infants consuming commercial formula. Infants consuming highest Trp dose sleep latencies were shorter than for infants in the human milk-fed condition. Infants fed high dose of Trp tended to be less alert, spent less time crying, and more time sleeping than infants fed lower levels of added Trp
Harada et al. (2007)	1055 infants (0–6 months), 751 young children (0.6–8 years), and 473 older children (9–15 years)	No intervention; index of Trp calculated at breakfast	Not available	Naturalistic study design	Sleep habits and mental symptoms (e.g. depression, anger)	Positive correlation between Morning–Evening scores and Trp index for infants and young elementary children; lower Trp index scores associated with increased levels of difficulty in infants falling asleep at bedtime and waking up in the morning

3. Effects of tryptophan loading on cognitive function

In the past decades, animal and human experiments have provided evidence that central 5-HT can modulate a wide array of cognitive processes, although the specific actions of 5-HT on distinct cognitive (sub) domains remains somewhat elusive. Much of the evidence is derived from psychopharmacological manipulations that increase or decrease 5-HT activity in the brain, either globally or via specific 5-HT receptors. An overview of the vast literature on animal studies investigating the role of 5-HT on cognition is beyond the scope of this paper. Several excellent reviews have recently been published on this topic (e.g. Monleon et al., 2008; Meneses, 2007a; Meneses and Perez-Garcia, 2007b; King et al., 2008; Fone, 2008).

In humans, most data originate from studies that have used acute tryptophan depletion (ATD) to induce an acute global reduction in 5-HT synthesis in the brain, or from studies using acute or sub-chronic administration of pro-serotonergic drugs, mostly antidepressants. Detailed recent reviews are available on the effects of ATD on human cognitive functioning (Mendelsohn et al., 2009) and human brain activation (Anderson et al., 2008; Evers et al., 2007; Fusar-Poli et al., 2006), as well as on the findings of pro-serotonergic drug research (Schmitt et al., 2006; Merens et al., 2007; Harmer, 2008). Studies examining the cognitive effects of 5-HT stimulation by TRP loading on human cognitive performance provide additional information on the role of 5-HT in human cognitive performance and these studies are reviewed (see Table 1). In the following sections, the TRP loading results for each of the investigated cognitive domains are discussed in the context of the relevant findings from human ATD and pro-serotonergic drug studies.

3.1. Tryptophan loading and memory

Research indicates that 5-HT is involved in specific memory processes. The most compelling evidence for this in humans has been obtained from ATD studies showing impaired long-term memory functioning following ATD. These seem to be specifically related to disturbed consolidation of new information in the long-term memory. The effects of ATD are most robustly observed in visual verbal learning tests, where delayed recall and/or recognition is impaired (Mendelsohn et al., 2009; Schmitt et al., 2006). However, a recent pooled analysis of nine ATD studies (Sambeth et al., 2007) revealed that ATD also impairs immediate recall, potentially through disruption of early consolidation and/or impairment of encoding of new information. The impairing effects were more pronounced in women. No consistent ATD-induced impairments were found on short-term or working memory (Mendelsohn et al., 2009). As for serotonergic stimulation, studies employing acute or sub-chronic administration of serotonergic drugs (i.e. SSRIs, 5-HT receptor agonists) in healthy volunteers show an inconsistent pattern of no effects, impairments and improvements of various memory functions (Schmitt et al., 2006). Although in depressed patients, successful serotonergic pharmacotherapy is generally associated with cognitive enhancement, the direct effects of 5-HT on memory and other cognitive functions cannot be easily disentangled from potential cognitive enhancement through alleviation of other depressive symptoms (mood, motivation and sleep disturbances) (see Schmitt et al., 2006).

A total of eight studies have examined the memory effects of Trp loading, with four measuring effects on long-term memory functioning. Sobczak et al. (2003) reported memory deficits following Trp loading in healthy adults and in healthy first-degree relatives of bipolar patients. Specifically, impairments in delayed word recall and recognition were found following an intravenous 7 g Trp challenge. However, the high dose of Trp (which increased plasma Trp–LNAA ratio by 1500%) also produced significant sedative effects that were apparent from the subjective rating scores. Moreover, sedation was positively correlated with memory decrements, suggesting that the memory impairment may be attributed to melatonin accumulation, a neuro-hormone that regulates the circadian cycle by chemically causing drowsiness and thus promoting sleepiness (Richardson, 2005; Vanecek, 1998, see Section 6).

During the premenstrual stage, women with premenstrual complaints manifest serotonergic abnormalities (Halbreich, 2003; Kouri and Halbreich, 1997), which may underlie, at least partially, certain symptoms, such as memory deficits (Schmitt et al., 2005). Interestingly, 40 g α-lactalbumin (plasma Trp–LNAA ratios increased between 6% and 25% from baseline) or a carbohydrate rich drink (Trp–LNAA increased ratio by 29%) resulted in improvements in long-term memory for abstract figures and long-term memory word recognition, respectively, in women with premenstrual complaints during the premenstrual stage (Sayegh et al., 1995; Schmitt et al., 2005).

In a more recent study, exploring the effects of Trp loading on cognitive performance in unmedicated recovered depressed patients and matched controls, Booij et al. (2006) found that an α-lactalbumin-rich diet (two chocolate drinks each containing a whey-protein fraction rich in α-lactalbumin; containing 12.32 g/kg Trp; Trp/ΣLNAA ratio of 8.7%) improved abstract visual memory (specifically, recognition and speed of retrieval from short- and long-term abstract visual memory), without affecting mood, in healthy controls and in recovered depressed subjects (plasma Trp–LNAA ratio increased 21% from baseline). These results indicate that the beneficial effects of Trp loading on memory are not limited to individuals vulnerable to 5-HT related disorders. Moreover, these findings are consistent with the ATD literature where memory consolidation deficits have been observed in healthy volunteers (Riedel et al., 1999; Schmitt et al., 2000). However, the beneficial effects of Trp loading on memory performance may be attributed to impaired memory performance that was observed in the placebo (casein) condition. Without a non-intervention control group this possibility cannot be negated. However, the change in plasma Trp–LNAA ratio in the casein condition is comparable to previous findings (Merens et al., 2005; Schmitt et al., 2005) and justifies the use of casein as a placebo.

The other four studies focused on working memory performance changes after Trp loading. Luciana et al. (2001) compared the effects of Trp loading with the effects of Trp depletion on various cognitive processes in healthy subjects. 10.3 g L-Trp loading increased total plasma Trp by tenfold (53.22 at baseline to 551.4 μmol/L), and resulted in decrements to working memory performance for verbal and affective stimuli relative to Trp depletion. As both Trp loading and Trp depletion resulted in decreased levels of positive affect, the authors argue that the memory impairments in the Trp loading condition are not likely to be attributed to changes in mood. However, as there was no placebo condition the results are difficult to interpret and may be inflated.

Improvements in short-term memory scanning have been observed in stress-vulnerable subjects following acute Trp loading (Markus et al., 1999, 2002). Increased serotonergic activity is an established consequence of stress (Joseph and Kennett, 1983; Stanford, 1993), and continual stress may lead to a shortage of the supply of this neurotransmitter. Consequently, serotonin activity may drop below the functional levels producing stress-related cognitive disturbances. In accordance with this, it would be expected that Trp loading would improve cognitive performance in stress-prone subjects following acute stress as the diminished serotonergic pools are replenished by Trp loading. Consistent with this, Markus et al. (1999) found short-term memory scanning

improvements, following laboratory acute stress, only in high stress-prone volunteers after a carbohydrate rich/protein poor diet. In low stress-vulnerable subjects, Trp loading had no effect on cognitive performance, as the serotonergic system was not compromised to begin with. Further support for the beneficial effects of Trp loading in high stress-prone subjects was found in a later study where increases in plasma Trp, following an α-lactalbumin-rich diet (two chocolate drinks each containing a 20 g whey-protein fraction rich in α-lactalbumin; containing 12.32 g/kg Trp; Trp/ΣLNAA ratio of 8.7%), were shown to improve memory scanning ability in healthy, stress-vulnerable subjects (Markus et al., 2002). As expected, and consistent with the previous study, this effect was not observed in the control group (low stress-vulnerable subjects) (Markus et al. (2002). Interestingly, in an earlier study Markus et al. (1998) failed to show improvements to short-term memory following a carbohydrate rich/protein poor diet in stress-prone subjects following laboratory stress. Although the diet significantly increased the plasma Trp–LNAA ratio by 42%, no memory scanning effects were found. The authors suggested that the lack of effects may be attributable to a higher level of the subject's control of the induced stress (Markus et al., 1999).

Hitherto, no studies addressing the chronic effects of Trp on human cognitive function have been performed. However, animal studies have produced some interesting results. In a recent study, it was shown that following 6 weeks of oral Trp administration (100 mg/kg body weight), spatial working memory was improved in Trp-treated rats (Haider et al., 2006). Similarly, Khaliq et al. (2006) reported improved memory following 6 weeks oral administration of Trp at doses of 50 and 100 mg/kg body weight in rats. At both doses plasma Trp, brain Trp, and 5-HT levels increased with Trp. The authors concluded that increases in brain 5-HT synthesis following long-term Trp administration may be involved in the observed memory enhancement. Haider et al. (2007) reported improvements in short- and long-term memory and in learning acquisition following 6 weeks administration of Trp at doses 50 and 100 mg/kg body weight in rats. These results further indicate that long-term administration of Trp as a dietary supplement may be beneficial to memory functioning. Future work should assess whether chronic consumption of Trp similarly improves memory function in humans.

In summary, improvements in long-term memory processes, memory scanning ability, and abstract visual memory following Trp loading have been shown in vulnerable and clinical populations where some serotonergic disturbances are known (i.e. females with premenstrual symptoms, recovered depressed patients, and in stress-vulnerable subjects following experimental stress). In contrast, in healthy volunteers the reports are inconsistent.

3.2. Tryptophan loading and attention

Sustained attention (vigilance) refers to the ability to direct and focus attention or alertness to a task over a prolonged period of time. There is consistent evidence from a series of studies with serotonergic antidepressants that 5-HT stimulation (acute and sub-chronic) impairs vigilance performance in healthy volunteers as measured by the Mackworth Clock Test (Riedel et al., 2005; Wingen et al., 2008; for review see Schmitt et al., 2006). In contrast, ATD generally does not affect sustained attention (Mendelsohn et al., 2009) as measured by a variety of tasks (although not the Mackworth Clock Test). Two studies have assessed the effects of Trp loading on sustained attention. Both Luciana et al. (2001) and Dougherty et al. (2007) observed fewer errors of omission during a vigilance task in the Trp loading condition (10.3 g L-Trp; 5.15 g Trp, respectively) relative to the Trp depletion condition, in healthy adults. These results suggest that Trp loading may improve sustained attention. However, as there was no placebo condition and results were compared only to Trp depletion, the results may be inflated.

Focused or selective attention refers to the ability to attend to relevant stimuli while simultaneously ignoring irrelevant information. ATD studies have provided evidence for 5-HTs involvement in focused attention (Mendelsohn et al., 2009; Schmitt et al., 2006). ATD has been shown to reduce interference on the Stroop test (a frequently employed measure of focused attention, response inhibition, and cognitive flexibility) and increase performance on focused attention components of dichotic listening tasks in healthy and depressed subjects (Booij et al., 2005; Rowley et al., 1998; Schmitt et al., 2000). Further substantiation for 5-HTs role in focused attention is found in ATD studies employing electrophysiological measures in healthy subjects (Ahveninen et al., 2002).

The few studies that have explored the effects of 5-HT loading on focused attention (Go/NoGo Task, Stroop Colour Word Test, Left/Right Choice Reaction Time, Dichotic Listening Task) have shown minimal effects. A 7 g intravenous Trp challenge resulted in performance decrements on the Go/NoGo Task and a Left/Right Choice Reaction Time task in subjects with a first-degree relative with bipolar disorder (Sobczak et al., 2003), which is consistent with the effects of ATD on focused attention. Although the deficit in focused attention could be explained by a corresponding sedative effect produced by the high Trp dose, a similar deficit in focused attention was not observed in the healthy control group. In addition, the impairment did not extend to other tests of focused attention (i.e. the Stroop test and dichotic listening). This is consistent with Booij et al. (2006) who reported no effects of α-lactalbumin (2 × 20 g whey-protein containing 12.32 g/kg Trp; Trp–LNAA ratio increase from baseline 21%) on the Stroop task in both recovered depressed patients and healthy controls.

There is no clear indication that Trp loading affects sustained or focused attention, although the data on both functions are scarce. No data are available on Trp loading effects on divided attention.

3.3. Tryptophan loading and executive functions

Executive functions is a general term that refers to a wide variety of cognitive processes such as planning, decision-making, monitoring and behavioural adaptation, reasoning, cognitive flexibility, and response inhibition (Chan et al., 2008). These functions are considered essential for purposeful, goal-directed, future-oriented behaviour.

Serotonin's contribution to executive functioning processes remains unclear. ATD studies have produced inconsistent results across most of the executive function domains. Although some treatment effects have been reported for planning ability, cognitive flexibility and decision-making (Murphy et al., 2002; Park et al., 1994; Rogers et al., 1999, 2003; Sobczak et al., 2002; Talbot et al., 2006), a considerable amount of research has shown no effects of ATD on planning, cognitive flexibility, decision-making abilities, response inhibition, and attentional set-shifting or reversal learning (Anderson et al., 2003; Booij et al., 2005; Evers et al., 2004, 2005; Gallagher et al., 2003; Hughes et al., 2003; LeMarquand et al., 1998; Roiser et al., 2007, 2008; Talbot et al., 2006).

Trp loading has not been shown to modulate planning or response inhibition in healthy adults (Booij et al., 2006; Morgan et al., 2007; Sobczak et al., 2003). Furthermore, in sub-group and clinical populations, Trp loading has similarly not modulated planning or response inhibition (Booij et al., 2006; Schmitt et al., 2005). However, Sobczak et al. (2003) observed significant decrements in planning functions (assessed with the Tower of London task) in healthy first-degree relatives of bipolar patients

following an intravenous 7 g Trp challenge, which was not observed in the control group. Interestingly, planning deficits have previously been reported in healthy first-degree relatives of bipolar patients following acute tryptophan depletion (Sobczak et al., 2002), which could suggest that these patients may be sensitive to any modulations to serotonergic functioning.

Overall, Trp loading – as well as ATD studies – has not shown clear evidence of serotonergic modulation of the various aspects of executive functioning. Although this may indicate 5-HT does not exert a meaningful influence on these functions, the inconsistencies may also be partly related to more general issues regarding executive function test sensitivity and reliability, particularly in repeated assessments where the level of novelty may confound the test outcomes (Rabbitt, 1997; Chan et al., 2008).

3.4. Tryptophan loading and emotional processing

Over past years, there has been an increasing interest in the role of serotonin in processing and classifying emotionally loaded information. Emotional processing is typically assessed by measuring response biases to positive or negative stimuli (words, pictures, reward, punishment) in attention, memory or reaction time tests, or by measuring perception and classification of emotionally loaded stimuli, such as emotional face expressions. Evidence for a serotonergic involvement in such processes has emerged, as human studies have shown that ATD can decrease recognition of facial emotions, particularly for fearful expressions, and leads to a response bias towards negative stimuli in healthy volunteers and vulnerable populations (Harmer, 2008), although an absence of ATD effects on facial recognition has also been reported (Cools et al., 2005; Fusa-Poli et al., 2007; Van der Veen et al., 2007). Serotonergic stimulation by acute SSRI administration produces generally opposite effects of those seen with ATD and enhances positive affective processing (see Harmer, 2008; Merens et al., 2007 for detailed overviews).

Although the number of studies is limited, the results suggest that Trp loading can modulate emotional information processing using facial emotion recognition tasks. Attenburrow et al. (2003) investigated the acute effects of pure Trp (1.8 g Trp) loading on facial expression recognition in healthy females. The authors found that Trp enhanced the perception of fearful and happy facial expressions relative to placebo. Consistent with this, Murphy et al. (2006), reported increases in the recognition of happiness and decreases in the recognition of disgust in healthy females following 14 days Trp intervention (1 g three times a day). Furthermore, Trp administration decreased attentional vigilance towards negative stimuli and reduced the baseline emotional startle response. These effects were not seen in males. Interestingly, modulations of emotional processing were observed in the absence of any change in subjective mood ratings. Thus, the authors argue that the decrease in attentional vigilance towards negative stimuli is a direct consequence of modulations to 5-HT levels in the brain that is independent to mood improvement.

In contrast, Scrutton et al. (2007) failed to find an effect of 40 g α-lactalbumin-rich drink (total Trp 1.8 g) on recognition of emotional facial expressions in healthy females. This discrepancy in results may be attributed to the considerably lower increase in plasma Trp–LNAA ratio that was achieved following α-lactalbumin (+80%) relative to the Trp–LNAA ratio achieved with pure Trp (approximately 6-fold increase; Attenburrow et al., 2003). The increase in Trp–LNAA ratio following α-lactalbumin may not have been sufficient to modulate emotional processing (Scrutton et al., 2007).

The available reports suggest that Trp loading in females can induce a positive bias in the processing of emotional stimuli, which is consistent with the effects of serotonergic antidepressants (Harmer et al., 2003, 2004, 2006). There is some indication that these changes occur following higher increases of the Trp–LNAA ratio, but the current dose–response data are very limited. The results also suggest that women may be more susceptible to serotonergic manipulations than men. However, given the small male sample size it is difficult to draw conclusions regarding the effect of Trp loading on emotional processing in men.

3.5. Tryptophan loading and psychomotor performance

Trp loading has consistently been shown to impair motor performance on a range of psychomotor tasks (specifically: Grooved pegboard test, Left/right choice reaction time task, Motor choice reaction time task, and the Symbol copying test), in both healthy adults (Booij et al., 2006; Luciana et al., 2001; Sobczak et al., 2003; Winokur et al., 1986) and in vulnerable populations (Booij et al., 2006; Sobczak et al., 2003) following both Trp loading (range 5–10.3 g) and administration of an α-lactalbumin-rich drink. In line with this, decrements in reaction time performance following Trp loading have also been consistently reported in healthy and sub-group volunteers following both Trp and a carbohydrate rich/protein poor diet (Cunliffe et al., 1998; Markus et al., 1998; Morgan et al., 2007; Murphy et al., 2006). These findings suggest that Trp has a mild sedative effect, which is consistent with previous sleep studies (refer to below Section 5). Although Cunliffe's et al. (1998) findings of a decreased Critical Flicker Fusion threshold (measure of central fatigue) appears to support Trp's sedative effects, the putative effects of pupillary changes were not accounted for. Previous research has shown that modulations to serotonergic activity through administration of SSRIs induce an acute and steady increase in pupil diameter (Schmitt et al., 2002), independently invoking CFF threshold increases. Nevertheless, the observed increase in subjective ratings of fatigue reported by Cunliffe et al. (1998) does lend support for Trp's sedative effects. Furthermore, the fact that decrements to psychomotor and reaction time performance have been reported across different study populations (i.e. healthy, sub-group, clinical populations), further suggests that psychomotor performance impairment may be attributed to the proposed sedative effects of Trp loading, that may be linked to increased melatonin production.

3.6. Conclusion

In summary, the beneficial effects of Trp loading on cognition are generally modest and not always found. Following Trp loading, improvements in long-term memory for verbal and abstract information, as well as memory scanning ability has been shown in vulnerable and clinical populations. In healthy volunteers the results are less consistent. However, Trp loading does appear to induce a positive bias in the processing of emotional stimuli in healthy women, which is consistent with the SSRI literature. Trp loading has also consistently been shown to impair psychomotor and reaction time performance across the different study populations (i.e. healthy adults and vulnerable populations). There is no clear indication that Trp loading affects attention or executive functions, but studies in this field are too limited and heterogeneous to allow any firm conclusions.

4. Effect of tryptophan loading on mood and alertness

Since lowered serotonergic functioning has been implicated in affective disorders (Arango et al., 2002; Deakin, 1998; Delgado, 2000; Mahmood and Silverstone, 2001), the role of 5-HT on mood has been extensively investigated. ATD studies have shown a significant, transient reappearance of depressive symptoms following ATD in both medicated and unmedicated depressed

patients in remission (Delgado et al., 1990; Smith et al., 1997), and in subjects with family histories of depression (Benkelfat et al., 1994). Although there have been reports of mood-lowering effects of ATD in healthy subjects (Young et al., 1985), the bulk of the literature indicates no effects in healthy volunteers (see Ruhe et al., 2007). Similarly, administration of pro-serotonergic drugs to healthy volunteers generally does not induce mood changes (Merens et al., 2007). Based on these results, it is expected that Trp loading may improve mood in vulnerable populations where dysfunction of the serotonergic system is known. However, in healthy subjects, it is likely that the effects of Trp loading on mood are less clear, with minimal effects expected.

4.1. Effect of tryptophan loading on mood in clinical populations

Over five decades ago Lauer et al. (1958) reported the first observation that Trp loading improves mood. Some of the earlier research investigated the use of Trp with other antidepressant treatments, demonstrating the ability of Trp (doses ranging from 3.5 to 18 g/day) to potentiate the action of monamine oxidase inhibitors and tricyclic antidepressants in depressed patients (Ayuso Gutierre and Lopez-Ibor Alino, 1971; Coppen et al., 1963; Glassman and Platman, 1969; Pare, 1963).

There are a substantial number of studies that have addressed the efficacy of Trp given alone as an antidepressant (Bowers, 1970; Chouinard et al., 1979, 1983; Mendels et al., 1975; Murphy et al., 1974; Steinberg et al., 1999; Thomson et al., 1982), and there are many reviews available on the topic (Baldessarini, 1984; Carroll, 1971; Cole et al., 1980). However, there is little consensus in terms of Trp's efficacy in treating depression as studies vary considerably in terms of sample size, study populations, dosages, study designs, and control conditions. For instance, in severely depressed inpatients, Trp has been shown to have little or no effect when compared with placebo (Chouinard et al., 1983). In contrast, Trp has been reported to be an effective antidepressant in mild to moderately depressed outpatients (Thomson et al., 1982). While in patients with premenstrual dysphoric disorder, Steinberg et al. (1999) found that 6 g L-Trp (given as 2 g three times a day for 17 days) was more effective than placebo in controlling extreme mood swings, dysphoria, irritability, and tension.

4.2. Effect of tryptophan loading on mood and alertness in healthy and vulnerable volunteers

Research investigating the effects of Trp loading on mood in healthy volunteers and in vulnerable subjects with presumed dysfunction of the serotonergic system (i.e. stress-vulnerable subjects, recovered depressed patients, unaffected first-degree relatives of bipolar disorder patients) has also produced varying results. Refer to Table 2 for summary of main findings relating to the effects of Trp loading on mood.

In a recent study in healthy adults, the effects of a hydrolysed protein on plasma Trp–LNAA ratio and mood was compared to other sources of Trp (α-lactalbumin, hydrolysed protein, pure Trp, a Trp-containing synthetic peptide), and a placebo protein (Markus et al., 2008). All of the interventions contained a similar amount of Trp (0.8 g Trp; excluding placebo), but differed in the content of other amino acids. The hydrolysed protein produced significantly faster and greater increases in plasma Trp–LNAA ratio (255%) compared to α-lactalbumin (67%) and pure Trp (191%). Mood (a total mood disturbance score obtained by summing all six factor scores of the POMS questionnaire; the six mood factors include: tension-anxiety, depression-dejection, anger-hostility, vigour-activity, fatigue-inertia, and confusion-bewilderment) was significantly improved 60 min following the hydrolysed protein and pure Trp. The most profound and durable mood enhancing effects were observed 210 min after intake of the hydrolysed protein. No significant mood effects were observed with α-lactalbumin or the synthetic Trp peptide. The lack of a mood effect in the α-lactalbumin condition is consistent with previous research that has only shown α-lactalbumin to reduce feelings of depression (Markus et al., 1998, 2000) and increase ratings of vigour (Markus et al., 1998) in stress-vulnerable subjects after acute stress exposure (Markus et al., 1998, 2000). These results indicate that larger increases in plasma Trp–LNAA ratio may be more likely to modulate mood, even in healthy adults.

In contrast, Sobczak et al. (2002, 2003) reported increased feelings of anger, depression, fatigue, tension, and decreased feelings of vigour (measured by an abbreviated version of POMS) and alertness (measured by Bond and Lader Visual Analogue Scale) in unaffected first-degree relatives of bipolar disorder patients and healthy controls following a single intravenous 7 g Trp challenge, when compared to placebo. The intervention led to a 1500% increase in plasma Trp–LNAA ratio. This increase in plasma ratio was considerably higher than what was observed by Markus et al. (2008) and this may have resulted in the opposite mood effect, specifically negative effects.

A carbohydrate rich/protein poor drink and an α-lactalbumin-rich drink (chocolate drink containing a whey-protein fraction rich in α-lactalbumin; 2 × 20 g whey-protein containing 12.32 g/kg Trp; Trp/ΣLNAA ratio of 8.7%) has been shown to reduce feelings of depression (measured by the depression subscale of the Profile of Mood States inventory; Markus et al., 1998, 2000) and increase ratings of vigour (measured by the vigour subscale of the Profile of Mood States [POMS] inventory; Markus et al., 1998) in high stress-vulnerable subjects, exposed to experimental stress, relative to a protein rich/carbohydrate poor drink or a casein diet. In contrast, no effect of Trp loading was observed on measures of mood and depressive symptoms in low stress-vulnerable subjects (control) exposed to experimental stress (Markus et al., 1998, 2000). The effects of the Trp-rich drink on ratings of depression and vigour were found when plasma Trp–LNAA ratio increased by only 48% (Markus et al., 1998, 2000). However, this increase in plasma Trp–LNAA ratio may not have been high enough to modulate mood in healthy adults, based on the findings by Markus et al. (2008) and Sobczak et al. (2002, 2003).

Merens et al. (2005) did not find Trp loading to significantly modulate ratings of depression, anger, fatigue, tension, and vigour (measured with the POMS), in stress-induced unmedicated recovered depressed subjects and healthy controls. Although there was a trend reduction in depressive ratings following α-lactalbumin (2 × 20 g whey-protein fraction rich in α-lactalbumin; containing 12.32 g/kg Trp; Trp/ΣLNAA ratio of 8.7%) in stress-induced unmedicated recovered depressed subjects, a similar decrease was also observed in the casein (placebo) condition. The authors argue that 1-day α-lactalbumin intervention may not be sufficient to prevent a stress-induced deterioration in mood in unmedicated recovered depressed subjects. Furthermore, plasma Trp–LNAA ratio increased by only 21%, which may not have been sufficient to significantly reduce depressive ratings in the recovered depressed subjects.

These findings are consistent with a later study that reported no effects of an α-lactalbumin-rich diet (two drinks containing a whey-protein fraction rich in α-lactalbumin; containing 12.32 g/kg Trp) on POMS subscales (i.e. depression, anger, fatigue, tension, vigour) in recovered unmedicated depressed patients and healthy controls (Booij et al., 2006), when plasma Trp–LNAA ratio increased by 21% from baseline.

Similarly, several other studies that have failed to show modulations to mood following Trp loading in healthy adults also reported relatively small increases in plasma Trp–LNAA ratio (relative to the plasma increases noted by Markus et al. (2008) and

Sobczak et al. (2002, 2003)). Specifically, Beulens et al. (2004) reported a 16% increase following an α-lactalbumin drink and Scrutton et al. (2007) reported an 80% increase following an α-lactalbumin-rich drink. Murphy et al. (2006) and Luciana et al. (2001) also did not find Trp loading to modulate mood in healthy adults. However, plasma Trp–LNAA ratio was not measured.

In conclusion, the effects of Trp loading on mood factors in healthy volunteers and in vulnerable subjects with presumably sub-optimal central serotonergic function are rather inconsistent, with some reports indicating improvements, other reports showing decreases, yet other studies showing no effect. However, it is plausible that differences in elevations of the plasma Trp–LNAA ratio may elucidate some of these inconsistencies. This will be addressed further in Section 6.

5. Effect of tryptophan loading on sleep

Trp has been shown to have direct effects on the homeostatic regulation of sleep (Minet-Ringuet et al., 2004), by increasing availability of brain 5-HT which has been implicated in the regulation of sleep (Bhatti et al., 1998; Hartmann and Greenwald, 1984). In the pineal gland, 5-HT serves as precursor of melatonin (Kleine and Moore, 1979), a neuro-hormone secreted during the night which acts as the signal for darkness in the internal milieu (Vanecek, 1998).

Nocturnal Trp administration is known to increase physiological concentrations of both serotonin and melatonin (Esteban et al., 2004). Melatonin production in the pineal gland is high during the night and inhibited by light. Therefore, in the evening the synthesis of melatonin is activated and serotonin is converted to melatonin (Richardson, 2005). Administration of Trp during the night can therefore be useful in facilitating sleep as Trp increases the release of melatonin (Hajak et al., 1991).

In addition, 5-HT has some direct effects on sleep. Electrophysiological, neurochemical and neuropharmacological studies have shown serotonergic activation promotes waking and inhibits slow-wave sleep and/or rapid eye movement (REM) sleep. Specifically, serotonergic neurons of the dorsal raphe nucleus fire at a steady rate during waking, but decrease their firing during slow-wave sleep, and almost cease activity during REM sleep (Monti and Jantos, 2008 for review).

The effects of Trp on sleep have been investigated for over four decades, with several older reviews available on this topic (Cole et al., 1980; Hartmann and Greenwald, 1984; Young, 1986). The first study to assess the effects of Trp on sleep, Oswald et al. (1966) reported that 5–10 g Trp decreased the time before onset of REM sleep in healthy adults. Since then much research has been conducted in both healthy and clinical populations, specifically insomniacs, to explore the effects of Trp loading on sleep parameters. Refer to Table 3 for summary of the main findings pertaining to the effects of Trp loading on sleep measures in healthy adults, vulnerable populations, and infants and children.

5.1. Effect of tryptophan loading on sleep parameters in insomniacs

The bulk of evidence indicates that doses as low as 1 g L-Trp significantly reduce sleep latency and increase subjective ratings of sleepiness in subjects with insomnia (Brown et al., 1979; Hartmann et al., 1974; Hartman and Spinweber, 1979; Körner et al., 1986; Spinweber, 1986). Doses below 1 g have shown trends towards decreased sleep latency in mild insomniacs (Hartman and Spinweber, 1979). Although in a recent study Hudson et al. (2005) demonstrated that 250 mg pharmaceutical grade Trp and protein sourced Trp (25 mg deoiled butternut squash seed meal containing 22 mg Trp/1 g protein mixed with 25 mg dextrose) significantly improved subjective and objective sleep measures in clinically diagnosed insomniacs. However, given the small sample size further research is warranted. Overall, these results indicate that Trp at doses as low as 1 g improve time to onset of sleep, and doses below 1 g produce trends in a similar direction.

Few studies have reported modulations to sleep stages in insomniac subjects following Trp loading. Hartman and Spinweber (1979) found Stage IV sleep (deep sleep) to be significantly increased following only 250 mg of L-Trp, with no modulations to sleep observed following 500 mg or 1 g Trp. In a dose–response study, Hartmann et al. (1974) found that 1–15 g of L-Trp decreased sleep latency, but only doses above 5 g increased slow-wave sleep and decreased REM sleep. Other studies have failed to demonstrate modulations to sleep stages (Brown et al., 1979; Spinweber, 1986), which may, in part, be attributed to varying Trp doses. Spinweber (1986) found that 3 g L-Trp did not alter sleep stages or brain electrical activity during sleep in chronic sleep-onset insomniacs. However, significant decreases in sleep latency on nights 4–6 of Trp administration were found relative to placebo. Similarly, Brown et al. (1979) did not report modulations to REM and slow-wave sleep following 1 and 3 g L-Trp compared to placebo in healthy females with mild falling asleep complaints. However, significant reductions in sleep latency following the 3 g Trp dose were observed. In summary, doses at low as 1 g L-Trp significantly reduce sleep latency and doses lower than 5 g do not appear to affect sleep stages.

5.2. Effect of tryptophan loading on sleep parameters in healthy volunteers

In normal subjects, who fall asleep easily, it would be expected that Trp loading would produce minimal hypnotic effects as sleep latency is already short and sleep quality is normal. Nevertheless, sleep parameters have been shown to improve in healthy subjects manifesting no sleep problems. Studies have shown that doses of 500 mg, 1 g (Chauffard-Alboucq et al., 1991), 1.2 g, 2.4 g (George et al., 1989), and 4 g (Spinweber et al., 1983) L-Trp significantly reduced sleep latency and increased subjective ratings of sleepiness in healthy adults during the day (George et al., 1989; Spinweber et al., 1983; Thorleifsdottir et al., 1989) and during the night (Chauffard-Alboucq et al., 1991). Interestingly, significant negative correlations have also been reported between plasma Trp level and sleep latency at 0, 60 and 120 min following 1.2 g and 2.4 g L-Trp administration in healthy adults (George et al., 1989). Similarly, an intravenous challenge of 3 and 5 g L-Trp showed a dose-dependent increase in the percentage of sleep observed during Stages I and II (light sleep) during the day compared to placebo in healthy males (Hajak et al., 1991). During nighttime sleep, sleep latency for Stages I and II and sleep efficiency improved following 1, 3, and 5 g L-Trp compared to placebo. Interestingly, during the nighttime condition plasma melatonin increased considerably higher following 1, 3 and 5 g Trp than during the daytime condition, lending support to the notion that time of day may influence melatonin synthesis (Hajak et al., 1991).

Chauffard-Alboucq et al. (1991) reported an increase in nighttime sleepiness and sedative effects in healthy females following 500 mg and 1 g L-Trp (combined with a carbohydrate load) relative to placebo. This effect was observed when plasma Trp–LNAA ratio increased 200% (500 mg) and 300% (1 g) from baseline, peaking 90 min after Trp administration. Interestingly, peak in perceived sleepiness was also found 90 min following Trp consumption. Although previous studies have reported sleepiness and sedative effects as early as 30 min following Trp administration (Hartmann et al., 1976; Yuwiler et al., 1981), this difference is likely attributed to the significantly higher doses consumed, specifically 4 g (Hartmann et al., 1976) and 50 mg/kg (Yuwiler et al., 1981) Trp.

Few studies have reported modulations to sleep stages following Trp loading. Furthermore, reports are somewhat inconsistent. Wyatt et al. (1970) found 7.5 g Trp decreased REM sleep and increased non-REM sleep in healthy subjects during the night. Nicholson and Stone (1979) observed an increase in the duration of Stage III (slow-wave sleep) sleep during the day following 4 g L-Trp in healthy males. However, no modulations to sleep stages were reported during nighttime sleep following 2, 4, and 6 g L-Trp. In contrast, Spinweber et al. (1983) did not find 4 g L-Trp to modulate sleep stages during the daytime sleep.

It has been argued that L-Trp may be an effective daytime hypnotic for healthy adults, by facilitating sleep onset at times outside the normal circadian rhythm. Spinweber et al. (1983) found that during waking EEG, 4 g L-Trp significantly increased alpha latency, theta latency, and theta amplitude, and decreased alpha frequency, indicating a reduction in wakefulness. However, no wave bands were modulated during sleep. Similarly, Thorleifsdottir et al. (1989) found that during the day, 2 g Trp increased theta amplitude and decreased alpha amplitude in healthy adults, characterising the EEG of drowsiness. In addition, increased subjective ratings of sleepiness were also reported following morning administration of Trp. Thus, it may be that during the day, during wakefulness, Trp loading has a relaxing and calming effect in healthy adults, whereas it has minimal effects on sleep in healthy adults who fall asleep easily.

5.3. Effect of sub-chronic tryptophan loading on sleep

In patients with severe insomnia, Trp loading seems to either lack the potency that other hypnotic drugs have, or doses have not been high enough to modulate sleep. However, reports indicate that interval and sub-chronic Trp treatment may be an effective approach for improving sleep in severe cases of insomnia.

Several studies have observed improved sleep quality and decreased sleep latencies during and several nights following Trp treatment in chronic insomniacs (Demisch et al., 1987a; Hartman et al., 1983; Schneider-Helmert, 1981). Interestingly, results are consistent across different lengths of treatment period. For example, following three nights of 2 g L-Trp administration, significant improvements to sleep were found to continue during a four night placebo period compared to the pre-Trp baseline (Schneider-Helmert, 1981). Reductions to sleep latency have also been shown 1 week after 1 g L-Trp treatment, but surprisingly not during the 7-day treatment (Hartman et al., 1983). Similarly, improvements in sleep were found following 4 weeks of 2 g L-Trp treatment in patients with chronic insomnia (Demisch et al., 1987a,b). During the control period (4 weeks following the 4 week Trp treatment period), where no Trp was administered, sleep deteriorated in half of the improved patients, i.e. 10 out of 19 subjects (Demisch et al., 1987a).

In healthy adults, five nights of 500 mg Trp administration has also been reported to decrease sleep latency and increase sleep depth, sleepiness, and calming effects, relative to five nights of placebo (Leatherwood and Pollet, 1984). Interestingly, younger females were found to be more sensitive to the sedating effects of Trp than other groups (Leatherwood and Pollet, 1984). These results suggest that in cases of severe insomnia or even in healthy adults, Trp loading may be an effective hypnotic when consumed sub-chronically or intermittently.

5.4. Effect of tryptophan loading on sleep and cognition

A benefit of Trp as a sleep aid is that it does not seem to impair performance the next day following administration as some more potent hypnotics have been shown to do (Johnson and Chernik, 1982; Vermeeren, 2004). In a recent study, the cognitive benefits of evening Trp loading on morning performance were assessed (Markus et al., 2005). As positive associations between Trp availability and sleep have previously been shown (see above), the aim of this study was to ascertain whether evening intake of α-lactalbumin improves morning cognitive performance due to improved sleep. The authors demonstrated that evening consumption of 2 × 20 g α-lactalbumin protein with an enriched Trp content of 4.8 g/100 g Trp, increased plasma Trp availability and the Trp–LNAA ratio by 130%, decreased feelings of sleepiness in the morning, and improved morning alertness and attention (measured by the P300 evoked related potential component) in subjects with and without mild sleep complaints. However, only in subjects with mild sleep complaints did evening consumption of α-lactalbumin improve vigilance performance the following morning. These findings provide support for the notion that Trp loading may improve cognition indirectly by improving sleep.

5.5. Effect of tryptophan loading on sleep in infants and children

The concentration of Trp in human milk varies in relation to the age of the lactating infant, the duration of the milking episode, and the time of day, where it has been shown to be higher during dark time (Cubero et al., 2005). Recently it was shown that oscillations in Trp concentration in maternal milk parallels oscillations in infant urinary 6-sulphatoxy-melatonin (Cubero et al., 2005), thus supporting Trp's important role in maternal milk as a regulator of the circadian rhythms of the infant. However, the internal clock that the oscillating levels of Trp in maternal milk provides, disappears with the use of commercial milk formulas, as the level of Trp found in infant formulas always remains constant (i.e. no difference in Trp level between daytime and nighttime formulas). Furthermore, maternal milk has been shown to contain higher levels of Trp than commercial formulas, which result in formula fed infants manifesting lower plasma Trp concentrations compared to human milk-fed infants (Heine, 1999).

The effect of Trp loading on sleep latency in newborns was first investigated by Yogman and Zeisal (1983). The authors found that newborn infants (2–3 days of age) fed Trp in glucose during an evening feeding manifested shorter sleep latencies, and entered rapid eye movement and quiet sleep (defined as quiescent state with eyes closed, absence of eye movements, little or no motor activity, and slow, regular respiration) sooner than when fed a commercial formula containing Trp. A limitation of this study was that the Trp was ingested in glucose solutions as a single dose, rather than as a constituent of milk which was chronically fed to infants.

Thus, in a later study, Steinberg et al. (1992) investigated whether infant formulas, varying only in Trp content (0, 294, 588 and 882 μmol/L), result in differences to plasma Trp concentration and Trp–LNAA ratio, and whether the Trp–LNAA ratio was predictive of infants' (gestational age of 37–42 weeks) sleep latency. Trp–LNAA ratio, and not plasma Trp concentrations, predicted differences in sleep latency across treatment conditions. Thus, sleep latency was shorter for infants with the highest Trp–LNAA ratios. Infants consuming the high Trp dose (882 μmol/L) manifested shorter sleep latencies than infants consuming human milk. Furthermore, infants receiving the high Trp dose tended to be less alert, spent less time crying, and more time sleeping than the infants fed lower levels of added Trp (0, 294, 588 μmol/L). These findings indicate that infant formula Trp composition can modulate sleep latency and wakefulness in infants.

In a recent study, Aparicio et al. (2007) demonstrated that milk formulas with Trp content that is appropriate to the light–dark variations in Trp level improve the circadian sleep/wake cycles in infants who are not breast fed. Specifically, infants (aged between 12 and 20 weeks) receiving a low Trp formula during the day and a

high Trp formula during the night, slept more, manifested better sleep efficiency, increased immobility time, had fewer night movements and waking episodes. No difference was found between the two control groups (control group 1: low Trp formula fed throughout the 24 h day; control group 2: low Trp formula fed from 18:00 to 06:00 h and tryptophan-enriched milk (3.4 g Trp/100 g protein) fed from 06:00 to 18:00 h) despite that the Trp content varied considerably (1.5% and 2.72% [note that the latter % is an average value]). These findings indicate that the use of different formulas for day and night feeding constitutes an interesting and novel approach to infant nutrition.

In a large Japanese sample, increased sleep latencies and difficulties waking up in the morning were shown to be associated with lower Trp index scores (Trp content consumed during breakfast) in infants and children (0–8 years; Harada et al., 2007). This effect was not similarly observed in older children (9–15 years). The authors concluded that Trp consumed at breakfast is important to sustain a healthy circadian rhythm and improve quality of sleep. A limitation of this study was that the Trp index was calculated based only on what was consumed at breakfast. Therefore, it cannot be excluded that the observed sleep modulation was due to whole-day or evening dietary Trp intake, of which breakfast measures may be a proxy.

5.6. Conclusion

Most of the beneficial effects of Trp loading on sleep have been shown in subjects with some sleep disturbances, such as patients with mild-moderate insomnia or healthy subjects reporting a longer than average sleep latency. In these subjects, Trp doses as low as 1 g have been shown to improve subjective measures of sleepiness and decrease sleep latency, and doses below 1 g have produced trends in a similar direction. However, modulations to sleep stages have only been observed with doses above 5 g Trp (see Fig. 3).

Interestingly, in healthy subjects who manifest no sleep disturbances, Trp loading has also been shown to increase subjective ratings of sleepiness and reduce sleep latency. Furthermore, Trp appears to be an effective daytime hypnotic for healthy adults, by facilitating sleep onset at times outside the normal circadian rhythm, and modulating sleep stages during the day. Thus, it may be that during wakefulness, Trp loading has a relaxing and calming effect in healthy adults.

In patients with severe sleep insomnia, Trp loading does not appear to be effective as a hypnotic. This may be because Trp lacks the potency that other hypnotic drugs have, or study doses have not been high enough to produce any sleep benefits. However, the literature does suggest that Trp loading may be an effective hypnotic for severe insomniac patients when consumed sub-chronically or intermittently.

In infants, reports indicate that Trp loading improves sleep. Moreover, it seems that varying the Trp content in milk formulas that are appropriate to light–dark variations (i.e. low Trp levels during daytime feeding and high Trp levels during nighttime feeding) improves the sleep/wake cycles of infants who are not breast fed.

6. Discussion

The beneficial effects of Trp loading on cognition are rather modest and not always found. ATD studies have provided the fundamental insights into which cognitive functions are susceptible to modulation of 5-HT and the direction of effects. It would be expected that Trp loading would produce opposite effects to that of ATD. However, this has not generally been the case. Reports vary considerably across the different cognitive domains, study designs, and populations. There are several possible explanations for this.

Given the few Trp loading studies that have been performed, there are considerable differences in methodology, such as, Trp doses, method of administration, treatment regimes, and variances in study populations, which all affect the central 5-HT effects that are achieved, and thus the behavioural outcome of the manipulation.

Variations in Trp dose will inevitably produce different results. However, in addition, variations in Trp found in plasma may also modulate the outcome. As metabolism differs across individuals and populations, the level of plasma Trp would seem to be an important aspect to be taken into consideration. However, only a limited number of studies address this point. It may be that variance in performance is dependent on the level of Trp found in blood rather than as a function of dose administered. Furthermore, plasma Trp–LNAA should also be considered as a means to identify that a specific amount of Trp has been transported to the brain in order to better understand the outcome. This notion is illustrated in Figs. 1 and 2. In healthy subjects, plasma Trp–LNAA increases up to 80% have not been found to modulate mood and memory (with

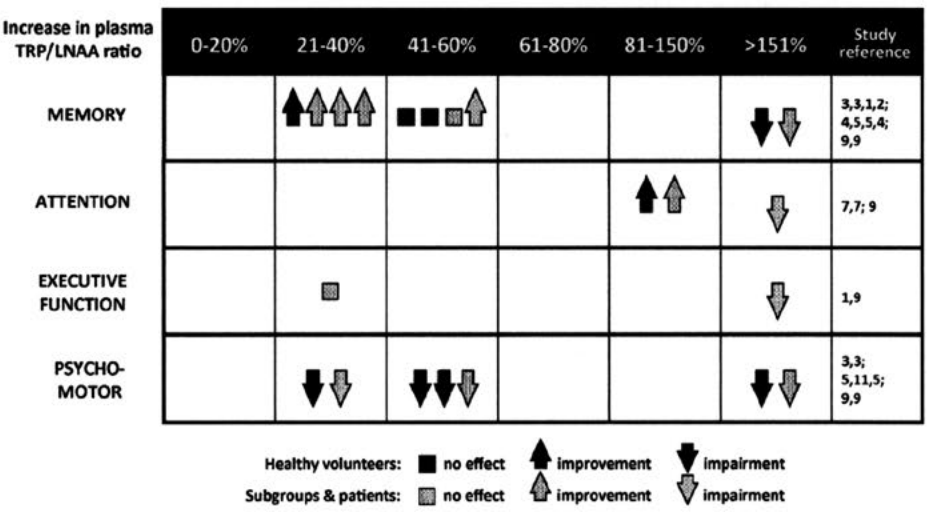

Fig. 1. Summary of the cognitive changes by increase in tryptophan versus large neutral amino acid (TRP–LNAA) ratios after tryptophan loading. Reference numbers are linked to the symbols in order of appearance in the rows and refer to the studies described in Table 1.

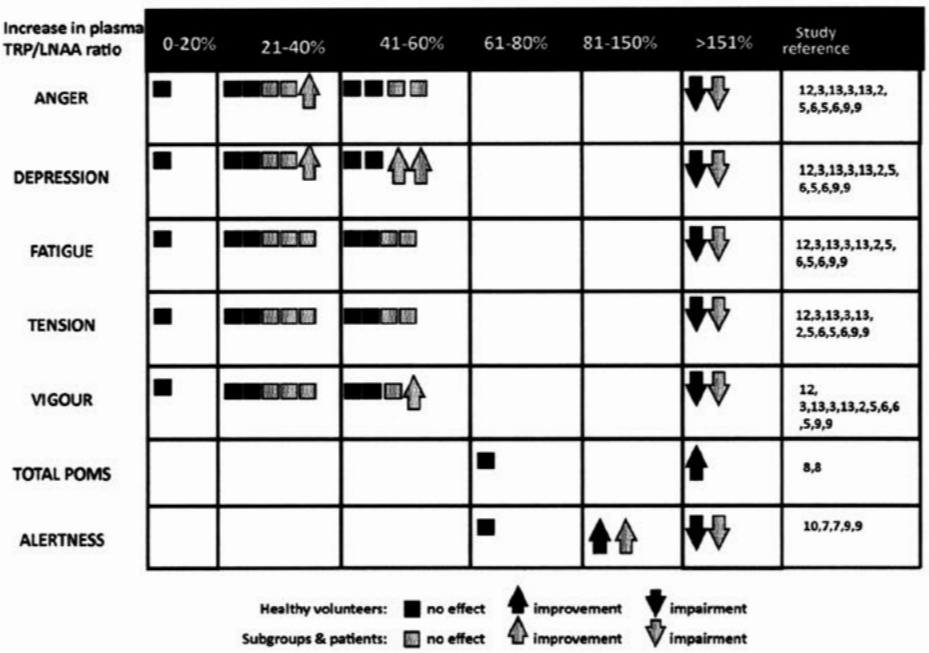

Fig. 2. Summary of the mood changes by increase in tryptophan versus large neutral amino acid (TRP–LNAA) ratios after tryptophan loading. Reference numbers are linked to the symbols in order of appearance in the rows and refer to the studies described in Table 2.

exception of one study). However, larger increases in plasma Trp–LNAA ratio (191–255%) have been shown to improve mood and attention (Markus et al., 2005, 2008). While, considerably large increases in Trp–LNAA ratio (up to 1500%) have been shown to impair memory performance and produce negative mood effects (Luciana et al., 2001; Sobczak et al., 2002, 2003). In vulnerable subjects, it appears that the most beneficial effects of Trp loading on memory, mood and alertness are attained when plasma Trp–LNAA ratio increases 20–60% from baseline. Significant increases in plasma Trp–LNAA ratio (i.e. above 151%) produce negative effects across the different cognitive domains. Note that psychomotor performance has consistently been shown to be impaired across all populations irrespective of the plasma Trp–LNAA ratio. This will be addressed later in the discussion. Future research should report changes in the plasma Trp–LNAA ratio as this may help elucidate the variance in Trp loading effects on cognitive functions in both normal and vulnerable individuals.

The effects of Trp loading may also be dependent on the initial state of the serotonergic system of the subject. Trp loading may either move serotonin towards the optimal level (if the subject has a hypo-serotonergic state to begin with (i.e. depressed, stress-vulnerable, women with premenstrual complaints), thus improving performance, or move serotonin beyond the optimal level if the subject is already at the optimal level to begin with (healthy subjects), thus decreasing performance or producing no effects. Trp loading seems to improve memory performance in vulnerable subjects (i.e. premenstrual symptoms, recovered depressed patients, stress-vulnerable subjects following experimental stress, mild insomniacs), whereas, minimal modulations to memory performance have been shown in healthy subjects. Moreover, based on the ATD and Trp loading literature, it appears to be easier to induce cognitive decrements than to enhance performance in healthy subjects who already have a close to optimal performance level. ATD always move 5-HT activity away from its optimum, thus it is relatively easy to find decrements. However, it seems rather difficult to improve already optimal function. These differences in the initial state of the serotonin system may explain the variance in reported effects, and the lack of mirrored behavioural effects of opposite 5-HT manipulations. Comparing the effects of Trp loading in individuals with low versus normal serotonergic states in the same study could provide a clearer picture as to the influence of initial serotonergic state on the effects of Trp loading. Only few studies have implemented such a design and the general pattern of results is rather inconsistent. Markus et al. (1999, 2002) found improvements in memory scanning ability and mood (Markus et al., 1998, 2000) in high stress-vulnerable individuals with no effects in low stress-vulnerable subjects. In contrast, Booij et al. (2006) reported improvements in abstract visual memory in both vulnerable and control populations. Sobczak et al. (2002, 2003) reported decrements in memory and psychomotor performance and mood in both unaffected first-degree relatives of bipolar disorder patients and healthy controls. Finally, Merens et al. (2005) found no effect on mood in either recovered depressed patients or healthy controls. Taken together, these comparative studies do not indicate that initial state serotonergic function is a strong general determinant of the effects of Trp loading. However, the large heterogeneity in Trp loading methodology and dose, outcome measures and 'vulnerable' study populations hampers a clear cut comparison between studies. Furthermore, the actual presence and extent of a lowered serotonergic state in the investigated populations' remains an assumption as 5-HT activity or vulnerability is not actually measured in the studies.

An important question that therefore needs to be addressed is how we define serotonergic vulnerability. In the scientific literature it generally refers to a vulnerability or sensitivity to natural or experimental modulations or dysregulations to the serotonergic system (Jans et al., 2007). There are a range of factors that can modulate the serotonergic system which subsequently may produce a hypo-serotonergic state. These include innate factors, such as genetics, gender, personality characteristics, prenatal stress; and environmental factors, such as stress and drug use. It has been proposed that the serotonergic functioning of an individual will determine the individual's vulnerability to develop a 5-HT related disorder (Jans et al., 2007). Specifically, the model suggests that as long as the number of innate and environmental factors that disrupt serotonergic functioning are

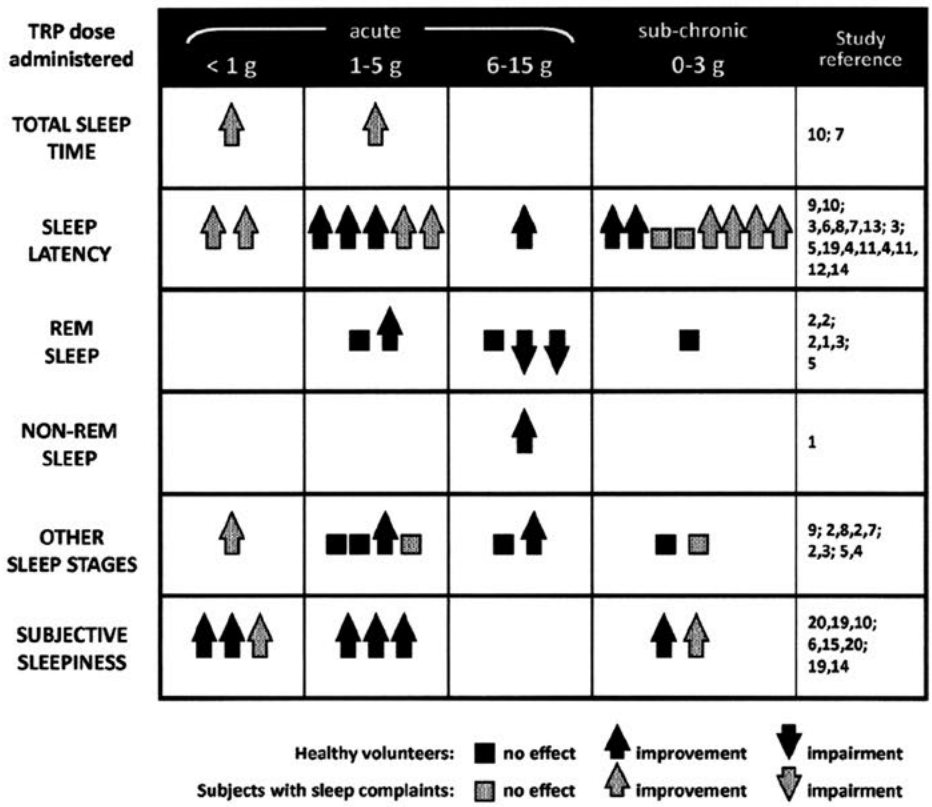

Fig. 3. Summary of the changes in sleep measures by dose of tryptophan administered acutely or sub-chronically. Reference numbers are linked to the symbols in order of appearance in the rows and refer to the studies described in Table 3.

limited, the disturbances they cause to the serotonergic system will be compensated for, and thus no overt behavioural changes should be observed. However, if several of these vulnerability factors occur, a threshold will be reached and the system will no longer be able to compensate (Jans et al., 2007). This is when overt behavioural changes will be observed and pathologies surface. The variance in performance following Trp loading may be related to what is proposed by this model and may also elucidate the lack of any mirror-behavioural effects with ATD reports. It is also important to note that studies implementing manipulations to the serotonergic system establish serotonergic vulnerability only as an outcome, an endpoint. Therefore, there will always be variations in the degree of serotonergic vulnerability within the study population (even if the population is seemingly homogenous i.e. depressed, stress-prone, healthy, etc.), and thus modulations in performance in response to 5-HT challenges will vary across subjects making interpretation of results difficult. Furthermore, differences in the form that serotonergic vulnerability presents itself, for example state or trait, may further complicate interpretation of the effects of various manipulations of the serotonergic system. Specifically, premenstrual syndrome in women or stress may be defined as a state level of serotonergic vulnerability as it is transient. Whereas, depression, a more stable and permanent form of hypo-serotonergic state, can be seen as trait. Thus, manipulations to the serotonergic system (5-HT challenges or ATD) may modulate serotonergic functioning differently for state or trait cases of serotonergic vulnerability, thus producing variance in behavioural outcomes.

It is unclear whether the relationship between serotonergic activity and cognitive function is a linear one or follows an inverted-U curve, where either too little or too much serotonin can impair performance. This may be true irrespective of the initial state of the serotonergic system of the individual. In vulnerable subjects, it seems that Trp loading improves performance when plasma Trp–LNAA ratio increases up to 150% from baseline. However, increases above this have been shown to negatively impact performance across various cognitive domains (Fig. 1). Similarly, in healthy subjects, the effects of Trp loading on memory and mood appear to follow the inverted-U curve hypothesis. Specifically, low doses of Trp attained from α-lactalbumin drinks have been shown to produce minimal effects on memory performance. Similarly, studies that have reported plasma Trp–LNAA increases up to 80%, have not reported mood to be modulated. However, large increases in plasma Trp–LNAA ratio (191–255%) have been shown to improve mood (Markus et al., 2008). Whereas, high doses of Trp (i.e. 7 g IV and 10.3 g) producing increases in Trp–LNAA ratio up to 1500% have been shown to impair memory performance and produce negative mood effects (Luciana et al., 2001; Sobczak et al., 2002, 2003). This could be related to both the initial serotonergic system of the individual (i.e. high serotonin function) or to the Trp dose administered (i.e. high doses). Independent of the positive association of 5-HT activity and function or the inverted-U curve hypothesis, ATD will always move 5-HT activity away from good functioning and thus it is easy to produce decrements. Currently there is insufficient evidence to either support or dismiss an inverted-U curve hypothesis. However, further research investigating this dose–response effect could provide insight into an optimal plasma Trp–LNAA ratio that may improve cognitive performance in healthy adults.

The effects of Trp loading differ across the cognitive domains. Specifically, Trp loading has quite consistently been shown to improve aspects of memory functioning in vulnerable subjects, yet impair motor and reaction time performance within the same population. This does not seem to be attributed to a dose effect as

the same doses and populations were employed across the cognitive domains. It may be that these cognitive functions are related to different neuroanatomical serotonergic pathways. The hippocampus has long been associated with learning and memory processes (van Strien et al., 2009) and serotonergic projections from the raphe nuclei innervate various hippocampal subregions (King et al., 2008). Long-term memory improvements observed following Trp loading may therefore be linked to increased serotonergic activity in the hippocampus. Animal studies have provided some evidence for memory enhancing effects of augmentation of 5-HT neurotransmission in the hippocampus (e.g. Haider et al., 2006, 2007) although other findings do not support this notion (e.g. Farr et al., 2000; Adams et al., 2008). Delineating the effects of global serotonergic manipulations on the hippocampus is difficult due to the complexity of serotonin receptor sub-types distribution on the different cell types in this region (Meneses, 1999). For example, agonist as well as antagonist 5-HT6 receptors in the hippocampus can improve cognition, and may be related to stimulation or inhibition (via GABAergic interneurons) actions on cholinergic and/or glutaminergic activity, depending on the localisation of the 5-HT6 receptors (King et al., 2008). In humans, decreased activation in the right hippocampus has been observed after ATD, but only during acquisition and not during retrieval of verbal information, and this has been hypothesised to reflect encoding and/or early consolidation deficits (Van der Veen et al., 2006). However, it must be noted that the serotonergic system also innervates other brain areas that are considered to be important for learning and memory, including the medial septum, entorhinal cortex and prefrontal cortext (King et al., 2008) and memory effects of serotonin may therefore result from a complex interplay of 5-HT actions on these as well as other brain areas.

In addition, part of the cognitive effects of Trp loading may be related to non-serotonergic mechanisms, particularly through melatonin. Melatonin production in the pineal gland varies dramatically in a circadian fashion that is internally controlled by the suprachiasmatic nuclei of the anterior hypothalamus with high levels of melatonin being synthesized and secreted during the dark phase and virtually no production during the light phase (Blask, 2009). Melatonin production is also inhibited by exposure to light of sufficient intensity (>200 lux) (Brzezinski, 1997). Nevertheless, Trp loading (3–5 g i.v.) has been shown to markedly and dose dependently increase circulating melatonin in humans during the day, albeit less pronounced as during the night (Hajak et al., 1991). The enterochromaffin cells of the gastrointestinal tract have been proposed as a significant source of circulating melatonin during the day (Bubenik, 2002) and particularly after Trp loading (see Huether, 1994). Interestingly, the melatonin production in the gastrointestinal tract appears to be regulated by food intake rather than photoperiodicity (Bubenik, 2002). Daytime conversion of Trp into melatonin may underlie mild sedating effects of Trp loading, especially at higher dosages, which may be most sensitively detected by psychomotor tasks. Finally, alteration of glutaminergic neurotransmission following Trp loading should be considered as potential mechanism of cognitive effects, as Trp may be metabolised via the kynurenine pathway, yielding quinolinic acid and kynurenic acid that can activate and antagonise the NMDA receptor, respectively (Ruddick et al., 2006).

Few studies have investigated the chronic effects of Trp loading on cognitive functioning in humans. This is an interesting avenue that should be explored further as it can provide insight into the potential long-term benefits of Trp loading. Animal studies have demonstrated that increases in brain 5-HT metabolism following long-term Trp administration enhance memory processes. In addition, human clinical trials assessing the effects of chronic Trp administration in the treatment of depression have demonstrated improvements to mood and a decrease in depressive symptoms. These results suggest that chronic administration of Trp does not necessarily decrease tolerance and desensitise the effects. It may be that Trp loading has more of an accumulative effect, and thus more robust cognitive effects may be seen following chronic administration in healthy adults.

Over the past decades, a role of the central serotonergic system in cognitive functioning has been revealed. In humans, these insights stem largely from experiments describing the consequences of reduced brain serotonergic function, often by means of tryptophan depletion. Now the challenge is to identify under which conditions an augmentation of serotonergic function can exert beneficial effects, particularly in 'healthy' individuals, i.e. without neuropsychiatric diseases, who nevertheless experience sub-optimal mental states (as discussed above). Optimizing mental health through restoration of state or trait serotonergic hypofunction in such individuals is obviously a compelling notion. In non-clinical populations a milder, non-pharmaceutical intervention such as oral Trp loading may be the preferred manner to achieve this. The current data seems to indicate that such an approach may be feasible and valuable, although the research has been rather fragmented and inconsistent in terms of methodologies. A structured approach, including investigations of dose–response relationships, chronic studies and comparative effects in well-characterised (presumably) serotonergically vulnerable populations will not only provide deeper fundamental insights into the role of serotonin in cognition and mood, but will also lead to evidence-based recommendations for the use of Trp to counteract cognitive and affective disturbances, particularly in non-clinical populations.

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Passiflora incarnata Linneaus as an anxiolytic before spinal anesthesia

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Abstract
Purpose Patients who undergo regional anesthesia experience anxiety in the preoperative period. *Passiflora incarnata* Linneaus is a plant that has traditionally been used as an anxiolytic and sedative. We aimed to investigate the effect of preoperative oral administration of *Passiflora incarnata* Linneaus on anxiety, psychomotor functions, sedation, and hemodynamics in patients undergoing spinal anesthesia.
Methods Under local ethics committee approval, 60 patients who were aged 25–55 years and ASAI–II and who were scheduled for spinal anesthesia were enrolled in this prospective, randomized, double-blind and placebo-controlled study. Thirty minutes before spinal anesthesia, baseline hemodynamic parameters, State-Trait Anxiety Inventory (STAI) score, sedation score, and psychomotor function test results were measured, then patients were randomly assigned to two groups: oral *Passiflora incarnata* Linneaus extract or placebo was given to the patients. Tests were repeated just before spinal anesthesia. Hemodynamics, sedation score, sensory-motor block and side effects were assessed during the operation. Psychomotor function tests were repeated at the end of the operation and 60 min after the operation.
Results There was a statistically significant difference between the two groups for the increase in State Anxiety Inventory (STAI-S) score obtained just before spinal anesthesia when compared to the baseline. There was no statistically significant difference in psychomotor function from the baseline for either group. A significant difference was not found between the two groups in demographics, psychomotor function, sedation score, hemodynamics, and side effects.
Conclusion Oral preoperative administration of *Passiflora incarnata* Linneaus suppresses the increase in anxiety before spinal anesthesia without changing psychomotor function test results, sedation level, or hemodynamics.

Keywords Anesthesia · Spinal · Anxiety · *Passiflora incarnata*

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Introduction

Regional anesthesia is popular and offers several benefits to the patient. On the other hand, some drawbacks are linked with regional anesthesia: pain at the puncture site, fear of needles, seeing the surgery, and recalling the procedure. Anxiolysis helps to achieve a calm and cooperative patient during placement of the block and decreases the response to needle puncture. Additionally, sedation usually reduces postoperative recall, which is important for many patients but can be undesirable [1].

Passiflora incarnata Linneaus is a plant that has traditionally been used as an anxiolytic and sedative throughout the world. The plant is widely used in phytotherapy due to its mild sedative and anxiolytic properties [2, 3]. Although some studies have demonstrated its anxiolytic properties [2, 4–9], two recent systematic reviews have emphasized that randomized controlled trials that compare the effectiveness of *Passiflora* with placebo or other types of

medication are needed [10, 11]. There is only one study in the literature that focuses on the anxiolytic effect of *Passiflora incarnata* Linneaus before general anesthesia [8], and there is no data on the preoperative oral administration of *Passiflora incarnata* Linneaus for anxiolysis before regional anesthesia.

We aimed to test the hypothesis that oral *Passiflora incarnata* Linneaus intake is an effective premedication before spinal anesthesia. The primary endpoint of the study was to investigate the effect of the preoperative oral administration of *Passiflora incarnata* Linneaus on anxiety levels of patients before spinal anesthesia. The other endpoints were to evaluate the level of sedation, psychomotor function, hemodynamics, and side effects in the *Passiflora* group and the placebo group.

Materials and methods

The study was approved by the appropriate Institutional Review Board, and written informed consent was obtained from all the patients. Sixty patients aged 25–55 years, ASA physical status I–II, who were scheduled for elective inguinal herniorrhaphy under spinal anesthesia were enrolled in this prospective, randomized, double-blinded and placebo-controlled study. Exclusion criteria included lack of cooperation, previous experience with surgery and spinal anesthesia, bronchial asthma, cigarette smoking habit, anxiety disorders, chronic consumption of alcohol or antidepressant, sedative, analgesic, antiepileptic, or anticoagulant drugs, and contraindications for spinal anesthesia.

At the preoperative visit, an investigator informed the patients about the tests used in the study. Forty-five minutes before spinal anesthesia, patients were brought to the premedication room in the operating suite. All patients were monitored with an electrocardiogram, pulse oximetry, and for noninvasive arterial blood pressure. No premedication was given to the patients. All patients were requested to complete both parts of the State-Trait Anxiety Inventory (STAI) questionnaire. The STAI consists of two distinct forms, the Trait Anxiety Inventory (STAI-T) and the State Anxiety Inventory (STAI-S) scores. The former measures basic anxiety, and the latter evaluates anxiety that can be induced or modified by changes in the environment [12]. Sedation level was measured using the Observer's Assessment of Alertness/Sedation (OAA/S) score [1]. Patient psychomotor function was assessed with the perceptive accuracy test (PAT) and the finger tapping test (FTT). Patients were asked to state the two or three digits number displayed on a calculator. The number of correct answers provided during a period of 2 min was recorded in PAT. The patients were asked to tap on the keyboard of the calculator. The number of times that the patient tapped on the keyboard in a 30 s period was taken as the finger tap score [13]. After the completion of the tests ($STAI\text{-}S_1$, $STAI\text{-}T_1$, PAT_1 and FTT_1), 30 min before spinal anesthesia, baseline hemodynamic parameters [heart rate (HR) and the systolic, diastolic, and mean arterial pressures (SAP, DAP and MAP)] were measured, and then patients were randomly assigned to two groups according to the numbers inserted into sealed envelopes: *Passiflora incarnata* Linneaus 700 mg/5 ml aqueous extract (*Passiflora* Syrup, Sandoz, Kocaeli, Turkey) was given to the patients in group P ($n = 30$), and the same volume (5 ml) of drinking water with mineral was given in group C ($n = 30$). Hemodynamic parameters, oxygen saturation (SpO_2), and the OAA/S score were noted every 10 min before spinal anesthesia. 8 ml/kg of sodium chloride 0.9% solution were infused intravenously during this period. Thirty minutes after drug administration, the patients were transported to the operating room and prepared for spinal anesthesia. All of the tests were repeated ($STAI\text{-}S_2$, $STAI\text{-}T_2$, PAT_2, and FTT_2) just before spinal anesthesia in the operating room. Tests were performed by an anesthesiologist who was unaware of the group in which the patient was involved. Spinal anesthesia was then performed with the patient in the sitting position, using a 25 G Quincke needle in the L_{4-5} interspace and a midline approach. Three milliliters of 0.5% hyperbaric bupivacaine were given via intrathecal injection. Hemodynamic parameters, SpO_2 value, sensory and motor blocks were assessed at 1, 3, 5, 7, and 10 min after the injection of the local anesthetic solution and then every 5 min. Sensory block was assessed using a pin-prick test. Motor block was assessed using modified Bromage scale (0 = no motor block, 1 = inability to raise extended legs, 2 = inability to flex knees, 3 = inability to flex ankle joints). The time to achieve a sensory block of T_{10}, the highest level of sensory block, the time to two-segment regression of the sensory block and the maximum degree of motor block were recorded. A 30% decrease from baseline SAP or SAP <90 mmHg was treated using incremental boluses of intravenous ephedrine 5 mg. Bradycardia was treated using intravenous atropine 0.5 mg. In the case of a failed spinal block, general anesthesia would be performed, and in the event of the need for sedation/analgesia, the patient would be discharged from the study. A decrease in SpO_2 to <93% in room air was defined as hypoxemia and treated with supplemental oxygen via a face mask.

Psychomotor function tests were repeated at the end of the operation (PAT_3, FTT_3) and 60 min after the end of the operation (PAT_4, FTT_4). In the postoperative care unit (PACU), patients received oxygen via a nasal cannula (4 l/min). Nausea and vomiting were treated with 10 mg metoclopramide intravenously. Patients were discharged from the PACU after the motor block was completely resolved. Paracetamol 1 g was given with a 15 min

infusion when the patient complained of pain in the postoperative period. The time to first analgesic requirement, time to discharge, and side effects were also noted. The patients were contacted at 24, 48, 72, and 168 h and asked to report backache, headache, or any transient neurological symptoms following the surgery.

Statistical analysis

It was calculated that a sample size of 30 patients per group was required to detect at least a five-score difference in STAI-S between the two groups with a power of 80% and $\alpha = 0.05$, based on a pilot study. Sample size was estimated using NCSS and the PASS (Hintze J., 2001, Number Cruncher Statistical Systems, Kaysville, UT, USA) statistical package program. All of the data were analyzed using SPSS 11.5 (SPSS Inc., Chicago, IL, USA) software. The Shapiro–Wilk test was used to test the normality of the distribution for continuous variables. Data are presented as mean ± SD, median (range), or number (incidence) as appropriate. Statistical analyses were performed using Student's t test for parametric data and the Mann–Whitney U test for nonparametric data. Fisher's exact test or chi-square test was used for categorical comparisons. Repeated measurements in groups were compared using a repeated measurements of variance analysis or the Friedman test, where applicable. The data for STAI-S and STAI-T scores and OAA/S scores were analyzed using the Wilcoxon signed-rank test. Statistical significance was set at the $p < 0.05$ level. Bonferroni adjustment was applied to control type I error in all possible multiple comparisons.

Results

Patient demographics are presented in Table 1. Spinal anesthesia was accomplished successfully in all patients.

Table 1 Demographic data

	Group P ($n = 30$)	Group C ($n = 30$)	p
Age (years)	50 (26–55)	44 (25–55)	0.434
Gender (F/M)	5/25	3/27	0.706
Weight (kg)	74.1 ± 11.4	75.6 ± 10.8	0.605
Height (cm)	169.2 ± 7.0	170.4 ± 8.3	0.538
BMI (kg m^{-2})	25.7 ± 3.4	26.4 ± 3.8	0.457
ASA I/II	19/11	18/12	0.791
Duration of operation (min)	62.8 ± 16.9	61.0 ± 20.4	0.480

Values are expressed as mean ± SD, median (minimum–maximum), or number of patients

BMI body mass index, *P Passiflora incarnata*, *C* control

Intraoperative sedation/analgesia was not administered. STAI-S_1 and STAI-T_1 scores were similar in both groups ($p = 0.728$ for STAI-S_1 and $p = 0.141$ for STAI-T_1). There was a statistically significant increase in STAI-S and STAI-T scores just before spinal anesthesia in group C (Table 2). A statistically significant difference from the baseline was found for the two groups in terms of the increase in STAI-S score obtained just before spinal anesthesia ($p = 0.004$ for STAI-S and $p = 0.293$ for STAI-T). PAT and FTT scores were similar in both groups ($p = 0.723$ and $p = 0.096$, respectively). There was no statistically significant difference from the baseline for either group in terms of the PAT and FTT scores (Table 3). The OAA/S scores for the groups were not significantly different during the study period (Mann–Whitney U test, $p > 0.05$). There was no statistically significant difference from the baseline for either group in terms of the OAA/S score (Wilcoxon signed-rank test $p > 0.05$ in each group) (Fig. 1). There was no statistically significant difference between the groups in terms of the characteristics of the sensory and motor blocks, the time to first analgesic requirement, the time to discharge (Table 4), and side effects. Nausea, vomiting, respiratory depression, shivering, or postoperative complications were not observed in any patient. The incidence of intraoperative hypotension was 3.3% (one patient) in group P and 10% (three patients) in group C ($p = 0.612$). The incidence of intraoperative bradycardia was 10% (three patients) in group P and 6.7% (two patients) in group C ($p = 1.00$). Hemodynamic parameters and SpO_2 values were similar in both groups during the study period.

Discussion

The results of the study demonstrate that preoperative oral administration of *Passiflora incarnata* Linneaus 700 mg/5 ml aqueous extract suppresses the increase in anxiety before spinal anesthesia, and *Passiflora incarnata* Linneaus is a safe and effective anxiolytic remedy.

We used the STAI scale and OAA/S scale to evaluate patients' anxiety and sedation levels, respectively. STAI is a well-established instrument for self-reporting anxiety [12], and the OAA/S scale may be the best choice if precise assessment of sedation is required [1]. Age, ASA physical status >III, history of smoking, and type of operation are important factors that affect preoperative anxiety [14]. We paid attention to these criteria before patient selection. Nonsmokers were enrolled in the study. Patients who chronically consume alcohol or antidepressant, sedative, analgesic, or antiepileptic drugs are prone to anxiety disorders, so we excluded these patients from the study.

Table 2 Anxiety scores in the two groups

	STAI-S_1	STAI-S_2	p	STAI-T_1	STAI-T_2	p
Group P ($n = 30$)	36.4 ± 10.9	35.7 ± 10.8	0.311	32.5 ± 9.5	33.4 ± 8.7	0.421
Group C ($n = 30$)	34.8 ± 8.4	36.6 ± 7.6	<0.001*	35.3 ± 8.3	38.1 ± 9.2	0.004†

Values are expressed as mean ± SD

STAI-S_1 Baseline State Anxiety Inventory score, *STAI-S_2* State Anxiety Inventory score obtained just before spinal anesthesia, *STAI-T_1* Baseline Trait Anxiety Inventory score, *STAI-T_2* Trait Anxiety Inventory score obtained just before spinal anesthesia

* $p < 0.001$ indicates a significant difference from the baseline score

† $p < 0.01$ indicates a significant difference from the baseline score

Table 3 The perceptive accuracy test (PAT) and the finger tapping test (FTT) scores in both groups

	Group P ($n = 30$)	Group C ($n = 30$)		Group P ($n = 30$)	Group C ($n = 30$)
PAT_1	98.0 ± 2.6	95.2 ± 16.4	FTT_1	67.4 ± 18.9	72.3 ± 14.1
PAT_2	98.0 ± 2.9	98.6 ± 2.3	FTT_2	68.1 ± 19.8	70.9 ± 13.0
PAT_3	98.6 ± 2.1	98.9 ± 2.4	FTT_3	67.4 ± 19.7	71.6 ± 12.6
PAT_4	99.1 ± 1.7	99.1 ± 1.4	FTT_4	67.6 ± 19.8	72.3 ± 13.1
p	0.138	0.565	p	0.151	0.172

Values are expressed as mean ± SD

PAT_1, *FTT_1* baseline score, *PAT_2*, *FTT_2* score obtained just before spinal anesthesia, *PAT_3*, *FTT_3* score obtained at the end of the operation, *PAT_4*, *FTT_4* score obtained 60 min after the end of the operation

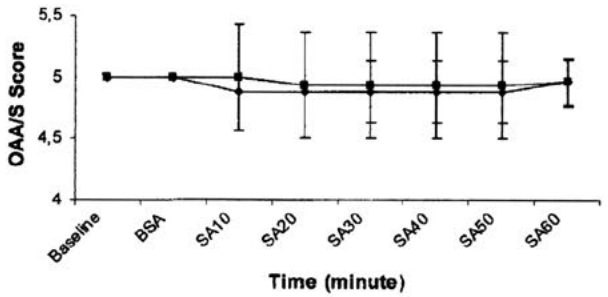

Fig. 1 Observer's Assessment of Alertness/Sedation (OAA/S) scores at different time intervals in the *Passiflora* group (*filled diamonds*) and in the control group (*filled squares*). *BSA* before spinal anesthesia, *SA10* 10 min after spinal anesthesia, *Group P Passiflora incarnata*, *Group C* control

Anxiolytic drugs and sedatives are administered before surgery for the purpose of calming patients. On the other hand, sedation involves some risks, especially the induction of respiratory depression, hemodynamic instability, or uncontrolled movements [1]. Using a safe and cheap herbal remedy with an anxiolytic effect as an alternative to conventional anxiolytic–sedative drugs for alleviating patient anxiety levels before regional anesthesia may seem worthwhile.

Amongst the 500 species of the genus *Passiflora*, *Passiflora incarnata* Linneaus is the one used most extensively for clinical applications throughout the world. *Passiflora incarnata* Linneaus is included in the nine plants for which there is considerable evidence of therapeutic effect, and is marketed in Western countries [15]. The sedative and anxiolytic activities of *Passiflora incarnata* Linneaus have been attributed to benzodiazepine and γ-aminobutyric acid receptor-mediated biochemical processes in the body [16, 17]. There are many different preparations of *Passiflora incarnata* Linneaus that vary considerably in their constituents. Although the compounds responsible for the therapeutic activity of *Passiflora incarnata* Linneaus are yet to be identified, phytomedicines should be made using plant material characterized by the typical flavonoid profile [18]. We used the aqueous extract of *Passiflora incarnata* Linneaus that contained 2.8 mg benzoflavone per 5 ml extract. The therapeutic dose of *Passiflora incarnata* Linneaus is 500–1000 mg three times daily [19]. We used the anxiolytic dose (700 mg/5 ml) that is suggested for adults in the prospectus. The peak anxiolytic activity of *Passiflora incarnata* Linneaus was noted to occur at 30 min after oral administration [8]. *Passiflora incarnata* Linneaus was given to the patients 30 min before spinal anesthesia in the study.

There is only one study in the literature on the use of *Passiflora incarnata* Linneaus before general anesthesia for its anxiolytic effect [8]. Movafegh et al. have used a tablet form of *Passiflora incarnata* Linneaus extract (500 mg) 90 min before general anesthesia [8]. Unlike in their study, we used a liquid extract of the plant 30 min before spinal anesthesia, and we investigated the effects of the extract in awake patients under spinal anesthesia. Movafegh et al. [8] suggested that oral administration of *Passiflora incarnata* Linneaus significantly reduces preoperative anxiety levels,

Table 4 Characteristics of spinal anesthesia in the two groups

	Group P ($n = 30$)	Group C ($n = 30$)	p
Time to T_{10} (min)	10.8 ± 6.1	9.6 ± 5.0	0.709
Highest level (dermatome)	T_7 (T_{10}–T_3)	T_7 (T_{10}–T_6)	0.221
Time to two-segment regression (min)	78.6 ± 27.6	83.5 ± 24.0	0.310
Maximum motor block degree (n) (Bromage 2/3)	2/28	1/29	1.000
Time to first analgesic requirement (min)	92.0 ± 53.7	88.0 ± 57.8	0.714
Time to discharge (min)	316.0 ± 58.8	319.0 ± 67.9	0.694

Values are expressed as mean ± SD, median (minimum–maximum), or number of patients

but does not affect the preoperative sedation level, recovery time, or psychomotor function test results after extubation. They found that psychomotor function test results were impaired 30 min after extubation but reached their preoperative values 90 min after extubation in both groups. In our study, we did not find any impairment in psychomotor function test results during the study period. We observed that *Passiflora incarnata* Linneaus does not affect discharge after spinal anesthesia.

It was stated that there is a significant relationship between the maximum extent of sensory block and the level of sedation during spinal anesthesia [20]. In our study, maximum sensory block levels were similar in both groups, and hence the maximum level of sensory block did not affect the results for the sedation levels.

There are a few reports of side effects of *Passiflora incarnata* Linneaus ingestion. Side effects include cutaneous vasculitis, urticaria, asthma, and rhinitis [21, 22]. These side effects are very rare, and only occurred after chronic usage [22]. Many herbal remedies influence platelet activity. It was suggested that aqueous extract of *Passiflora incarnata* Linneaus inhibits human platelet aggregation by only 1.3% in platelet-rich plasma induced by ADP. The maximum inhibitory effect (90%) was observed with garlic, followed by alfalfa (73%), onion (71%), fresh nettle (65%), and chamomile (60%) [23]. We used a single dose of aqueous extract before spinal anesthesia, and no side effect was seen after the procedure. Hemodynamic parameters did not change after the administration of *Passiflora incarnata* Linneaus when compared to those of the placebo. Lack of intraoperative sedation and respiratory depression is an another advantage of this plant. According to our results, *Passiflora incarnata* Linneaus is a safe and effective anxiolytic remedy that can be used before spinal anesthesia.

One limitation of this study is that there are statistically significant but small differences in STAI scores. Subsequent investigations of the clinical usefulness of *Passiflora incarnata* Linneaus may be useful to support our findings. Another limitation is that it is difficult to produce a placebo with the same color and taste as *Passiflora incarnata* Linneaus. To exclude this limitation, the drug or the placebo was given to the patient in a dark colored glass. The patients were asked whether they noticed the group in which they were enrolled. In the event of a patient being aware of which group they were in, they would have been excluded from the study. However, none of the patients noticed the group in which they were enrolled.

It was concluded that preoperative oral administration of 700 mg/5 ml of *Passiflora incarnata* Linneaus aqueous extract suppresses the increase in anxiety levels of patients before spinal anesthesia without changing their sedation level, psychomotor function test results, or hemodynamics.

Conflict of interest No conflict of interest exists.

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$GABA_A$ receptors as *in vivo* substrate for the anxiolytic action of valerenic acid, a major constituent of valerian root extracts

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ABSTRACT

Valerian extracts have been used for centuries to alleviate restlessness and anxiety albeit with unknown mechanism of action *in vivo*. We now describe a specific binding site on $GABA_A$ receptors with nM affinity for valerenic acid and valerenol, common constituents of valerian. Both agents enhanced the response to GABA at multiple types of recombinant $GABA_A$ receptors. A point mutation in the β2 or β3 subunit (N265M) of recombinant receptors strongly reduced the drug response. *In vivo*, valerenic acid and valerenol exerted anxiolytic activity with high potencies in the elevated plus maze and the light/dark choice test in wild type mice. In β3 (N265M) point-mutated mice the anxiolytic activity of valerenic acid was absent. Thus, neurons expressing β3 containing $GABA_A$ receptors are a major cellular substrate for the anxiolytic action of valerian extracts.

1. Introduction

Various extracts from the roots of plants of the genus *Valeriana* (Valerianaceae) are used in herbal medicine of many cultures as mild sedatives and tranquilizers. Valerenic acid is a main constituent of *Valeriana officinalis*, most widely used in Europe and USA. In animal experiments, valerenic acid or extracts from valerian showed tranquilizing and/or sedative activity (Bent et al., 2006; De Feo and Faro, 2003; Hendriks et al., 1985). Activation of adenosine receptors has been implicated in the action of valerian ingredients (Müller et al., 2002; Schumacher et al., 2002). Valerenic acid was also shown to modulate or – at high concentrations – activate $GABA_A$ receptors as shown for recombinant receptors expressed in *Xenopus* oocytes (Khom et al., 2007) or neonatal brain stem neurons (Yuan et al., 2004). However, the molecular mechanism of action of any of the valerian ingredients *in vivo* is yet unknown. We have now identified a specific binding site for valerenic acid and valerenol, common constituents of valerian, on $GABA_A$ receptors. In addition, it was demonstrated that valerenic acid mediates anxiolytic activity via $GABA_A$ receptors containing the β3 subunit. Mice containing a single amino acid point mutation in the β3 subunit [β3(N265M)] failed to show anxiolytic activity to valerenic acid, but maintained an anxiolytic response to diazepam.

2. Materials and methods

2.1. Chemicals

Valerenic acid was purchased from Extrasynthese, Lyon (France). TTX was purchased from Alomone Labs (Israel). All other chemicals were from Sigma–Aldrich (Switzerland). The α1 subunit-specific antibody is described in Benke et al. (1991).

2.2. Synthesis of valerenic acid derivatives

Valerenol (**2**), valerenal (**3**), and *O*-methyl valerenol (**4**) were synthesized according to Scheme S1.

2.2.1. Valerenol (2)

Valerenic acid (**1**) (275 mg, 1.17 mmol, 1 eq) was dissolved in THF (10 ml) under Ar. A 1 M solution of $LiAlH_4$ in THF was then added (1.1 ml, 1.1 mmol, 1 eq) and the reaction mixture was stirred at room temperature for 2 h. The reaction was quenched with diluted aq H_2SO_4 and extracted with diethyl ether. The organic phase was dried over Na_2SO_4 and concentrated *in vacuo*. The resulting crude product was purified by flash chromatography (FC) in AcOEt/hexane 1/4, yielding 238 mg (92%) of valerenol (**2**) as colourless oil. AcOEt = ethyl acetate.

R_f (AcOEt/hexane 1/4): 0.36 (vanilline/H_2SO_4) ^{1}H NMR (500 MHz, $CDCl_3$): 5.75 (d, J = 9.3 Hz, 1H), 4.01 (s, 2H), 3.46 (dd, J_1 = 4.9 Hz, J_2 = 8.5 Hz, 1H), 2.94–2.92 (m, 1H), 2.20 (t, J = 7.6 Hz, 2H), 1.97–1.96 (m, 1H), 1.89–1.76 (m, 3H), 1.73 (s, 3H), 1.65 (s, 3H), 1.59–1.49 (m, 1H), 1.40–1.27 (m, 2H), 0.77 (d, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): 147.5, 135.1, 133.0, 129.1, 69.2, 47.3, 37.4, 33.4, 33.1, 26.1, 24.5, 28.5, 13.7, 13.5, 12.0.

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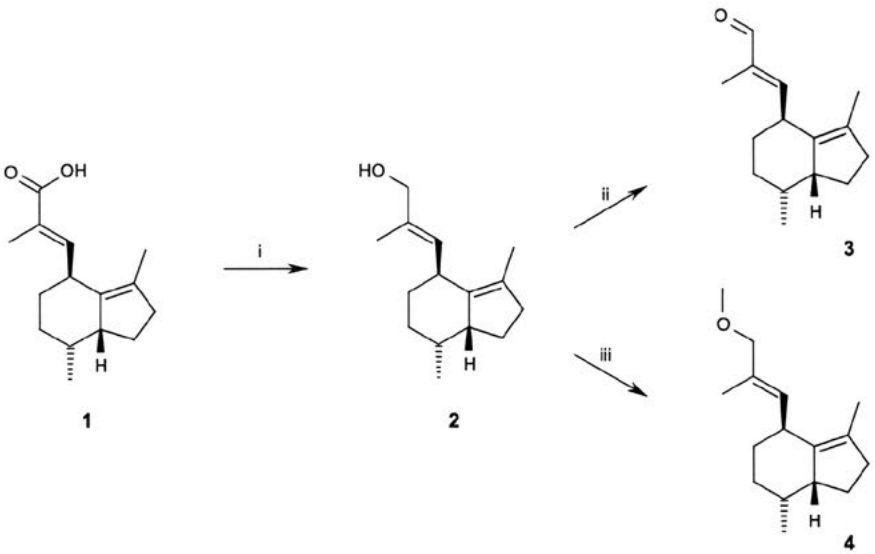

Scheme S1. (i) $LiAlH_4$, THF, rt, 2 h, 92%. (ii) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −78 °C → RT, 30 min, 89%. (iii) CH_3I, NaH, ether, −78 °C → RT, 84%.

*2.2.2. Valerenal (**3**)*

A solution of oxalyl chloride (9 µl, 0.102 mmol, 1.5 eq) in CH_2Cl_2 (0.5 ml) under Ar was cooled to −78° C and DMSO (7.5 µl, 0.102 mmol, 1.5 eq) was added. After 15 min a solution of valerenol (**2**) (15 mg, 0.068 mmol, 1 eq) in CH_2Cl_2 (0.1 ml) was added followed by triethylamine (125 µl, 0.340 mmol, 5 eq; after 15 additional min). The cooling bath was removed after 5 min and stirring was continued at room temperature for 30 min. Then the reaction was quenched with water and the mixture extracted with diethyl ether. The organic layer was separated, dried over Na_2SO_4, and concentrated *in vacuo*. The resulting crude product was purified by FC (ether/pentane 1/10), yielding 13.3 mg (89%) of valerenal (**3**) as a colourless oil.

R_f (ether/pentane 1/2): 0.39 (vanillin/H_2SO_4). ^{1}H NMR (500 MHz, $CDCl_3$): 9.38 (s, 1H), 6.75 (qd, J_1 = 9.8 Hz, J_2 = 1.3 Hz, 1H), 3.74–3.69 (m, 1H), 2.92–2.98 (m, 1H), 2.24–2.19 (t, J = 7.4 Hz, 2H), 2.06–1.97 (m, 1H), 1.89–1.81 (m, 3H), 1.80 (d, J = 1.3 Hz, 3H), 1.65 (s, 3H), 1.61–1.53 (m, 1H), 1.50–1.42 (m, 2H), 0.80 (d, J = 7.3 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): 195.9, 156.1, 139.9, 137.3, 132.4, 47.3, 37.2, 34.5, 32.7, 28.6, 25.0, 24.3, 13.4, 11.7, 9.1.

*2.2.3. O-methyl valerenol (**4**)*

To a solution of valerenol (**2**) (19 mg, 0.09 mmol, 1 eq) in diethyl ether (1 ml) was added sodium hydride (2.3 mg, 0.10 mmol, 1.1 eq) at −78 °C under Ar. After 15 min methyl iodide (6 µl, 0.10 mmol, 1.1 eq) was added and the mixture stirred at room temperature for 1 h. The reaction was then quenched with water and extracted with diethyl ether. The organic layer was separated, dried over Na_2SO_4, and concentrated *in vacuo*. The crude product was purified by FC (AcOEt/hexane 1/10), yielding 16 mg (84%) of *O*-methyl valerenol (**4**) as a colourless oil.

R_f (AcOEt/hexane 1/10): 0.76 (vanillin/H_2SO_4). ^{1}H NMR (500 MHz, $CDCl_3$): 5.73 (dq, J_1 = 9.1 Hz, J_2 = 1.3 Hz, 1H), 3.83–3.76 (m, 2H), 3.48–3.43 (m, 1H), 3.28 (s, 3H), 2.96–2.89 (m, 1H), 2.19 (t, J = 7.6 Hz, 2H), 2.00–1.92 (m, 1H), 1.88–1.77 (m, 2H), 1.75–1.67 (m, 1H), 1.70 (d, J = 7.6 Hz, 3H), 1.65–1.63 (m, 3H), 1.57–1.50 (m, 1H), 1.40–1.34 (m, 2H), 0.77 (d, J = 6.9 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): 135.1, 130.2, 129.8, 129.0, 78.9, 57.2, 47.3, 37.4, 33.3, 33.2, 28.6, 26.2, 24.5, 13.7, 13.2, 12.0.

2.3. Preparation of crude neuronal membranes

Crude membranes were prepared from adult rat brain tissue. Following homogenization in 10 volumes of 10 mM Tris–HCl, pH 7.4, 0.32 M sucrose, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and centrifugation at 1000g for 10 min the resulting supernatant was centrifuged for 20 min at 12,000g to obtain the crude membrane pellet. The crude membranes were resuspended in buffer and stored at −80 °C until used. For [^{3}H]valerenic, [^{3}H]flunitrazepam and [^{3}H]flumazenil binding, crude membranes were thawed and washed three times in 50 mM Tris pH 7.4. For [^{3}H]muscimol binding, crude membranes were resuspended in 20–40 volumes of 5 mM Tris–HCl 7.4, frozen in liquid nitrogen, thawed and centrifuged for 20 min at 45,000g. This procedure to eliminate endogenous GABA was repeated two times. Radioligand binding using [^{3}H]muscimol, [^{3}H]flunitrazepam and [^{3}H]flumazenil was performed as described previously (Benke et al., 1991).

2.4. [^{3}H]valerenic binding assay

To establish a radioligand binding assay, valerenic acid was custom-labeled by tritium using random catalytic exchange of hydrogen by tritium (6.5 Ci/mmol; RC Tritec, Teufen, Switzerland). For [^{3}H]valerenic acid binding, crude rat brain membranes were washed three times in 50 mM Tris–HCl pH 7.4 and incubated (200 µg protein) with 300 nM [^{3}H]valerenic acid in a total volume of 0.2 ml for 45 min at room temperature. Subsequently, the reactions were filtered though glass microfiber filters and washed with ice cold buffer (50 mM Tris–HCl pH 7.4). Filters were then processed for liquid scintillation counting using a Tricarb 2500 liquid scintillation analyzer. Nonspecific binding was assessed in parallel by co-incubation with 100 µM unlabelled valerenic acid. Binding of [^{3}H]valerenic acid (300 nM) to well-washed membranes increased linearly with the protein concentration up to 1.5 mg/ml. Equilibrium of binding was reached after 20 min at room temperature (measured at 50 and 300 nM [^{3}H]valerenic acid and 200 µg protein). Saturation binding experiments were performed with 3–600 nM [^{3}H]valerenic acid and 200 µg protein per assay. Binding data were analyzed using the programs Kell (Biosoft, UK) and GraphPad Prism 4.0 (GraphPad Software, USA).

2.5. Immunoprecipitation

Crude membranes prepared from rat brain tissue were washed once with 10 mM Tris–HCl pH 8, 150 mM NaCl, 1 mM EDTA, 200 mg/l bacitracin, 0.1 mM phenylmethylsulfonyl fluoride, 2.3 mg/l aprotinin, 1 mM benzamidine and resuspended in the same buffer to a protein concentration of 5 mg/ml followed by addition of sodium deoxycholate to a final concentration of 0.5%. After incubation for 30 min at 4 °C, insoluble material was removed by centrifugation for 30 min at 100,000g. For immunoprecipitation of $GABA_A$ receptors, aliquots (0.5 ml) of the deoxycholate extract were incubated with α1 subunit-selective antiserum overnight at 4 °C (Benke et al., 1991). Receptor–antibody complexes were precipitated by incubation with 50 µl of protein A–agarose for 60 min. After extensive washing, the precipitates were resuspended in 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 200 mg/l bacitracin, 0.1 mM phenylmethylsulfonyl fluoride, 2.3 mg/l aprotinin, 1 mM benzamidine, 0.2% Triton X-100 and subjected to radioligand binding experiments using 300 nM [^{3}H]valerenic acid (see above), 6 nM [^{3}H]flumazenil and 10 nM [^{3}H]Ro 15-4513 as described previously (Benke et al., 1991).

2.6. cDNAs and site-directed mutagenesis

Rat α1, α2, α3, α4, α5, β2, β3 and γ2s $GABA_A$ receptor subunit cDNAs were used and transferred in human embryonic kidney cells (HEK) as described previously (Siegwart et al., 2002). For the β3 subunit, site-directed mutagenesis substituting an asparagine residue at position 265 with a methionine residue, β2(N265M) and β3(N265M) was performed as described (Siegwart et al., 2002, 2003).

2.7. Electrophysiology

Current recordings from recombinant $GABA_A$ receptors in HEK 293 cells were performed using the whole cell patch-clamp technique as described previously (Benson et al., 1998). Primary cultures of hippocampal neurons were prepared from wild type E18 rat brain (van Rijnsoever et al., 2005) and whole cell voltage-clamp recordings were made using standard techniques (Hamill et al., 1981). Briefly: cultures were continuously superfused with ACSF containing (in mM: 125 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, and 11 glucose, oxygenated with 95% O_2–5% CO_2), TTX was added to block Na channels and APV (100 µM) and NBQX

(20 μM) were added to isolate miniature inhibitory postsynaptic currents (mIPSCs). Whole cell voltage-clamp recordings of spontaneous mIPSCs from pyramidal cells were obtained at room temperature with a holding potential of −60 mV and a high chloride containing internal solution (in mM: 100 CsCl, 2 MgCl2, 1 EGTA, 2 ATP, 0.3 GTP, and 40 HEPES, pH 7.2, 300 mOsm).

2.8. Mutant mice

The generation and breeding of homozygous mutant mice containing a (N265M) mutation in the $GABA_A$ receptor β3 subunit on a 129/Sv × 129X1/SvJ background was performed as described previously (Jurd et al., 2003).

2.9. Behavioral tests

The anxiolytic drug action was tested in the light/dark choice test and in the elevated plus maze test as described (Löw et al., 2000; Rudolph et al., 1999). The animals were maintained on a 12:12 h reversed light/dark cycle, with *ad libitum* food and water. All manipulations described here had been approved by the Cantonal Veterinary Office of Zurich; they conformed to the ethical standards required by the Swiss Act and Ordinance on Animal Protection and the European Council Directive 86/609/EEC.

2.10. Statistics

Data are expressed as mean ± SEM or mean ± SD as indicated in the legend to the figure. If required, data were analyzed for statistical significance using the test indicated in the respective figure legend. p Values less than 0.05 were considered significant.

3. Results

3.1. Allosteric interaction of valerenic acid with the GABA and benzodiazepine binding site

Using radioligand binding, we aimed at identifying the binding site for valerenic acid. In a first set of experiments the potential interaction of valerenic acid with the benzodiazepine and GABA binding site was analyzed. [^{3}H]flumazenil, [^{3}H]flunitrazepam and [^{3}H]muscimol binding to crude rat brain membranes was dose-dependently increased by valerenic acid to a maximum of 200–500% with EC_{50} values of 23 ± 1, 7 ± 1 and 42 ± 6 μM, respectively (Fig. 1). Valerenol also potentiated benzodiazepine binding to $GABA_A$ receptors with an EC_{50} of 16 ± 7 μM as tested in the [^{3}H]flunitrazepam binding assay (not shown). These results indicate that valerenic acid and valerenol allosterically interact with the benzodiazepine and GABA binding sites of $GABA_A$ receptors.

3.2. [^{3}H]valerenic acid binding

To directly assess the valerenic acid binding site of $GABA_A$ receptors, valerenic acid was radiolabeled by random catalytic exchange of hydrogen by tritium. [^{3}H]valerenic acid binding to brain membranes revealed both a high affinity binding site (K_D = 25 ± 20 nM) and a low affinity site (K_D = 16 ± 10 μM). [^{3}H]valerenic acid was displaced not only by valerenic acid but also by valerenol with even higher potency (IC_{50} = 3 ± 2 nM, Table 1). A 1000-fold lower displacing potency was found for hydroxy-valerenic acid whereas *O*-methyl valerenol, valerenal and valeric acid (each at 100 μM) were completely inactive (Table 1).

To verify that $GABA_A$ receptors harbour the binding site for [^{3}H]valerenic acid, $GABA_A$ receptors were immunoprecipitated from deoxycholate extracts of rat brain membranes using an antibody directed against the α1 subunit. Radioligand binding to the well-washed immunoprecipitates demonstrated that the valerenic acid binding site was coimmunoprecipitated with the classical benzodiazepine sites as demonstrated by the specific [^{3}H]flumazenil, [^{3}H]Ro 15-4513 and [^{3}H]valerenic acid binding to the precipitate (Fig. 2). Thus, high affinity [^{3}H]valerenic acid binding sites are located on $GABA_A$ receptors.

To test whether the [^{3}H]valerenic acid binding site corresponds to any of the well established modulatory binding sites of the $GABA_A$ receptor, competition experiments were performed. Ligands for the binding sites of GABA (THIP, SR95531), benzodiazepines (flunitrazepam), barbiturates (phenobarbital), channel blocker (picrotoxinin) and loreclezole (each up to 100 μM) did not affect [^{3}H]valerenic acid binding (not shown). However, [^{3}H]valerenic acid binding was enhanced in the presence of propofol (680 ± 126%), etomidate (190 ± 21%) and alphaxolon (200 ± 19%) (each at 100 μM), suggesting an allosteric interaction with sites for these anaesthetics. The anti-inflammatory agent mefenamic acid, which shares structural similarities with loreclezole and etomidate (Halliwell et al., 1999), was found to inhibit [^{3}H]valerenic acid binding with an IC_{50} of 7 ± 3 μM. These results suggest that valerenic acid may bind to a previously unrecognized site on $GABA_A$ receptors that is allosterically linked to sites for anaesthetics and mefenamic acid.

3.3. Effect of valerenic acid of recombinant receptors expressed in HEK 293 cells

Valerenic acid was recently shown to enhance the GABA-induced chloride currents of recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes (Khom et al., 2007). Here we used a different expression system (HEK 293 cells) to confirm a positive allosteric potentiation of recombinant $GABA_A$ receptors by valerenic acid. Valerenic acid strongly enhanced a submaximal

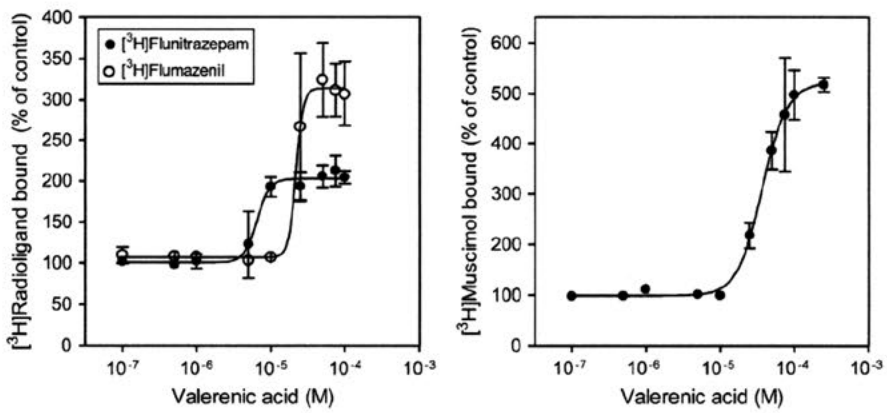

Fig. 1. Valerenic acid increases radioligand binding to the benzodiazepine and GABA binding site. Well-washed rat brain membranes were incubated with increasing concentrations of valerenic acid in the presence of [^{3}H]flunitrazepam (0.5 nM), [^{3}H]flumazenil (0.25 nM) and [^{3}H]muscimol (10 nM), respectively. Nonspecific binding was determined by co-incubation with clonazepam (10 μM) for [^{3}H]flunitrazepam and [^{3}H]flumazenil binding or with GABA (100 μM) for [^{3}H]muscimol binding. Data represent the mean ± SEM of three independent experiments.

Table 1
Inhibition of [^{3}H]valerenic acid binding

Drug	IC_{50} (μM)	n
Valerenol	0.003 ± 0.002	5
Hydroxy-valerenic acid	61 ± 19	3
O-methyl valerenol	>100	2
Valerenal	>100	2
Valeric acid	>100	2

The potencies of various agents in inhibiting binding of [^{3}H]valerenic acid were determined by incubating rat brain membranes with [^{3}H]valerenic acid (300 nM) in the presence of increasing concentrations of the respective compounds. Nonspecific binding was determined in the presence of excess cold valerenic acid. Data represent the mean ± SD of the indicated number (*n*) of independent experiments.

GABA response (EC_{10} at 3 μM) on $GABA_A$ receptors expressed in HEK 293 cells, as demonstrated for the subunit combinations α1β2γ2 (EC_{50} = 5.6 ± 0.6 μM), α2β2γ2 (11.8 ± 0.9 μM), α3β2γ2 (8.1 ± 1.3 μM) and α5β2γ2 (8.6 ± 1.8 μM) with similar Hill coefficients (Hill 1.7 ± 0.2; 1.6 ± 0.2; 1.7 ± 0.4; 1.7 ± 0.5, respectively) (Fig. 3A). The GABA response at α2β3γ2 was likewise enhanced by valerenic acid as tested at 10 μM (Fig. 3D). Valerenic acid (10 μM) enhanced the GABA response also at α4β2γ2 receptors (350 ± 50% of control) while diazepam but not bretazenil (Benson et al., 1998) was inactive (Fig. 3B).

In the absence of GABA, there was no direct activation of $GABA_A$ receptors by 10 μM valerenic acid as tested at α1β2γ2 receptors (not shown). At higher concentrations of valerenic acid (≥30 μM) a direct activation of α1β2γ2 receptors was previously observed (Khom et al., 2007).

At α1β2 $GABA_A$ receptors, valerenic acid (up to 50 μM) failed to enhance the GABA response, while alphaxolone and propofol (each at 10 μM) retained their potentiating activity (Fig. 3C).

Valerenol, a congener of valerenic acid, likewise enhanced the GABA response as tested at α2β3γ2 receptors (Fig. 3D). However, structurally different compounds such as valeric acid, valeric anhydride, valeronitrile, δ-valerolactame and γ- or δ-valerolactone (each at 10 μM) failed to alter the GABA response as measured at α1β2γ2 receptors (not shown).

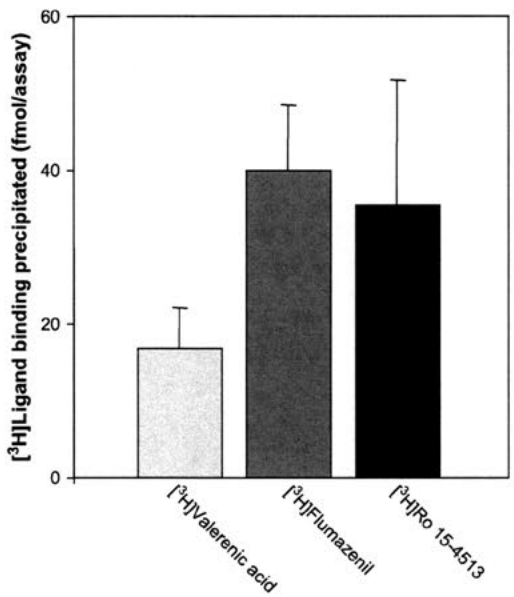

Fig. 2. Co-immunoprecipitation of [^{3}H]valerenic acid binding sites with $GABA_A$ receptors. $GABA_A$ receptors were immunoprecipitated from solubilized rat brain membranes using α1 subunit-selective antibodies and probed for the presence of [^{3}H]valerenic acid (300 nM), [^{3}H]flumazenil (6 nM) and [^{3}H]Ro 15-4513 (10 nM) binding. Data represent the mean ± SD of three independent experiments.

Based on its mutation to serine, the asparagine residue 265 in the β2 subunit was suggested to play an important role for the activity of valerenic acid on β2 containing recombinant receptors (Khom et al., 2007). We used β2 and β3 subunit cDNAs, in which the asparagine residue 265 was mutated to methionine (Siegwart et al., 2002). When recombinant $GABA_A$ receptors were expressed in HEK 293 cells in the subunit combination α1β2(N265M)γ2 or α2β3(N265M)γ2, the ability of valerenic acid to enhance the GABA response was strongly diminished (Fig. 3D). The GABA potentiating effect of valerenol (10 μM) was likewise strongly reduced as tested in α2β3(N265M)γ2 receptors, as compared to the corresponding wild type receptor (Fig. 3D). This result suggests that the integrity of the valerenic acid binding site or the transduction of the GABA potentiating effect is impaired when the N265M mutation is present in either the β2 or β3 subunit.

3.4. Potentiation of synaptic GABAergic responses by valerenic acid

Valerian extracts and valerenic acid are known to reduce the discharge rate in muscimol-sensitive brain stem neurons of neonatal rats (Yuan et al., 2004). However, it was not clear whether valerenic acid would enhance the amplitude or the kinetics of the GABA response. Whole cell patch-clamping experiments were therefore performed on cultured hippocampal neurons and miniature inhibitory postsynaptic currents (mIPSPs) were measured. The synaptic GABAergic response in hippocampal neurons was enhanced by valerenic acid as shown by an increase of the peak amplitude of the GABAergic mIPSCs from 37.5 ± 4.8 to 53.1 ± 4.5 pA (mean ± SEM, $n = 7$, $p < 0.01$) without an apparent change in kinetics (Fig. 4A, B). These results demonstrate that valerenic acid potentiates the GABA response not only at recombinant receptors but also on synaptic $GABA_A$ receptors *in situ*.

3.5. The anxiolytic activity of valerenic acid is mediated by $GABA_A$ receptors containing the β3 subunit

To assess the relevance of $GABA_A$ receptors in mediating the effects of valerenic acid *in vivo*, the anxiolytic activity was chosen as pharmacodynamic parameter. In the light/dark choice test, valerenic acid (1, 3 and 6 mg/kg i.p. or 10 mg/kg p.o.) showed a significant effect by decreasing the aversion of wild type mice to the lit area (Fig. 5A, C). Valerenol (1, 10, 30 mg/kg i.p. or 1, 10 mg/kg p.o.) likewise decreased the aversion to the lit area in the light/dark test (Fig. 5B, D). The anxiolytic activity of valerenic acid (10 mg/kg p.o.) was also demonstrated in the elevated plus maze by the enhancement of the time spent on the open arms (Fig. 5E). There was no indication for sedation induced by valerenic acid (10 mg/kg p.o. and 30 mg/kg i.p.) as tested by analyzing locomotor activity in an actimeter for at least 1 h (not shown). Thus, a higher receptor occupancy may be required for a potential sedative effect of valerenic acid compared to its anxiolytic action.

To verify that the anxiolytic action of valerenic acid was exclusively mediated via $GABA_A$ receptors *in vivo* and not by a yet unknown target, the compound was tested in mice containing the N265M point mutation in the β3 subunit gene of $GABA_A$ receptors. In the β3(N265M) mice, in contrast to wild type mice, valerenic acid (3 mg/kg i.p.) failed to show anxiolytic-like activity in the light/dark test (Fig. 5F). After oral administration, valerenic acid (10 mg/kg) likewise displayed strongly reduced anxiolytic-like activity in this test in the β3(N265M) mice compared to controls (not shown). As a control, it had to be verified that the β3(N265M) mutation would not interfere with the animals principal ability to display an anxiolytic-like response to a $GABA_A$ receptor drug. When diazepam (1.5 mg/kg i.p.) was tested in the β3(N265M) mice, it retained its

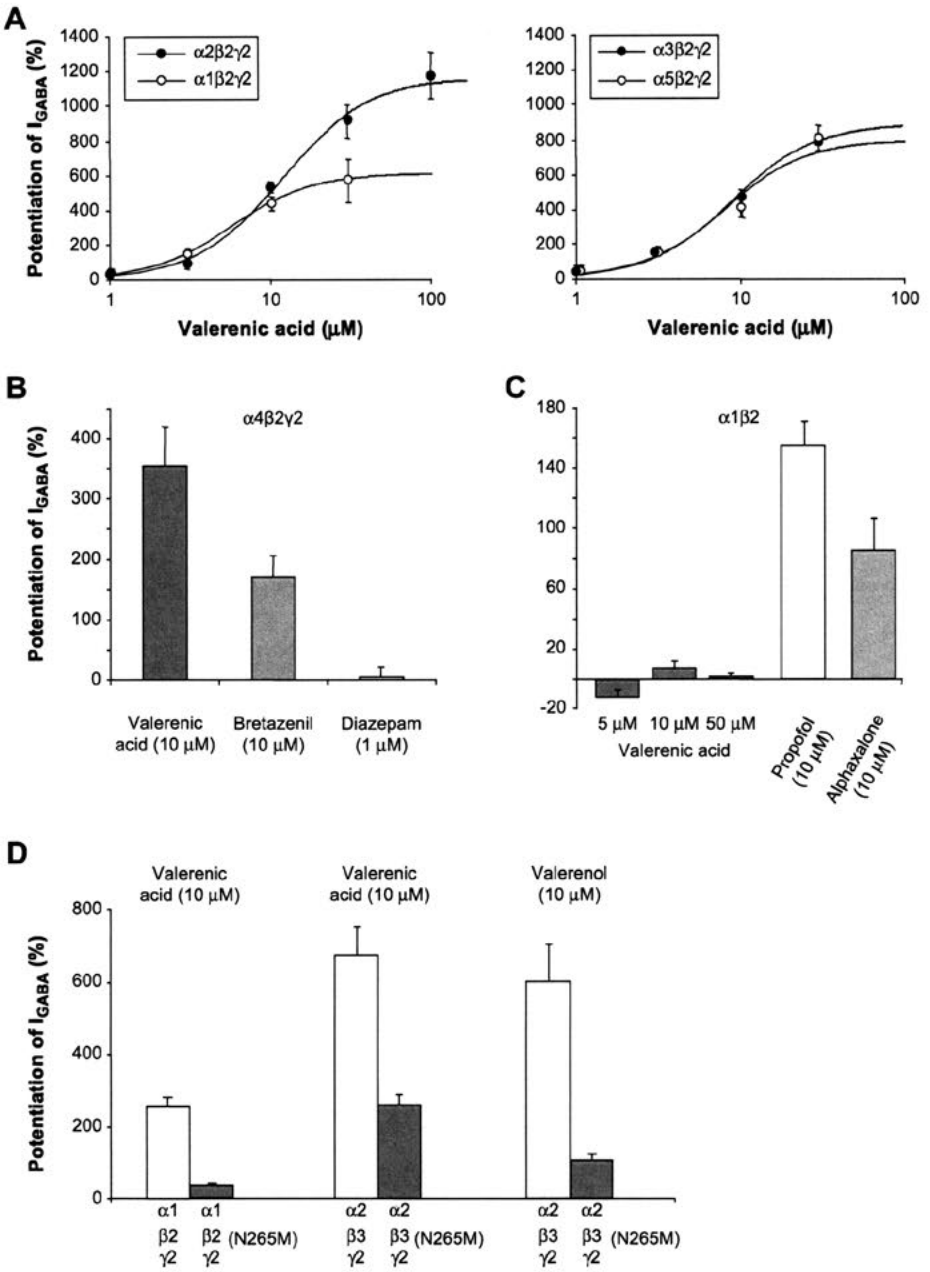

Fig. 3. Effect of valerenic acid and valerenol on whole cell currents of HEK 293 cells expressing the indicated $GABA_A$ receptor subunit combinations. (A) Valerenic acid potentiated GABA-induced currents (3 μM) in HEK 293 cells expressing the subunit combination α1β2γ2 ($EC_{50} = 5.6 \pm 0.6$ μM, $n_H = 1.7 \pm 0.2$) α2β2γ2 ($EC_{50} = 11.8 \pm 0.9$ μM, $n_H = 1.6 \pm 0.2$), α3β2γ2 ($EC_{50} = 8.1 \pm 1.3$ μM, $n_H = 1.7 \pm 0.4$) and α5β2γ2 ($EC_{50} = 8.6 \pm 1.8$ μM, $n_H = 1.7 \pm 0.5$). Data represent the mean peak currents ± SEM ($n = 4–8$). Error bars smaller than the symbol are not shown. Data were fitted using the equation: enhance (y) = 1/{1 + (EC_{50}/[valerenic])Hill}. (B) Valerenic acid (10 μM) and bretazenil (10 μM), but not diazepam (1 μM), increased GABA-activated currents (3 μM) of α4β2γ2 receptors. Data represent the mean peak currents ± SEM ($n = 4–8$). (C) GABA-activated currents (3 μM) at α1β2 receptors were potentiated by propofol (10 μM) and alphaxalone (10 μM), but not by valerenic acid (5–50 μM). Data represent the mean peak currents ± SEM ($n = 4–8$). (D) Recombinant receptors containing the N265M mutation in the β2 or β3 subunit displayed a strongly reduced sensitivity to the modulatory effects of valerenic acid and valerenol (3 μM GABA for α1β2γ2, α2β3γ2; 7 μM GABA for α1β2[N265M]γ2, α2β3[N265M]γ2). Data represent the mean peak currents ± SEM ($n = 6–14$).

anxiolytic activity as shown by the significantly increased time spent in the lit area in the light/dark test (Fig. 5F). Thus, the point mutation (N265M) in the β3 subunit of $GABA_A$ receptors is sufficient to completely prevent the ability of valerenic acid to display anxiolytic-like activity *in vivo*. This result strongly argues for β3 subunit containing $GABA_A$ receptors to be the molecular target for the anxiolytic-like action of valerenic acid. By implication, neuronal circuits expressing β3 containing $GABA_A$ receptors qualify as cellular targets.

4. Discussion

$GABA_A$ receptors containing the β3 subunit have been identified as the major target which mediates the anxiolytic action of valerenic acid, a major constituent of valerian root extracts. This result is based on several lines of evidence.

1. By [^{3}H]valerenic acid binding to brain membranes, a novel site was discovered which displayed high nM affinity for valerenic

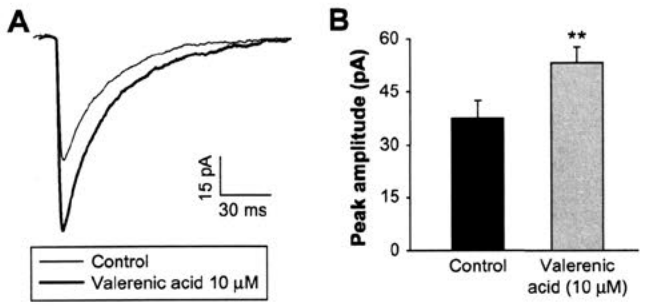

Fig. 4. Valerenic acid (10 μM) increases the peak amplitude of GABAergic mIPSCs without affecting their kinetics in cultured hippocampal neurons. (A) Averaged and superimposed mIPSCs (every trace represents the mean of at least 40 events) in control conditions (thin line) and in presence of valerenic acid (thick line). (B) Summary of the mIPSCs peak enhancement induced by valerenic acid (control, 37.5 ± 4.8 pA; valerenic acid, 53.1 ± 4.5 pA; mean ± SEM $p < 0.01$, paired t-test, $n = 7$).

acid and valerenol but not for structurally close derivatives for valerenic acid. This binding site was located on $GABA_A$ receptors as shown by the enhancement of [^{3}H]valerenic acid binding by various known $GABA_A$ receptor ligands. In addition, when $GABA_A$ receptors were solubilized and immunoprecipitated with anti-α1 subunit antibodies, [^{3}H]valerenic acid binding was enriched in the immunoprecipitate together with the benzodiazepine binding site. Judging by the ligand selectivity, the binding site appeared to differ from the known drug modulatory sites on $GABA_A$ receptors. Valerenic acid enhanced radioligand binding to the benzodiazepine and GABA site, while [^{3}H]valerenic acid binding was potentiated by anaesthetics like propofol, etomidate and alphaxolon, indicating an allosteric interaction with these sites. The only drug tested that inhibited [^{3}H]valerenic acid binding, albeit only at μM concentrations, was the anti-inflammatory agent mefenamic acid. This compound shares structural similarities with loreclezole and etomidate and was suggested to interact with the same or overlapping sites (Halliwell et al., 1999). Therefore, the binding sites for valerenic acid and merfenamic acid may be overlapping or allosterically linked.

2. Valerenic acid and valerenol were shown to act as positive allosteric modulators of $GABA_A$ receptors as demonstrated for both recombinant receptors and receptors *in situ*. On recombinant receptors (Fig. 3) valerenic acid acted as positive allosteric modulator not only on the classical benzodiazepine-sensitive $GABA_A$ receptors, characterized by the α1, α2, α3 or α5 subunit, but differed from classical benzodiazepines in that valerenic acid enhanced the action of GABA also on α4β2γ2 receptors. These results confirm previous findings on recombinant receptors of Khom et al. (2007). On α1β2 receptors, valerenic acid (up to 50 μM) failed to potentiate the GABA response. This finding is at variance with the observation of a potentiation ($EC_{50} = 5.2 \pm 2.4$ μM) of the GABA response by valerenic acid as tested on α1β2 receptors expressed in *Xenopus* oocytes (Khom et al., 2007). Although this discrepancy is presently unexplained, it is not of major relevance in the present context, since αβ receptors are a negligible complement of $GABA_A$ receptors *in vivo*. The lack of response of valerenic acid in our experiments in HEK 293 cells (Fig. 3D) is not due to a failure of α1β2 receptor expression, since, as expected, propofol and alphaxolone potentiated the GABA response at α1β2 receptors (Fig. 3C).
3. The β2(N265M) and the β3(N265M) point mutations inhibited the GABA potentiating effect of valerenic acid on recombinant receptors. This result implies that it is the β2 or β3 subunit in the $GABA_A$ receptors which determines the activity of valerenic acid. This result does not necessarily imply that the binding site of valerenic acid includes the residue in position 265. The β2(N265M) and the β3(N265M) point mutations are known to impair to various degrees the effects of diverse drugs such loreclezole, propofol or etomidate (Rudolph and Antkowiak, 2004). Thus, the point mutation may interfere with drug binding and/or with signal transduction within the $GABA_A$ receptor complex. Our results refer to $GABA_A$ receptors containing the β2 or the β3 subunit and thereby extend previous findings, which were restricted to the N265S mutation in the β2 subunit (Khom et al., 2007).
4. The results on recombinant receptors were substantiated by whole cell current recordings in hippocampal neurons in culture. Valerenic acid significantly enhanced the peak amplitude of the GABA-induced current without apparent changes in current kinetics (Fig. 4). This result is in keeping with the ability of valerenic acid (100 μM) to enhance the effect of muscimol in rat brain stem neurons (Yuan et al., 2004). In hippocampal CA1 pyramidal cell somata, the fast phasic currents in hippocampal CA1 pyramidal cell somata are known to be predominantly mediated by α2 $GABA_A$ receptors (Prenosil et al., 2006). Thus, the hippocampal neuronal somatic response to valerenic acid was most likely due to an enhancement of α2 $GABA_A$ receptors.
5. In order to elucidate the mechanism of action of valerenic acid *in vivo*, it was necessary to ascertain that the *in vivo* activity was indeed mediated via $GABA_A$ receptors and not by another yet unknown target. Therefore, the anxiolytic activity of valerenic acid was determined *in vivo* in wild type mice in comparison to β3(N265M) mice. This mutant mouse strain was chosen based on prior experience. On recombinant receptors, both the β2(N265M) and the β3(N265M) mutations strongly reduced the activity of valerenic acid and of valerenol (Fig. 3D). However, judging by the response of diverse $GABA_A$ receptor drugs tested previously on β2(N265M) or β3N265M) mutant mice, the β3(N265M) mutation generally showed a stronger differentiation of drug responses than the β2(N265M) strain *in vivo* compared to wild type (Rudolph and Antkowiak, 2004). Therefore, the mutant mice containing the β3(N265M) point mutation were chosen for the analysis of the mechanism of action of valerenic acid *in vivo*. In the light/dark choice test, an anxiolytic activity was demonstrated in wild type mice for valerenic acid administered either i.p. or p.o. (Fig. 5A, C) as well as for valerenol (Fig. 5B, D) at a potency comparable to that of diazepam in this test (Löw et al., 2000). Similarly, valerenic acid (10 mg/kg p.o.) showed anxiolytic activity in the elevated plus maze test, as shown by the significant increase in the time spent on the open arm (Fig. 5E). Most importantly, valerenic acid failed to display anxiolytic activity in mice containing the β3(N265M) mutation as tested in the light/dark choice test (Fig. 5F) in a concentration which produced a robust response in wild type mice (Fig. 5A). The failure of the β3(N265M) mice to display the anti-aversive activity of valerenic acid treatment is not due to the inability of the point-mutated animals to perform in this test. When the β3(N265M) mutants were treated with diazepam, a clear anti-aversive effect of the drug was apparent (Fig. 5F). At recombinant receptors containing the β3(N265M) mutation, valerenic acid displayed a weak residual activity (Fig. 3D). This finding is not reflected *in vivo*, where the anxiolytic effect of valerenic acid was absent in the β3(N265M) mutant mice. Furthermore, the lack of an anxiolytic response of valerenic acid in the (β3N265M) mice suggests that $GABA_A$ receptors containing the β2 subunit do not play a major role for the anxiolytic activity of valerenic acid. Thus, neurons expressing $GABA_A$ receptors containing the β3 subunit are

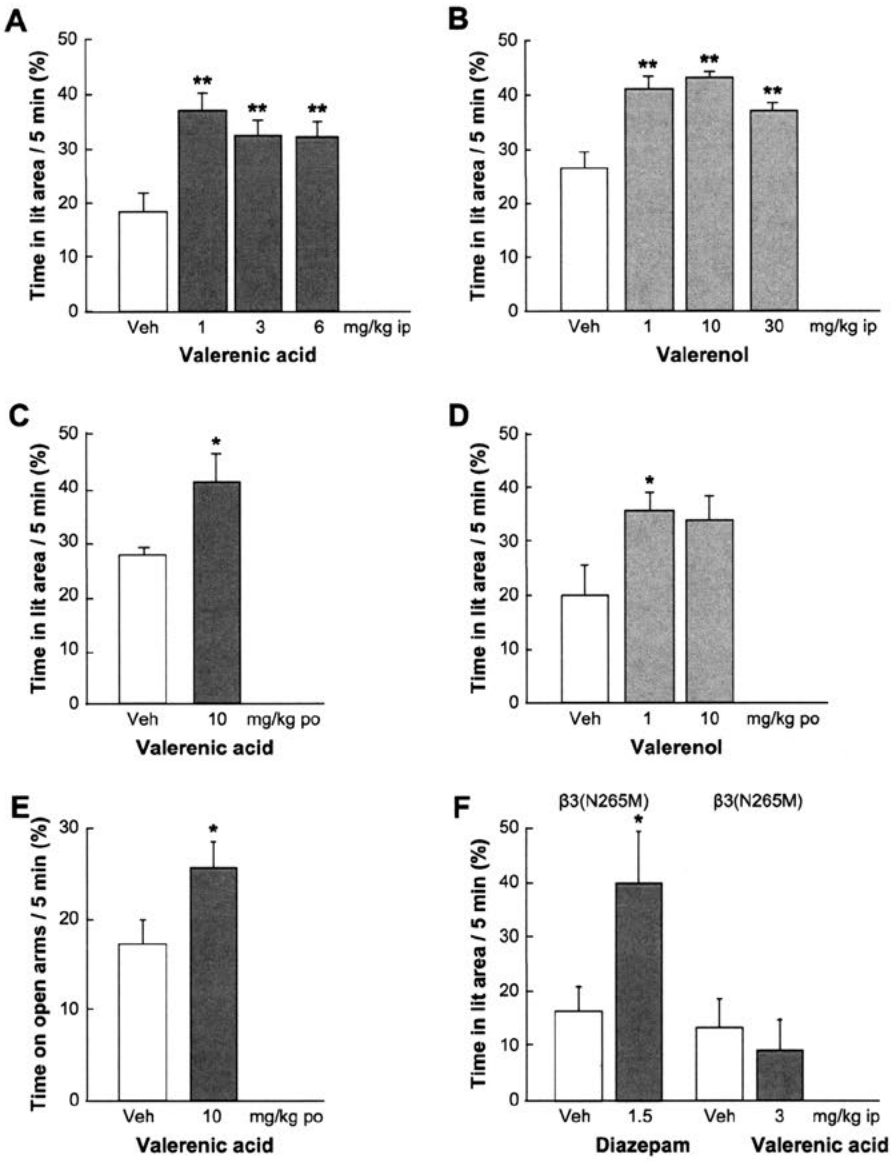

Fig. 5. Behavioral effects of valerenic acid and valerenol in the light/dark choice test and the elevated plus maze in mice. (A) Anxiolytic-like effects of valerenic acid (1, 3 and 6 mg/kg given i.p. 15 min before test) in the light/dark choice test. At all doses tested, valerenic acid treatment increased the proportion of time 129X1/SvJ mice spent in the lit area during the 5-min test session (ANOVA, $F(3, 30) = 7.14$, $p < 0.001$, $n = 8–9$ per group). (B) Likewise, valerenol administered i.p. at the dose range of 1–30 mg/kg decreased significantly the aversion to the lit area ($F(3, 25) = 12.42$, $p < 0.001$, $n = 7–8$ per group). (C) Oral administration of valerenic acid at the dose of 10 mg/kg was effective in reducing the aversion to the lit area ($U = 6$, $p < 0.05$ as compared to vehicle, Mann–Whitney, $n = 5–8$ per group). (D) Valerenol administered orally at the doses of 1 and 10 mg/kg increased the proportion of time spent in the lit area; this effect achieved significance at the dose of 1 mg/kg ($F(2, 17) = 3.68$, $p < 0.05$, $n = 6–7$ per group). (E) In the elevated plus maze, valerenic acid treatment (10 mg/kg orally) increased significantly the proportion of time 129X1/SvJ mice spent onto the open arms ($U = 34$, $p < 0.05$, $n = 11–12$ per group). (F) Mice containing the β3(N265M) mutation failed to display the anti-aversive effect of valerenic acid (3 mg/kg i.p.) ($U = 26$, *not significant*, $n = 7–8$ mice per group) as tested in the light/dark test. Nevertheless, diazepam (1.5 mg/kg i.p.) enhanced the time spent in the lit area in the β3(N265M) mice compared to vehicle ($U = 13$, $p < 0.05$, $n = 8–9$ per group). Results are expressed as means ± SEM; Veh, Vehicle. $^{*}p < 0.05$. $^{**}p < 0.01$ as compared to vehicle, Dunnett's tests after ANOVA, Mann–Whitney (U) tests.

major mediators of the anxiolytic activity of valerenic acid. Previously, the anxiolytic activity of benzodiazepines has been shown to be mediated via α2 $GABA_A$ receptors (Löw et al., 2000). These receptors largely contain β3 subunits in addition to a γ2 subunit. Thus, benzodiazepines and valerenic acid share, at least in part, a common anxiolytic molecular and cellular substrate in those neuronal circuits which express α2β3γ2 $GABA_A$ receptors.

Valerenic acid amounts up to about 2% of valerian extracts and was found in commercial preparations at a mean concentration of 3.5 mg/g powder capsules with the highest values of 6.3 mg/g (Shohet et al., 2001; Gao and Björk, 2000). The potency of valerenic acid in two widely used anxiolytic animal test models (Fig. 4A, E) was comparable to that of diazepam (Löw et al., 2000). Assuming that the pharmacokinetic properties in man are likewise comparable to diazepam, the daily dose of valerenic acid can be expected to be sufficient to induce anxiolytic activity. Valerenic acid is the main constituent in valerian extracts to convey an enhancement of $GABA_A$ receptors (Trauner et al., 2008). Thus, valerenic acid and valerenol would largely account for the anxiolytic action of valerian extracts.

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Research Report

Valerian extract and valerenic acid are partial agonists of the 5-HT_{5a} receptor in vitro

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Abstract

Insomnia is the most frequently encountered sleep complaint worldwide. While many prescription drugs are used to treat insomnia, extracts of valerian (*Valeriana officinalis* L., Valerianaceae) are also used for the treatment of insomnia and restlessness. To determine novel mechanisms of action, radioligand binding studies were performed with valerian extracts (100% methanol, 50% methanol, dichloromethane [DCM], and petroleum ether [PE]) at the melatonin, glutamate, and $GABA_A$ receptors, and 8 serotonin receptor subtypes. Both DCM and PE extracts had strong binding affinity to the 5-HT_{5a} receptor, but only weak binding affinity to the 5-HT_{2b} and the serotonin transporter. Subsequent binding studies focused on the 5-HT_{5a} receptor due to the distribution of this receptor in the suprachiasmatic nucleus of the brain, which is implicated in the sleep–wake cycle. The PE extract inhibited [^{3}H]lysergic acid diethylamide (LSD) binding to the human 5-HT_{5a} receptor (86% at 50 μg/ml) and the DCM extract inhibited LSD binding by 51%. Generation of an IC_{50} curve for the PE extract produced a biphasic curve, thus GTP shift experiments were also performed. In the absence of GTP, the competition curve was biphasic (two affinity sites) with an IC_{50} of 15.7 ng/ml for the high-affinity state and 27.7 μg/ml for the low-affinity state. The addition of GTP (100 μM) resulted in a right-hand shift of the binding curve with an IC_{50} of 11.4 μg/ml. Valerenic acid, the active constituent of both extracts, had an IC_{50} of 17.2 μM. These results indicate that valerian and valerenic acid are new partial agonists of the 5-HT_{5a} receptor.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Serotonin receptors

Keywords: Circadian rhythm; Insomnia; Serotonin; 5-HT_{5A}; Valerian; Valerenic acid

1. Introduction

Insomnia is considered the most frequent sleep complaint and affects nearly all populations throughout the world, particularly the elderly [28]. Menopausal women often suffer from sleep disturbances with frequent awakenings, likely due to disturbed expression of daily biological rhythms [4,30]. Some of the more commonly used hypnotic drugs to treat insomnia are benzodiazepines, which are associated with adverse events such as tolerance, dependence, and morning sleepiness [28]. Many menopausal women prefer not to use benzodiazepines but use valerian preparations instead, and report that these products are well tolerated, effective for the treatment of insomnia, while not inducing impairment of vigilance or cognitive and psychomotor performance [17,29,30].

Extracts from the roots of valerian (*Valeriana officinalis* L., Valerianaceae) have long been used in alternative medicine for the treatment of insomnia and are the most well recognized herbal sedatives worldwide. Approximately 15 controlled clinical trials have assessed the efficacy of various valerian extracts, and the German Commission E has approved its use for the treatment of restlessness and

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sleeping disorders [4]. Numerous clinical trials support the use of valerian and have demonstrated an improvement in sleep latency and quality in both healthy volunteers and patients with sleep disorder [4,29,33]. Currently, there is no scientific agreement on the mechanism of action of valerian's sedating activity or the compounds responsible. Many potential mechanisms for the pharmacological activity of valerian have been proposed, including agonistic activities on the GABA, adenosine, barbiturate, and benzodiazepine receptors [34,40,46]. However, the concentrations used in these investigations were extremely high in the older reports and more recent reports put some of these data into question [12,40].

Although serotonin is well known to modulate a variety of physiological and behavioral processes including sleep–wake cycles, and circadian rhythms, the effect of valerian on serotonin receptor binding has not been completely elucidated [9,24]. Serotonin (5-HT, 5-hydroxytryptamine) impacts numerous sensory, motor, and cortical functions by activating multiple 5-HT receptor subtypes [9,24,35]. Abnormalities of these receptor systems have been implicated in many psychiatric disorders including anxiety, depression, as well as disorders of cognition, stress, and sleep [9,28,35]. Serotonin was initially thought to be a true neuromodulator of sleep because the destruction of 5-HT neurons of the raphe system or the inhibition of 5-HT synthesis with p-chlorophenylalanine induced severe insomnia that could be reversed by restoring 5-HT synthesis [2]. More recent experiments suggest that the release of 5-HT during the sleep–wakefulness cycle initiates a cascade of genomic events in some hypnogenic neurons located in the preoptic area and the neighboring suprachiasmatic nucleus including vasoactive intestinal polypeptide and GABAergic mechanism [1,3,9,15,28,35].

Our previous work has shown that dichloromethane and petroleum ether extracts of valerian bind weakly (51% at 50 μg/ml) to the 5-HT_{2b} receptor but have significant binding (80%) at the 5-HT_{5a} receptor [12]. When compared with other serotonin receptors, little is known about the 5-HT_{5a} receptor, but it is believed to be involved in circadian (sleep–wake) rhythms, anxiety, and explorative behavior [15,18,20,42]. Distribution of the 5-HT_{5a} receptor is widespread throughout the rat brain, but the receptor concentrations are particularly intense in the suprachiasmatic nucleus of the hypothalamus, the site of the biological clock that drives circadian rhythms. Although the involvement of the 5-HT_{5a} receptors is a more recent hypothesis, support for the 5-HT_{5a}-mediated function in the SCN has been shown by strong distinct immunoreactivity in three neural components of the circadian timing system—the intergeniculate leaflet, median raphe, and dorsal raphe nucleus, in addition to the SCN [42].

Considering that our previous work indicated that valerian extracts bind predominantly to the 5-HT_{5a} receptor, the purpose of this work was to determine if the valerian extract acts as an agonist of this receptor. To this end, bioassay-guided fractionation of the DCM and PE extracts to isolate and identify the chemical constituents responsible for this activity was also performed.

2. Materials and methods

2.1. Materials and reagents

All chemicals and reagents were purchased from Fisher (Hanover Park, IL) or Sigma (St. Louis, MO) unless otherwise indicated. All cell culture media were obtained from Life Technologies (Carlsbad, CA). FBS was acquired from Atlanta Biologicals (Norcross, GA). [^{3}H]Lysergic acid diethylamide (LSD), [^{3}H]hydroxytryptamine (5-HT), and [^{3}H]-8-hydroxy-2-(di-*N*-propylamino)tetralin (8-OH-DPAT) were obtained from NEN Life Science Products (Boston, MA). 5-HT_{5a} membranes were purchased from Perkin-Elmer (Shelton, CT), while valerian reference compounds (valerenic acid, hydroxyvalerenic acid, and acetoxyvalerenic acid) were obtained from ChromaDex (Santa Ana, CA). The roots and rhizomes of valerian were obtained and identified from the field station of the University of Illinois at Chicago. Voucher specimens have been deposited at the University of Illinois at Chicago, College of Pharmacy.

2.2. Extraction and isolation

Valerian extracts for screening purposes were prepared from dried powdered underground parts of *V. officinalis* L. by successively extracting 100 g dried powdered material each time three times at 500 ml of the following solvents and an ultraturrax at ambient temperature: (1) dichloromethane (DCM), (2) petroleum ether (PE), (3) methanol (MeOH), and in the end with methanol 50% (MeOH 50%). Moreover, an extract was prepared by extracting 20 g dried powdered underground parts of *V. officinalis* only with methanol using the same procedure. Bioassay-guided fractionation: 1300 g of *V. officinalis* underground parts was exhaustively extracted in two parts each time three times at 1500 ml petroleum ether and an ultraturrax at ambient temperature. The extraction yield was 1.3% and 1.4%, respectively. The dried petroleum ether extract was separated by silica gel vacuum column chromatography with a petroleum ether and chloroform gradient (2.5% to 5.0% steps). The active fraction (13.3 g) was further fractionated by a second silica gel vacuum column with hexane and ethyl acetate as gradient (2.5% to 5.0% steps). Valerenic acid (4.2 mg) was isolated from subfraction 5 by semipreparative HPLC with a water/acetonitrile gradient (75–85% acetonitrile in 28 min). Besides valerenic acid, the separation resulted in two compound mixtures (0.9 mg and 1.4 mg) and one isomeric mixture (1.4 mg). The content of valerenic acid was determined by HPLC using the method with an external standard curve for valerenic acid [7]. RP-Column a 1:1 combination of the dichloroform (DCM) and

PE extract, a 1:2 mixture of the methanol (MeOH) and MeOH 50% and a 100% MeOH.

2.3. Serotonin receptor binding assays

Initial radioligand binding studies were performed by MDS Pharma Services (Bothell, WA) as previously described for serotonin receptor subtypes 1A, 1B, 2B, 2C, 3, 5A, 6, 7, serotonin transporter [5,6,8,23,31,32,37–41,43–45,47]. Significant binding affinity was only observed at the 5-HT_{5a} receptor (>50%) for the PE and DCM extracts at a concentration of 50 μg/ml [12]. Similar results were reported by Schumacher et al. [40]. Therefore, this investigation focused only on the 5-HT_{5a} receptor assay and GTP shift experiments.

For the 5-HT_{5a} receptor binding experiments, additional assays were performed with minor modifications using human 5-HT_{5a} receptor expressed in Chinese hamster ovary (CHO-K1) cell membranes. The human 5-HT_{5a}-transfected CHO-KI cells were grown in Ham's F12 media supplemented with 10% FBS, MEM sodium pyruvate (1 mM), gentamicin (50 mg/ml), and penicillin/streptomycin (50 U/ml). Cells were scraped from culture dishes at full confluence. These cells were homogenized and centrifuged twice at 12,000 $\times g$ for 20 min. The pellets were dissolved in TEM buffer (75 mM Tris, 1 mM EDTA, 12.5 mM of MgCl2, pH 7.4) and stored at −80 °C. Protein concentrations were determined by the Lowry method using bovine serum albumin as standard. All preparations were kept on ice. [^{3}H]LSD (1.64 nM) incubated at 37 °C for 60 min in incubation buffer (50 mM TRIS (base), 10 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM EDTA). After a 1-h incubation at 37°C, the mixtures for both receptors were filtered over 934-AH Whatman filters that had been presoaked in 0.5% polyethylenimine (PEI) and washed two times in ice-cold 50 mM Tris buffer (pH 7.4) using a 96-well Tomtec-Harvester (Orange, CT). Each filter was dried, suspended in Wallac microbeta plate scintillation fluid (Perkin-Elmer Life Sciences, Boston, MA), and counted with a Wallac 1450 Microbeta liquid scintillation counter (Perkin-Elmer Life Sciences, Boston, MA). 5-Hydroxytryptamine (serotonin, 5-HT) (250 nM) was used to define nonspecific binding, which accounted for <10% of the total binding. The percent inhibition of [^{3}H]-LSD binding to the 5-HT_{5a} receptor was determined as $[1 - (dpm_{sample} - dpm_{blank})/(dpm_{DMSO} - dpm_{blank})] \times 100$. The inhibition of [^{3}H]-LSD binding (%) of the sample was calculated in comparison with the inhibition of 1 μM 5-HT (100%). For the most potent compounds, IC_{50} values were determined by evaluation of the percent inhibition of [^{3}H]-LSD binding in a number of serial dilutions. The data represent the average of triplicate determinations.

2.4. GTP shift experiments

Inhibition of binding of [^{3}H]LSD (68.2 Ci/mmol, Perkin-Elmer) by the petroleum ether extract was measured in the presence and in the absence of 100 μM GTP. 5-HT (500 μM) was used to define nonspecific binding [8,40]. The assay was carried out under the same conditions as described for the assay above.

2.5. Data analysis

The data obtained in our laboratory represent the average ± SD of at least triplicate determinations. Curve fitting was performed using Akaike's Information Criteria (AICs), GraphPad Prism version 4.01 for Windows, GraphPad Software, San Diego CA, www.graphpad.com. Kd, IC_{50}, and Ki values were determined using the same program.

3. Results

Extracts of valerian (100 MeOH, 50% MeOH, DCM and PE) were initially screened to determine if these extracts contained any potential ligand(s) of the melatonin, serotonin, benzodiazepine, glutamate, and GABAA receptors, or serotonin transporter [12]. No significant binding was found for any of the extracts at the 5-HT_{1A}, 1_B, 2_C, 3, 6, 7, melatonin, benzodiazepine, or glutamine receptors. Only the PE and the DCM extracts (50 μg/ml) were active in the 5-HT_{2b}, 5-HT_{5a} receptor assays, and the serotonin transporter assay. A combination of the PE/DCM extracts bound to the serotonin 5-HT_{2b} (51% binding), serotonin transporter (53% binding), and in the 5-HT_{5a} receptor binding assay (80% binding) at a concentration of 50 μg/ml (Fig. 1). When tested separately, the PE extract exhibited 86% binding at the 5-HT_{5a} receptor as compared with a 51% binding of the DCM extract at a concentration of 50 μg/ml. None of the other extracts tested were active (data not shown). The 100% methanol and 50% methanol extracts were not active in any of the receptors or transporter tested.

Due to the dense distribution of the 5-HT_{5a} receptor to areas in the brain responsible for circadian timing and the sleep–wake cycle, the binding of the PE extract to the 5-HT_{5a} receptor was investigated in further detail. To validate

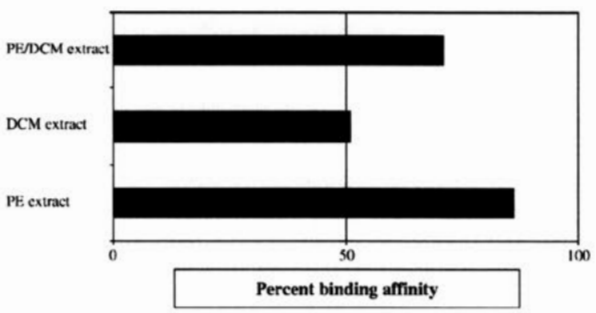

Fig. 1. Percent binding affinity of the dichloromethane/petroleum ether extract combination, the dichloromethane extract, and petroleum ether extract to the 5-HT_{5a} receptor. Extracts were tested at concentrations of 50 μg/ml in duplicate.

this method, serotonin (5-HT) was tested as an inhibitor [^{3}H]LSD binding to the 5-HT$_{5a}$ receptor. A binding assay was established and validated by creating a saturation curve with [^{3}H]LSD. Experimentally generated Ki values for [^{3}H]LSD were consistent with the literature (Ki = 1.8 nM). The PE extract displaced the [^{3}H]LSD from the 5-HT$_{5a}$ receptor with an IC$_{50}$ curve that depicted a biphasic shape and best fit a two-site competition equation using the Akaike's Information Criteria (GraphPad Prism 4.01 for Windows). The PE extract displaced radioligands from the 5-HT$_{5a}$ receptor with an IC$_{50}$ of 15.7 ng/ml (Ki: 9.48 ng/ml) for the high-affinity state and 27.7 μg/ml (Ki: 16.7 μg/ml) for the low-affinity state (Fig. 2). Since the 5-HT$_{5a}$ receptor belongs to the G-protein-coupled transmembrane proteins, GTP converts two classes of binding sites to a single lower affinity class of sites and leads to a right shift in case of an agonistic activity at the receptor. The IC$_{50}$ curves for the PE extract in the absence and presence of GTP are depicted in Fig. 3. GTP can cause an uncoupling of the receptor from the G-protein leading to a shift of the receptor from the high- to the low-affinity state for agonists [40]. The addition of GTP resulted in a rightward shift of the PE extract binding curve to a low-affinity state or monophasic with an IC$_{50}$ of 11.4 μg/ml (Ki = 6.88 μg/ml). These data suggested that chemical components of the PE valerian extract function as an agonist of the human 5-HT$_{5a}$ receptor.

In order to identify the active chemical compounds from valerian that bound to the 5-HT$_{5a}$ receptor, both the PE and DCM extracts were fractionated and pure compounds were isolated. In contrast to the methanol extracts tested, both the DCM and PE extracts contained valerenic acid (Fig. 3), with the PE extract characterized by its high valerenic acid content. In addition to valerenic acid, the PE extract also contained trace amounts of acetoxy-valerenic acid (Fig. 3) and a mixture of valepotriates. Hydroxyvalerenic acid (Fig. 3) was not found in the PE but did occur in the DCM

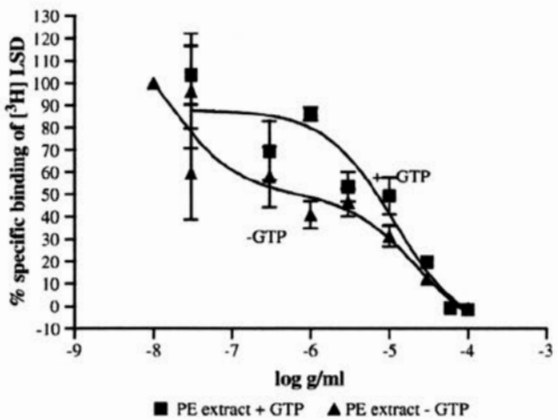

Fig. 2. Binding of the PE extract of valerian to human 5-HT$_{5a}$-transfected CHO membranes in the presence of [^{3}H]LSD and in the presence or absence of GTP (100 μM). GTP results in a right-hand shift of the IC$_{50}$ curve, indicating that the extract acts as an agonist at the 5-HT$_{5a}$ receptor.

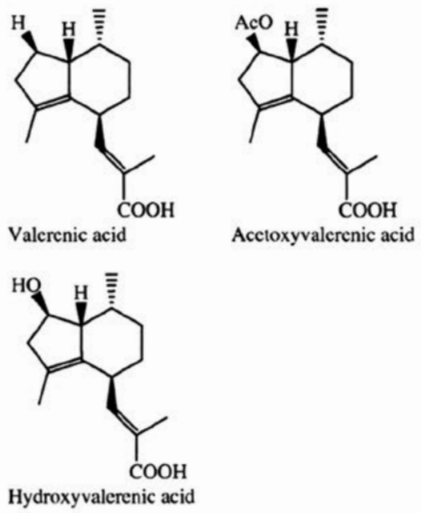

Fig. 3. Major sesquiterpenes present in the PE and DCM valerian extracts. (1) Valerenic acid, (2) acetoxyvalerenic acid, and (3) hydroxyvalerenic acid.

extract. To isolate the active 5-HT$_{5a}$ receptor binding ligand from the PE extract, bioassay-guided fractionation was conducted leading to several active fractions, all containing valerenic acid. Receptor binding comparisons of the three major sesquiterpenes, valerenic acid, hydroxyl valerenic acid, and acetoxy-valerenic acid, confirmed that valerenic acid was the active constituent (see Fig. 4 and Table 1). The semipreparative HPLC fractionation of the active fraction yielded valerenic acid as the major active compound, as well as valepotriate isomeric mixture, which also exhibited some activity (62%) at a concentration of 50 μg/ml in the 5-HT$_{5a}$ receptor binding assay. Valerenic acid exhibited significant affinity for the 5-HT$_{5a}$ receptor (80% at 50 μg/ml), with an IC$_{50}$ value for valerenic acid of 17.2 μM (Ki: 10.7 μM).

To correlate the valerenic acid content of various valerian extracts with the 5-HT$_{5a}$ receptor binding activity, we tested the PE and DCM extracts of three different batches of plant materials. The results clearly demonstrate that there is a direct correlation between the valerenic acid content of these

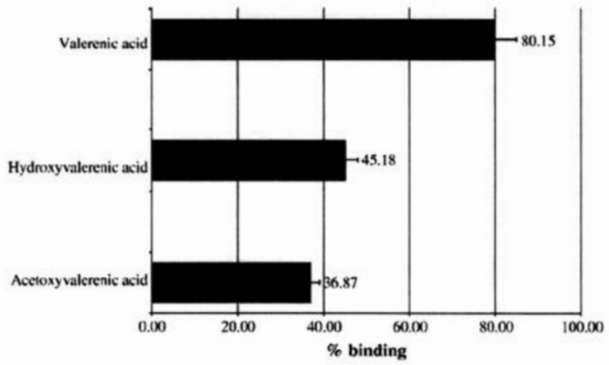

Fig. 4. Binding affinity of the sesquiterpenes valerenic acid, hydroxyvalerenic acid, and acetoxyvalerenic acid to the 5-HT$_{5a}$ receptor tested at a concentration of 50 μg/ml, n = 3.

Table 1
Correlation between the valerenic acid concentration and the 5-HT_{5a} receptor binding activity of the PE- and DCM extracts of different *V. officinalis* plant samples

Extract	Milligram valerenic acid/100 mg extract	Activity in 5-HT_{5a} assay, percent specific binding
Petroleum ether (PE) extracts were tested at a concentration of 20 μg/ml		
PE 1	7.33	64.93
PE 2	8.21	76.02
PE 3	6.23	40.4
Dichloromethane (DCM) extracts were tested at a concentration of 50 μg/ml		
DCM 1	2.88	70.88
DCM 2	3.39	71.38
DCM 3	1.94	46.83

The valerenic acid content was determined by external calibration (r = 0.96), every data point was at least obtained by triplicate determination.

extracts and the 5-HT_{5a} receptor binding activity (Table 1). Extracts prepared from valerian roots having a high concentration of valerenic acid were associated with a higher significant affinity for the 5-HT_{5a} receptor.

4. Discussion

Valerian has been used as a sedative for the treatment of insomnia and restlessness since the second century [26, 27,29,33]. Currently, valerian is approved in Europe as a sedative by the German Commission E. Approximately 15 controlled clinical trials have been published to date and suggest that treatment with valerian extracts improves sleep structure and sleep perception of insomnia in healthy patients and in patients suffering from sleep disorders, without producing the traditional sedative side effects [10,13–16,21,22,25,45]. Valerian extracts have also been used for the relief of sleep disorders associated with aging and menopause [19,43]. Interestingly, Vorbach et al. [45] reported that approximately 2–4 weeks of therapy with valerian is needed to achieve significant improvements in sleep disturbances. Clinically, valerian does not cause dependency, and there does not appear to be any additive effects with alcohol [4]. Thus, the mechanism by which valerian exerts its sedative activity may be unlike other sleep aids.

Many potential mechanisms for the pharmacological activities of valerian have been proposed, including agonistic activities on the GABA, adenosine, barbiturate, and benzodiazepine receptors [4,29,48]. However, newer studies have questioned these data [12,40]. Schumacher et al. [40] found no significant binding of valerian compounds to either benzodiazepine or GABAA receptors in concentrations up to 100 μM. These data are supported by our previous work [12]. Furthermore, the concentrations of valerian extracts used in at least one study were extremely high [48]. This study reported that valerian extracts bound to the GABA receptor in vitro; however, the concentration of valerian extract used was 3 mg/ml (29.6%) [48]. Thus, considering the concentration used and the low binding affinity, the results are questionable.

In contrast to other plausible mechanisms, the effects of valerian on the serotonin receptor have not been fully investigated. Serotonin is well known to modulate sleep–wake cycles and circadian rhythms in the human brain via a G-protein-coupled receptor family [24,28]. Reports on the binding affinity of valerian to serotonin receptors are few. In one previous investigation, several compounds isolated from a valerian extract were reported to have no significant affinity for the 5-HT_{1a} receptor [40]. This is also in agreement with our work, as none of our valerian extracts bound to the 5-HT_{1a} receptor in concentrations up to 50 μg/ml [12]. Only the DCM and PE extracts of valerian had high binding affinity for the 5-HT_{5a} receptor. Binding of valerian extracts to the 5-HT_{5a} receptor has not been previously reported, and thus may represent a new mechanism of action. Valerenic acid, one of the major constituents of valerian, is the primary active compound; however, a synergistic mechanism involving other compounds present in the PE extract cannot currently be ruled out. Valerenic acid was present in both the DCM and PE extracts of valerian; however, the PE extracts contained a higher concentration of valerenic acid than the DCM extracts, thus explaining the higher binding affinity to the 5-HT_{5a} receptor. Since the binding affinity of the extracts was dependent on the concentration of valerenic acid, and neither methanol nor 50% methanol extracts contained valerenic acid, they were not active in this assay.

Due to of the association of G-proteins with the 5-HT_{5a} receptor, GTP binding studies were used to determine if valerian extracts exhibited agonist or antagonist activity. While agonists stimulate the binding of GTP to the G-protein, neutral antagonists have no effect, and antagonists with inverse agonistic activity reduce GTP binding. Thus, addition of GTP induces an uncoupling of the receptor from the G-protein leading to a shift of the receptor from the high- to the low-affinity state for agonists [40]. In our experiments, the binding curve for the PE extract was biphasic in the absence of GTP, and addition of GTP resulted in a rightward shift of the PE extract binding curve to a low-affinity state. These data support the hypothesis that valerian extract and valerenic acid are partial agonists of the 5-HT_{5a} receptor, and suggest an entirely novel mechanism to explain the sedative effects of valerian. The 5-HT_{5a} receptor is expressed in many brain regions, including several important neural components of the circadian timekeeping system, namely the suprachiasmatic nucleus (SCN, in the hypothalamus), the intergeniculate leaflet, the dorsal raphe nucleus, and the median raphe nucleus [11,15,36]. The SCN is thought to tag the 24-h endogenous circadian clock that regulates sleep and wakefulness [11]. Thus, it has been proposed that the 5-HT_{5a} receptor may play a role in the serotonergic regulation of circadian timekeeping [15,18,36,42]. Confirmation of this mechanism of action should be performed in vivo using 5-HT_{5a} receptor knockout mice [20].

Acknowledgments

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Valerian Use for Sleep Disturbances Related to Rheumatoid Arthritis

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Complementary therapies are becoming increasingly popular, particularly for symptoms such as sleep disturbance. The herb valerian may be useful as a mild sleep aid in clinical populations, such as persons with rheumatoid arthritis. This article reviews valerian to inform healthcare providers of potential uses and safety considerations. **KEY WORDS:** *complementary and alternative therapies, rheumatoid arthritis, sleep disturbances, valerian* *Holist Nurs Pract* 2004;18(3):120–126

The use of complementary and alternative products and practices (CAPPs) is becoming increasingly popular in the United States, particularly for the management of chronic symptoms such as sleep disturbance and pain.[1] A national survey found that arthritic persons experiencing sleep disturbances were more likely to use CAPPs than were persons experiencing other symptoms.[2] Among CAPPs, herbal supplements are often used for therapeutic purposes. Unfortunately, much of the existing research is difficult to evaluate because of weak research designs, availability only in foreign languages, or failure to synthesize findings in a comprehensive or clinically useful manner. Thus, the purpose of this review is to discuss the herb valerian, which is commonly used as a sleep aid, with particular reference to the clinical population of persons with rheumatoid arthritis (RA). The discussion focuses on RA because the disease involves significant sleep disturbance, and valerian may be specifically useful for the type of sleep disturbance experienced. However, many of the points in the discussion are relevant to a variety of chronic conditions.

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REVIEW OF THE LITERATURE

Biological basis of the effects of valerian

The word *valerian* is thought to be derived from the Latin *valere*, meaning "to be healthy or strong."[3] The German name for valerian is baldrian,[3] which is commonly found in research literature concerning the herb. Valerian, also known as *garden heliotrope*, is a common plant with roots having well known medicinal properties, as well as a distinctive, unpleasant odor. Valerian has been used orally for centuries as a sedative, sleep aid, antispasmodic, and digestive aid.[3] Evidence from animal and human studies of valerian supports sedative, anxiolytic, and spasmolytic effects.[4] The German Commission E, known internationally as a leading authority on the therapeutic use of herbs, approved the use of valerian as a sleep aid.[4]

Although many species of valerian exist, 3 are most commonly used medicinally: *Valeriana officinalis, Valeriana wallichii* DC (also called Indian valerian or *Valeriana jatamansi* Jones), and *Valeriana edulis* Nutt. (also called Mexican valerian or *Valeriana mexicana* DC).[5,6] The species most commonly used in the United States is *V officinalis* L (also called Radix valerianae).[3,4] The root of the valerian plant contains many chemical constituents that are responsible for the medicinal properties of the herb, including valepotriates, valerenic acid, amino acids, and lignans.[4,6] Valerian has been shown to increase

activity at gamma-aminobutyric acid (GABA) receptors, which are involved in regulating normal sleep.[7] This effect is similar to benzodiazepines and nonbenzodiazepine hypnotics, which are prescription sleep aids that induce sleepiness by binding with GABA receptors.[8] However, there is evidence that valerian affects other neuroreceptors involved in sleep regulation, particularly adenosine and serotonin receptors.[9,10] Thus, valerian may have different effects on sleep than may conventional sleep aids. For a scientific review detailing the contributions of specific constituents to the biologic effects of valerian, see reference 4.

Standardization, preparation, and dosing

In selecting a valerian product, the species, standardization, form, and dose are important considerations. Several species of valerian differ on the content of active constituents. An important consideration in selecting a valerian preparation is the content of valepotriates, which differs between species.[5] Valepotriates are active constituents of valerian that have sedative and spasmolytic effects, but these chemicals also have demonstrated cytotoxic effects. Thus, safety concerns exist regarding possible carcinogenic effects of valepotriates.[4,11] A recent study demonstrated that DNA damage occurred with high doses of valepotriates, but not with low doses.[12] Thus, preparations that minimize valepotriates are recommended.

V officinalis (*Radix* valerinae) is the most common species used in commercial products. *V officinalis* is the species with the lowest valepotriate content.[5] Valerenic acid is considered the most important active constituent in *V officinalis*, and the percentage of valerenic acid is used to standardize preparations. A common industry standard is 0.8% valerenic acid.[13]

The purity and standardization of valerian is not mandated by the Food and Drug Administration (FDA), although the FDA does regulate labeling herbs under the Dietary Supplement Health and Education Act (DSHEA) of 1994 (http://www.fda.gov/opacom/laws/dshea.html#sec4). This dictates that labels may state the purported effects of herbs on the structure or function of the body but cannot claim to treat a specific medical condition. Because consumers bear the responsibility of identifying pure, standardized preparations, it is useful to be aware of several resources. First, the company should use current good manufacturing practices (CGMPs) to produce the valerian. CGMPs are mandatory minimum requirements set forth by the FDA for manufacturing products under sanitary conditions, with accurate standardization and labeling, with adequate purity, and that meet the physical standards of the production facilities.[14] Additionally, consumers can check for company membership in the American Herbal Products Association (AHPA) (member companies are listed at http://www.ahpa.org/companies.htm). The APHA maintains a code of ethics and business conduct for all member organizations. Finally, ConsumerLab.com, an independent consumer-based organization, tests standardization of herbal supplements that are voluntarily submitted by companies for analysis. The ConsumerLab.com Web site lists valerian products that adequately meet the specified criteria for standardization, but individuals must subscribe for a fee to view the full list.

The preparation technique used affects the composition of valerian products, and subsequent recommended dosing. Valerian may be prepared for ingestion as whole dried root, dried root extract (extracted by water or ethanol), tea, or alcoholic tincture. Capsules and tablets prepared by extraction, as well as teas prepared with hot water, were found to have negligible valepotriate content.[5] Because valepotriates are highly unstable compounds, the content is greatly reduced in alcoholic tinctures that have been stored for 3 weeks.[5] Concern remains regarding the safety of preparations using the whole herb that do not reduce valepotriate content.[5] It should also be noted that in some commercial preparations, particularly those using species other than *V officinalis*, the main active constituents are valepotriates, even though the safety of these constituents remains in question.

There are no standard dosage recommendations for valerian. The doses of dried valerian extract used in most studies ranged from 400 mg to 900 mg.[15] In research, this dose range has appeared safe and sufficient to achieve at least mild sedative effects.[13,16] Other cited doses in the literature include 0.5 to 1 tsp tincture,[13] 1 to 3 g/day whole herb,[13] and 2 to 3 g whole herb soaked in hot water and strained after 10 minutes for tea.[17] However, the safety of these preparations should be considered, as discussed. Given that the sedative effects of valerian have shown to be dose-dependent, dosage should be titrated within the recommended range, starting at a lower dose and increasing as needed. The onset of the effects of

valerian begin within an hour of administration,[18–20] supporting administration of valerian approximately 1 hour before bedtime to reduce sleep latency. Polysomnographic effects have shown to persist at least 3 to 4 hours following administration,[19,20] indicating that valerian might not prevent early awakening, but also indicating that valerian is unlikely to contribute to residual morning sedation.

Side effects and safety

Evidence generally supports valerian as a safe herb. Of the 28 clinical trials reviewed, no serious adverse events occurred in participants taking valerian. Specific side effects were very rare, but included vivid dreams, headache, gastrointestinal discomfort, slight dizziness, heavy sleep, depression, paradoxical stimulation, and residual sleepiness.[13,19,21–27] Research indicated that valerian carried little to no risk of residual sedation the morning after use at doses ranging from 160 mg to 900 mg,[6,15,16,18,22–24,27–29] which is an advantage in comparison to other sleep aids (benzodiazepines and diphenhydramine) for which residual sedation is a common side effect.[8,30] As with any sedative, individual responses to valerian may vary. Although residual sedation appears to be uncommon, persons taking valerian should be alerted to the potential effect and cautioned not to drive if they feel excessively sleepy the morning after valerian use.

Some concern exists regarding the contribution of herbs to liver toxicity, particularly following several reports of severe liver dysfunction with the use of the sedative herb kava kava. A few cases of liver dysfunction have been reported in persons using valerian, but evidence implicating valerian as the causal factor is tenuous. Liver dysfunction has been reported in 11 persons using valerian.[31–33] In 5 cases, the individuals used valerian in combination with skullcap or chaparral (both implicated in other cases of liver dysfunction).[34,35] These individuals recovered fully after use of the herbal product was discontinued. Another individual using valerian tea daily developed liver dysfunction, but recovered after ceasing to ingest the tea.[13,36] In 5 other cases, insufficient information was available to determine whether valerian contributed to the hepatic dysfunction.[33]

Evidence from intentional valerian overdoses generally indicates that the toxicity profile of valerian is mild. In 29 intentional overdoses of valerian in Hong Kong, liver function tests were normal.[37,38] In another case report, a young woman who ingested 20 g of valerian, which grossly exceeded the recommended to 1- to 3-g dose, had stable vital signs and normal liver function tests,[39] and her symptoms of weakness, abdominal pain, tremors, and drowsiness resolved in 24 hours. Similarly, one individual using valerian and wild lettuce intravenously had elevated liver function tests (LFTs) and developed symptoms of flank pain, tremors, stupor, and tachycardia.[40] The LFTs returned to normal and symptoms resolved within 3 days. Finally, one case reported acute transient withdrawal symptoms similar to benzodiazepine withdrawal following discontinuance of excessive daily doses of valerian (2.65 to 10 g of valerian daily for "many years").[41]

As is the case with both phytopharmaceuticals and conventional pharmaceuticals, interactions with other substances may occur. First, valerian should not be taken with other sedatives, such as benzodiazepines or alcohol, to avoid additive sedation. Within recent years, it has been demonstrated in vitro that valerian is a mild inhibitor of a subtype of cytochrome P450,[42] a family of enzymes in the small intestine and liver that are involved in first-pass metabolism of many orally ingested medications.[43] Valerian mildly inhibits the action of cytochrome P450 subtype CYP3A4,[42] which could increase plasma levels of the drugs metabolized by this protein. Such effects may not be problematic for medications with wide therapeutic ranges, but would be dangerous for medications with narrow ranges and high toxicity profiles. Notably, levels of antifungals, lipid-lowering HMG Co-A reductase inhibitors ("statins"), and certain antiarrhythmics (diltiazem and digoxin) are increased by inhibition of CYP3A4. Given that these drugs have severe side effects, it is advisable that persons on these drugs avoid valerian or other herbs that inhibit CYP3A4 until further information regarding the impact of herbs on the metabolism of these drugs is available. Other classes of medications metabolized by CYP3A4 that are particularly relevant to RA include benzodiazepines, certain opioids, and certain immunosuppressants. The reader is referred to reference 42 for a list of herbs that inhibit CYP3A4.

In summary, valerian appears to be a safe herb. However, it may cause adverse effects and patients should be warned. Use of other herbs or conventional pharmaceuticals should be considered before recommending valerian. Additionally, persons at risk for liver dysfunction should avoid the herb. Valerian

should not be taken by pregnant or lactating women, given that one study in mice demonstrated mildly reduced fetal development.[44] Finally, persons using valerian for sleep should be cautioned not to drive or operate machinery in the evening after taking valerian.

SLEEP, RHEUMATOID ARTHRITIS, AND VALERIAN

Sleep disturbances associated with rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammatory form of arthritis affecting about 1% of the adult population,[45] causing a wide array of symptoms, such as pain, sleep disturbance, depression, physical disability, and fatigue, that impair individuals' quality of life. One of the most common reasons for the use of CAPPs by persons with RA is the failure of conventional pharmacologic treatments to relieve symptoms adequately.[46] Sleep disturbances are highly prevalent among persons with RA, with studies reporting sleep disturbances in 54% to 70% of participants.[47] Various aspects of RA contribute to sleep disruption, including disruption of normal circadian rhythms, tendencies toward primary sleep disorders, chronic pain, and excess production of inflammatory cytokines.[48] Pro-inflammatory cytokines are molecules produced by immune cells that promote both the inflammatory response and sickness behaviors, including sleep. Also associated with the disease process in RA is disruption of the normal secretion patterns of the hormones cortisol and melatonin, both of which are involved in the onset and timing of normal sleep.[49,50] Primary sleep disorders, specifically periodic limb movements and sleep apnea are common in RA.[48] These disorders are associated with frequent awakenings and arousals, difficulty returning to sleep, and daytime sleepiness.[51,52] Finally, persistent pain is an important cause of sleep disturbances in persons with RA.[47]

A limited number of studies provide evidence on specific sleep disturbances associated with RA. Studies objectively measuring sleep indicate that persons with RA experience frequent awakenings and arousals; much time spent awake after the initial onset of sleep [wake after sleep onset (WASO)]; and low sleep efficiency (the ratio of time spent asleep to total time in bed). Studies of persons with RA reported averages of 19 to 46 awakenings throughout the night.[53,54] Arousals, which are transitions from deep sleep to light sleep or wakefulness, also occurred frequently over the course of the night.[51,53] Sleep efficiency is an overall index of how well an individual slept, with higher sleep efficiency indicating better sleep. Researchers have reported that persons with RA generally had sleep efficiencies between 61% and 81%, which is markedly lower than the 90% to 100% that is considered good sleep.[53,55] Objectively measured sleep latency was slightly prolonged in persons with RA, with reported latencies ranging from 20 to 30 minutes.[51,54,56] These individuals generally reported moderate to poor sleep quality, regardless of whether objective sleep disturbances were evident.[51,57] Overall, these studies indicated that persons with RA sleep poorly and experience the subjective symptom of sleep disruption.

Few trials have investigated medications for improving sleep in persons with RA. A 3-month trial of the NSAID tenoxicam resulted in improvement in nocturnal pain and morning stiffness but did not improve sleep disturbances relevant to RA.[58] These findings suggest that attenuation of pain and inflammation may not be adequate to improve sleep in persons with RA. In other studies, a benzodiazepine (triazolam) and a nonbenzodiazepine hypnotic (zopiclone) improved subjective sleep outcomes by reducing self-reported sleep latency and number of awakenings.[56,59] However, neither medication improved objective measures of sleep efficiency or WASO. Additionally, triazolam reduced slow wave sleep (SWS, non-REM stages 3 and 4), which is a common effect of benzodiazepines.[30,56] This effect may not have been beneficial because SWS appears to serve a restorative role in RA,[60] and because SWS deprivation has shown to induce musculoskeletal pain,[61] which is already a reason for sleep disturbances in RA. Given that none of the medications appeared adequate for treating sleep disturbances in RA, research on other interventions, including valerian, is warranted.

Effects of valerian on sleep

Although there are no published studies concerning the effects of valerian on sleep in persons with RA, evidence from studies of healthy persons and those with insomnia indicates that valerian may be of particular benefit for the types of sleep disturbances associated with RA. Both objective and subjective sleep outcomes of valerian trials provide evidence that valerian has at least mild effects on human sleep. The

following discussion of valerian research first addresses objective findings, followed by subjective findings. When articles were not available in English, secondary sources in English were used. Both the primary and secondary sources are cited.

Objective findings in samples of healthy persons and those with insomnia have provided some evidence of the effectiveness of acute use of valerian when the herb was used for 3 or fewer nights. In one study, acute use of valerian reduced objective sleep latency, indicating greater ease in falling asleep, compared to a placebo.[62] However, 2 studies found no difference in objective sleep latency.[63,64] Two studies of valerian showed improvement in sleep efficiency compared to baseline, but the results did not reach statistical significance in either study.[19,63] These studies were limited by nonequivalence of the placebo and valerian groups at baseline,[63] occurrence of an exceptionally large placebo effect in the control group,[19] and ceiling treatment effects resulting in a small and nonsignificant magnitude of change in a sample of healthy persons.[62]

Objective findings on repeated nightly dosing of valerian for 1 to 6 weeks have been favorable. Studies demonstrated that persons receiving repeated dosing of valerian experienced reduced time between going to bed and the first cycle of deep sleep (SWS) compared to those receiving a placebo.[65,66] In addition to reduced sleep latency, repeated use of valerian also reduced WASO and increased sleep efficiency compared to the placebo.[25]

Subjective sleep measures also improved with both acute and repeated dosing of valerian. With acute use of valerian, both healthy individuals and persons with insomnia experienced improvement in subjective sleep latency and sleep quality compared to placebo treatments.[19,21,62,67] Repeated dosing of valerian also improved subjective sleep latency.[6,13,66,67] In one study, participants experienced no change in sleep quality after acute or repeated dosing, although this group was elderly persons reporting poor sleep, who may have perceived sleep quality differently than insomniacs or healthy persons.[63]

Comparison of valerian to other sleep interventions

Several studies have administered a conventional sleep aid rather than a placebo to the control group to compare the effectiveness of valerian to these medications. Subjective sleep quality showed improvement with valerian that was comparable to benzodiazepines after a single dose or repeated dosing.[18,28,68,69] Although none of these studies used objective sleep outcomes, the findings suggest that valerian is at least as helpful as benzodiazepines in managing the subjective symptom of poor sleep.

Valerian also appears more favorable than other CAPPs for sleep disturbances related to RA. Other herbs, including kava kava, passionflower, chamomile, hops, skullcap, and melatonin, have been used for sleep disturbance.[48] Research provides little evidence that passionflower, kava kava, and hops may be mildly sedating, but evidence on skullcap and chamomile is lacking.[48] Additionally, kava kava has been linked to several cases of serious liver dysfunction.[70] Melatonin has also been shown to facilitate sleep, but this substance has immunostimulatory effects that could potentially enhance the autoimmune process in RA.[71] Finally, other complementary practices may improve sleep, including guided imagery and selected devices within the category of bioelectromagnetics. However, these approaches generally require daily use for weeks to months before therapeutic effects are obtained. In contrast, evidence suggests that valerian may be effective after a few doses, and additional benefits of repeated dosing are obtained within days. Therefore, other complementary modalities may be useful in long-term management of RA symptoms, but valerian may have a different role in short-term management of sleep disturbances.

SUMMARY AND CONCLUSIONS

In summary, the evidence indicates that valerian may be beneficial for persons with RA, and perhaps may be superior to other sleep aids, both conventional and complementary. Despite limitations, existing research suggests that valerian improves objective and subjective sleep beyond a placebo effect. Evidence on acute dosing indicated that valerian may improve sleep outcomes relevant to RA, including sleep quality, sleep latency, and sleep efficiency, but these findings were not all significant and several of the studies were methodologically flawed.[16] Studies of repeated dosing also suggest that valerian may improve subjective sleep quality, reduce sleep latency measures, and improve sleep efficiency.

In addition to demonstrated effects on sleep, the biological effects of valerian indicate that the herb may be beneficial for those with RA. Valerian may

have some analgesic and spasmolytic effects that would facilitate sleep by reducing musculoskeletal pain in addition to central somnogenic effects.[4] In contrast to benzodiazepines, which suppress SWS, valerian specifically facilitates SWS.[6] Therefore, valerian may provide specific benefit for those with RA, given that increased SWS appears to be a homeostatic response to inflammatory RA flares.[60] Because valerian affects multiple physiological mechanisms, it could potentially improve RA-related sleep disturbances in a manner not achieved by benzodiazepines. Finally, research indicates that there is little residual sedation, or "hangover effect," with valerian use,[16] which is important in RA because these individuals already experience difficulty in the morning because of joint stiffness.

Healthcare practitioners must attend to the growing trend of the use of CAPPs in the United States. Sedative effects of the herb valerian have been demonstrated in research, although the investigation has been conducted in limited populations. The properties of the herb, as well as its general safety, suggest that it may be useful as a mild sleep aid in clinical populations, such as persons with RA. However, before recommending the use of herbal supplements, practitioner awareness of existing evidence and recommended uses, as well as possible risks, is important.

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Highly Potent Modulation of $GABA_A$ Receptors by Valerenic Acid Derivatives

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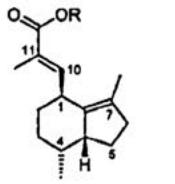
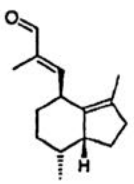
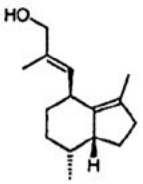
γ-Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the mammalian CNS, with as many as 30% of synapses being GABA-ergic in nature.[1] While two distinct receptor types for GABA exist ($GABA_A$ and $GABA_B$) many of its important physiological effects are mediated through interactions with ionotropic $GABA_A$ receptors, which largely determine the timing of neuronal firing and the sculpting of neuronal oscillations.[1] In light of the central role of the GABA-ergic system in the human brain, it is not surprising that drugs enhancing the GABA-induced chloride ion flux at $GABA_A$ receptors play a major role in the treatment of a variety of CNS-related disorders, such as generalized anxiety and panic disorders or sleep disturbances.[1,2] However, current $GABA_A$ modulators, most of which are benzodiazepines (BZ), are generally nonselective and act indiscriminately on all BZ sensitive $GABA_A$ receptor subtypes.[1b,3] In contrast, based on recent advances in our understanding of $GABA_A$ receptor physiology and pharmacology, subtype selective agents would be expected to exhibit a more selective therapeutic profile with fewer side effects.[3,4] Thus, the identification of new types of lead structures for $GABA_A$ receptor modulation represents an important objective in drug discovery directed at different types of CNS-based disorders, in particular in the area of anxiety.[3,4] In this context we[5] and others[6] have recently reported that valerenic acid (**1**), which is a major constituent of common valerian (*Valeriana officinalis*), is a potent modulator of $GABA_A$ receptors expressed in *Xenopus* oocytes[6] or HEK293[5] cells with EC_{50} values in the 5–20 μM range; concentrations >30 μM were found to lead to direct receptor activation.[6]



R = H: Valerenic acid (**1**)
R = CH_3: **4**
R = C_2H_5: **5**



Valerenal (**2**)



Valerenol (**3**)

Valerenic acid (**1**), at high μM concentrations, was found to enhance GABA-induced ion currents in oocyte[6] and HEK293[5] cell membranes up to fivefold and >tenfold, respectively. While the binding of **1** to $GABA_A$ receptors occurs with nM binding constants,[5] its binding site has not been characterized in detail. It is clear, however, that no direct interaction occurs with the BZ binding site;[5,6] rather, **1** may exploit the site for loreclezole[6] or a hitherto unrecognized site that is allosterically linked to binding sites for loreclezole, anesthetics and mefenamic acid.[5] In addition to these in vitro effects, both **1** as well as its natural congener valerenol (**3**) have been shown to exhibit anxiolytic activity in vivo either after i.p. or p.o. administration.[5] Collectively, these findings suggest that **1** is an attractive lead structure for the development of new $GABA_A$ receptor modulators.[7] At the same time, no structure–activity relationship (SAR) has yet been established for the $GABA_A$-modulatory activity of **1**, except that **3** has been shown to exhibit comparable activity to **1**, while valerenal (**2**), which is a minor constituent of *V. officinalis*, and the methyl ether of **3** (**11**, vide infra) do not bind to $GABA_A$ receptors.[5] In order to develop a broader understanding of the structural requirements for $GABA_A$-modulatory activity by **1** and related derivatives, we have embarked on a program to synthesize and biologically evaluate analogues of **1** with the ultimate objective to identify more potent and also subtype specific allosteric modulators at the $GABA_A$ receptor. In this report we describe a first series of analogues of **1**, which serve to probe the potential for modifications of the negatively charged carboxyl group of **1** and to assess the importance of the side chain double bond and the C11 methyl group for biological activity. As a result of this initial work we have identified a number of compounds that modulate GABA-induced ion currents with substantially higher potency than **1** itself.

Starting from valerenic acid (**1**) we have prepared valerenol (**3**) through direct reduction with LAH (as previously described).[5,8] Swern oxidation of **3** then provided valerenal (**2**).[5,8] DCC/DMAP-mediated[9] coupling of **1** with ammonia, methylamine, or dimethylamine gave the corresponding amides **6**–**8** in excellent yields (80–100%; Scheme 1).

Treatment of primary amide **6** with oxalyl chloride/DMF in THF[10] provided nitrile **9** (Scheme 1), which could be further elaborated into tetrazole **10** in 64% yield by heating with in situ prepared Bu_3SnN_3[11] at 100 °C for 6 days. A number of azide sources were investigated for the cycloaddition reaction with **7**, but Bu_3SnN_3 proved to be the most efficient with regard to yield and purity of the product, even though the reaction was very slow and took several days to go to comple-

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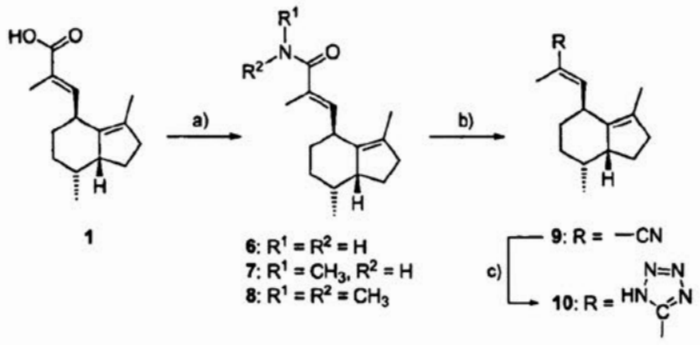
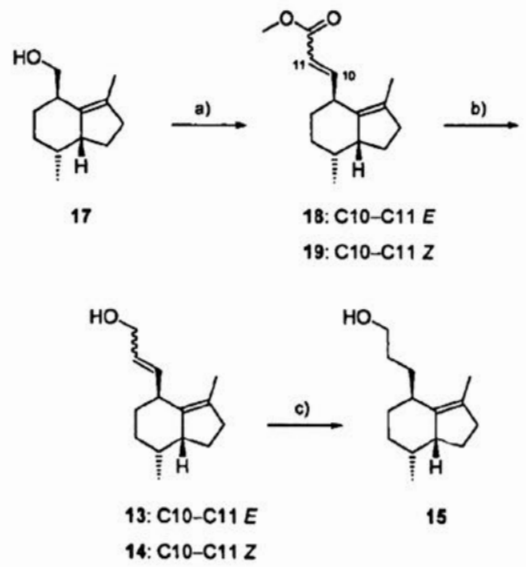


Scheme 1. a) $R^1R^2NH_2^+Cl^-$, DCC, DMAP, Et_3N, CH_2Cl_2, 0 °C→RT, 6 h, 80–100%; b) for **6**: $(COCl)_2$, DMF, THF, RT, 30 min, 98%; c) $NaN_3/BuSn_3Cl$, toluene, 100 °C, 6 days, 87%.

tion. Ethyl ester **5** is an intermediate in our recent total synthesis of **1**,[8,12] while methyl ester **4** was prepared through acid-catalyzed esterification of **1** with MeOH.

The C11-desmethyl valerenol analogues **13–15** were prepared from alcohol **17** (Scheme 2) for which we have developed an efficient synthesis as part of our total synthesis work on valerenic acid (**1**).[8] Oxidation of **17** with DMP provided the corresponding aldehyde, which was directly (i.e., without purification) submitted to HWE reaction with trimethyl phosphonoacetate. The resulting mixture of *E* and *Z* isomers could be readily separated by flash chromatography, thus furnishing the pure diastereomers **18** and **19** in 27 and 22% yield, respectively (based on **17**). Reduction of esters **18** and **19** with DIBAL-H gave the corresponding allylic alcohols **13** and **14**, respectively.

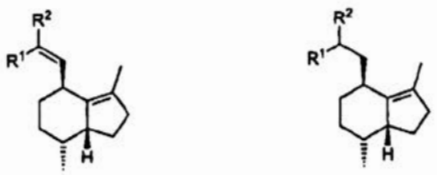


Scheme 2. a) 1. DMP, CH_2Cl_2, 0 °C, 2 h; 2. $(C_2H_5O)_2P(O)CH_2COOCH_3$, *n*BuLi, THF, 0 °C, 13 h, 27% (**18**)/23% (**19**; 2 steps); b) DIBAL-H (1 M in toluene), CH_2Cl_2, −78 °C, 2 h, 66% (**18**→**13**)/62% (**19**→**14**); c) H_2, 10% Pd-C, EtOAc, 4 h, RT, 83% (from **13**).

Careful treatment of *E* allylic alcohol **13** with H_2 over Pd/C (ethyl acetate, atmospheric pressure, room temperature, 4 h) allowed its transformation to the saturated 11-desmethyl valerenol analogue **15** in 83% yield, without affecting the endocyclic double bond in the cyclopentene ring. Finally, hydrogenation of **1** under similar conditions as for **13** (1.5 h) gave the partially saturated valerenic acid analogues **16** (Table 1) as an inseparable mixture of isomers, which also contained small amounts of fully saturated products as minor impurities that could not be removed by conventional chromatography.

The modulation of GABA activity on $GABA_A$ receptors by valerenic acid (**1**) and the different valerenic acid derivatives **2–16** was investigated on recombinant α1β2γ2 $GABA_A$ receptors expressed in *Xenopus* oocytes by means of two electrode voltage clamp measurements.[13,14] As illustrated by the data summarized in Table 1, and quite intriguingly, the replacement of the carboxylate moiety in **1** in general does not produce a loss in modulatory activity on α1β2γ2 receptors. On the contrary, reduction of **1** to valerenol (**3**) leads to substantially enhanced activity, with a >eightfold stronger potentiation of GABA-induced chloride ion influx at 1 μM modulator concentration

Table 1. Potentiation of I_{GABA} mediated by α1β2γ2 $GABA_A$ receptors by valerenic acid (**1**) and compounds **2–16**.



Cpd.	R^1	R^2	Potentiation I_{GABA} [%][a] 0.1 μM[b]	1.0 μM[b]	10 μM[b]
1	CH_3	COOH	–	25±10	474±176
2	CH_3	CHO	–	16±4	337±22
3	CH_3	CH_2OH	24/21	226±37	>2000[c]
4	CH_3	$COOCH_3$	–	22±8	315±58
5	CH_3	$COOC_2H_5$	–	25±12	107±45
6	CH_3	$C(O)NH_2$	11±6	177±44	>2000[c]
7	CH_3	$C(O)NHCH_3$	15±4	137±22	>2000[c]
8	CH_3	$C(O)N(CH_3)_2$	–	−15±26	127±19
9	CH_3	CN	7±4	137±22	1574±758
10	CH_3	tetrazol-5-yl	52±9	554±98	>2000[c]
11	CH_3	CH_2OCH_3	–	9±2	126±39
12	CH_3	CH_2OBn	–	1±3	7±9
13	H	CH_2OH (*E*)	–	11±9	409±66
14	H	CH_2OH (*Z*)	–	25±14	434±199
15	H	CH_2OH	–	10±8	138±56
16	CH_3[d]	COOH	–	3±5	9±7
diazepam			130[16]	220[16]	–

[a] Potentiation of the chloride ion flux triggered by GABA concentrations eliciting between 1 and 4% of the maximal current amplitude (EC_{1-4}; 1–2 μM) in *Xenopus* oocytes expressing α1β2γ2 receptors. Data represent the mean ±SD for three experiments. [b] Concentration of test compound. [c] At GABA concentrations eliciting EC_{1-4} the maximal theoretical potentiation cannot exceed 2500–10000%. [d] Diastereomeric mixture.

than **1** (226% as compared to 25% for **1**) and a detectable enhancement of the GABA effect even at a concentration of **3** as low as 0.1 μM. A significant activity increase over **1** is also observed for primary and secondary amides **6** and **7**, respectively, as well as nitrile **9**, while valerenal (**2**) and esters **4** and **5** appear to be slightly less active than **1**. Dimethyl amide **8** at 10 μM concentration shows similar activity as methyl ester **5**, but at 1 μM is significantly less active than any other ester or amide derivative. Most significantly, a rather spectacular increase in GABA-modulatory activity (>20-fold at 1 μM modulator concentration) is associated with the substitution of a tetrazole moiety for the natural carboxyl group in **1**, with the corresponding analogue **10** being by far the most active allosteric modulator at the $GABA_A$ receptor investigated in this study. As a tetrazole moiety is a classical bioisostere for a carboxyl group,[15] compound **10** might have been expected to be roughly equipotent with **1**; however, the magnitude of the activity enhancement suggests that the role of the tetrazole moiety in **10** goes beyond the simple isosteric replacement of a carboxyl group and may involve specific interactions with amino acid side chains in the receptor binding pocket. This is also supported by the fact that analogues **3**, **6**, **7**, and **9** are more effective allosteric modulators on the $GABA_A$ receptor than **1** itself, although they all lack acidic hydrogen atoms.

In contrast to the limited activity change associated with the conversion of valerenic acid (**1**) to its methyl ester **5**, methylation of valerenol (**3**), to produce ether **11**, leads to a substantial reduction in modulatory activity; increasing the size of the ether group from methyl to benzyl (analogue **12**) then results in a complete loss of allosteric modulation on the $GABA_A$ receptor. Removal of the methyl group attached to C11 in **3** again caused a significant decrease in activity (analogue **13**); surprisingly, however, the concomitant change in the geometry of the C10=C11 double bond does not cause any further loss in potency (if anything, *Z* derivative **14** appears to be a somewhat more effective allosteric modulator on the $GABA_A$ receptor than **13**). Compared with **13**/**14** the activity of 11-desmethyl-10,11-dihydro-valerenol (**15**) is further reduced, although the compound at 10 μM concentration is still capable to enhance GABA-induced chloride influx by about twofold. In contrast, no modulation of GABA activity on the $GABA_A$ receptor was evident for the corresponding partly saturated valerenic acid derivatives **16**. Overall, the activity data obtained for compounds **11**–**16** clearly point to the significance of a free hydroxyl group (in **3**) and the need for a C11 methyl group and a C10=C11 double bond for maximum $GABA_A$-modulatory activity of valerenic acid derivatives.

In light of the potent modulatory activity of analogue **10** at $\alpha1\beta2\gamma2$ $GABA_A$ receptors, the modulation of GABA-induced chloride influx was also investigated for other receptor subtypes. As shown in Figure 1, tetrazole **10** was an even more effective allosteric modulator at the $\alpha1\beta3\gamma2$ receptor, whereas no potentiation of GABA-induced currents was observed for the $\alpha1\beta1\gamma2$ subtype. Similar findings have been reported for the activity of valerenic acid (**1**) on $\alpha1\beta3$ and $\alpha1\beta1$ receptors;[6] as for analogue **10**, receptors based on the β1 subunit were virtually insensitive to the effect of **1**.[6] In addition, the replacement of Asn265 in the β2 subunits of $\alpha1\beta2\gamma2$ receptors by Ser leads to a significant decrease in the modulatory effect of **10** (Figure 1), which parallels previous findings for **1** on $\alpha1\beta2$ or $\alpha1\beta2\gamma2$ channels with a N265S[6] or N265M[5] mutation in the β2 subunit, respectively. The similar response of **10** and **1** to changes in the subunit composition of $GABA_A$ channels and to specific mutational changes clearly suggests that both compounds bind to at least overlapping sites on the $GABA_A$ receptor.

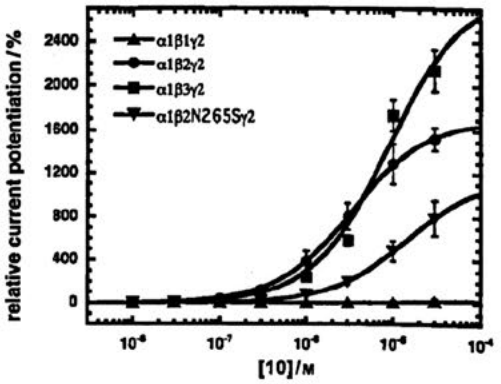


Figure 1. Cumulative concentration response curves of the potentiation of GABA-induced ion currents by tetrazole **10** in oocytes expressing different subtypes of the $GABA_A$ receptor. GABA concentrations were at 1–4% of its EC value (EC_{1-4}; 1–2 μM). Mean ± SD is indicated for three experiments.

In conclusion, we have prepared a series of valerenic acid derivatives and we have evaluated their modulatory effect on the $\alpha1\beta2\gamma2$ $GABA_A$ receptor subtype. Based on these experiments it is clear that a negatively charged carboxylate is not a crucial requirement for the $GABA_A$-modulatory activity of valerenic acid derivatives, while the loss of the methyl group at C11 or the olefinic double bond between C10 and C11 each lead to a substantial reduction in potency. Of the 15 analogues investigated valerenol (**3**) and tetrazole **10** proved to be the most potent allosteric potentiators of GABA-induced ion currents; the activity of tetrazole **10** exceeds the activity of valerenic acid (**1**) by at least one order of magnitude and, at least at higher concentrations, is more potent than diazepam (Table 1). The effects of changes in receptor subtype composition and of mutation of Asn265 in the β2 subunit on the activity of **10** are similar to those observed for **1**, thus suggesting that both compounds bind to the receptor at overlapping sites. Further modification of **10** may lead to even more potent and perhaps subtype-specific modulators of GABA-induced ion currents, which would be attractive drug candidates with a reduced potential for side effects.

Keywords: allosteric modulators · drug discovery · natural products · structure–activity relationships · valerenic acid

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