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Emergence of anti-conflict effects of zolpidem in rhesus monkeys following extended post-injection intervals

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Abstract

Rationale—Zolpidem is a hypnotic drug that binds to γ -aminobutyric acid type A (GABA_A) receptors but lacks consistently demonstrable anxiolytic efficacy.

Methods—Rhesus monkeys (N=4) were trained under a multiple schedule in which food-maintained responding was programmed (18-response, fixed ratio) for a 5-min period, followed by a 5-min period in which the food-maintained responding was suppressed by response-contingent electric shock (20-response fixed ratio). Doses of zolpidem (range= 0.03 to 1.0 mg/kg, i.v.) were administered 5 minutes before the session and responding was re-assessed at 3 additional 20 minute intervals. A similar experiment also was carried out with the non-selective benzodiazepine, triazolam, over a dose range of 0.001 to 0.1 mg/kg, i.v.

Results—Zolpidem did not engender a significant increase in average rates of suppressed responding at earlier time points; however, rates of non-suppressed responding were robustly decreased. At 45- and 65-min post-injection, zolpidem treatment resulted in a dose-dependent increase in rates of suppressed responding. In contrast, the non-selective benzodiazepine triazolam increased rates of suppressed responding in a dose-dependent manner at all 4 time points, although decreases in non-suppressed responding were less at the later time points.

Conclusions—These findings suggest that zolpidem has anxiolytic-like effects, but only >25 min after i.v. injection in this rhesus monkey conflict model. It was hypothesized that time-dependent effects on the response rate suppressing properties of zolpidem become tolerant (i.e., acute tolerance). Because anxiolytic-like effects remain stable throughout the session, the absence of rate-decreasing effects may “unmask” anti-conflict effects.

Zolpidem in generic or controlled-release form (Ambien CR®) is one of the most commonly-prescribed drugs for treatment of insomnia worldwide (Morlock et al. 2006). This drug exerts its actions by binding to sites on the γ -aminobutyric acid type A (GABA_A) receptor. GABA_A receptors in the central nervous system are pentamers constituted from structurally distinct proteins, with each protein family consisting of different subunits (for review, see Rudolph et al., 2001). The majority of GABA_A receptors are composed of α , β , and γ subunits, each of which has structurally distinct subtypes. Zolpidem, as well as benzodiazepine anxiolytics and hypnotics, bind to GABA_A receptors containing α 1, α 2, α 3

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subunits, with most benzodiazepines also sensitive to $\alpha 5$ subunit-containing receptors. However, zolpidem has a pharmacological profile distinct from most benzodiazepines, which generally is attributed to its selectivity for the $\alpha 1$ subunit-containing GABA_A receptor (Sanger et al. 1994; Griebel et al. 1996; Sanger 2004; Rowlett et al. 2005).

In addition to the treatment of sleep disorders, drugs that act at GABA_A receptors also have a prominent clinical role in the treatment of anxiety disorders (Nutt 2005). Zolpidem does not consistently induce anxiolytic-like effects in animal models, consistent with accumulating evidence indicating that the $\alpha 1$ -containing GABA_A receptor does not play a key role in the anxiolytic effects of benzodiazepines. For example, in rodents zolpidem is inactive in the open field test, punished operant responding procedures, the light/dark transition test, defensive behaviors test, and stress-induced hypothermia (e.g., Sanger and Zivkovic 1988; Griebel et al. 1996; Vinkers et al. 2009), although zolpidem is effective in some tests of anxiolytic-like activity (e.g., punished drinking; Depoortere et al. 1986; Guisti et al. 1991; Massotti et al. 1991). Moreover, zolpidem lacked anxiolytic-like effects in non-human primates in some studies (Rowlett et al. 2005; 2006; see below) although not in others (Paronis et al. 2001). Finally, in a recent study with healthy human volunteers, zolpidem did not reduce subject-rated measures of anxiety when volunteers were exposed to a relatively high concentration of CO₂, although it did reduce these measures at a lower concentration of CO₂ (Bailey et al. 2009). The non-selective benzodiazepine alprazolam, in contrast, reduced anxiety measures at both CO₂ concentrations.

The original purpose of the study presented here was simply to determine the time-course of action for zolpidem in a conflict procedure. Conflict models are commonly used to evaluate anxiolytic-like effects, and characteristically consist of positively-reinforced behavior that is suppressed by response-contingent administration of a noxious stimulus (for review, see Millan 2003). Compounds with anxiolytic effects in people typically increase rates of responding suppressed by noxious stimuli, and we have developed a conflict procedure using rhesus monkeys as subjects (Rowlett et al. 2006). With this procedure, we have shown that zolpidem has no effect or modestly increases rates of punished responding (Rowlett et al. 2005; 2006). Instead, this $\alpha 1$ -selective compound consistently and robustly attenuates rates of operant responding. Our previous studies used cumulative dosing procedures, which necessarily result in higher doses administered at later time points and might be complicated by the pharmacokinetic properties of the drug. Therefore, we sought to determine the time-course of action of zolpidem using single doses. Surprisingly, rates of suppressed responding emerged at later time points after zolpidem injection, concomitant with a decrease in the ability of zolpidem to attenuate rates of non-suppressed responding. For this report, a control compound (the non-selective hypnotic, triazolam) was examined under similar conditions. Different mechanisms of action are discussed, including the possibility that acute tolerance to the response rate-decreasing effects of the drugs occurred.

Materials and Methods

Subjects and surgical procedures

The subjects were four adult male rhesus monkeys (*Macaca mulatta*), two of which had experience with benzodiazepine-type compounds prior to these studies with the other animals being experimentally naïve (no behavioral differences between experienced and new monkeys was evident in these studies). Monkeys were maintained at 85–95% of their free-feeding weights, individually housed with water available continually, and maintained on a 12 hr lights-on/12 hr lights-off cycle (lights on at 0600 hr). Monkeys received commercial monkey diet (Teklad) and supplemental feeding (fruits, vegetables, and commercially available primate treats) daily and were given toys and video stimulation when not in an experimental session. The weights of the monkeys ranged from 8.1 to 9.2 kg

at the beginning of the studies, and remained relatively constant throughout the experiments. The animals in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and the “Guide for Care and Use of Laboratory Animals” (National Research Council, Department of Health, Education and Welfare Publication No. (NIH) 85-23, revised 1996).

Each monkey was prepared with a chronic indwelling venous catheter according to the procedures described by Platt et al. (2005). A polyvinyl chloride catheter (inner diameter: 0.65 mm; outer diameter: 1.35 mm) was implanted in a jugular (internal or external), femoral, or brachial vein under isoflurane anesthesia and aseptic conditions. The proximal end of the catheter terminated above the right atrium, and the distal end was passed subcutaneously to exit in the midscapular region. Monkeys were treated postoperatively with antibiotics and analgesics, and experimental sessions began at least 7 days after surgery.

Apparatus and procedure

Monkeys were seated in custom-made primate chairs (Crist Instrument Co., Hagerstown, MD) that were placed into ventilated and sound-attenuating chambers. A single response lever (model ENV-610M; Med Associates, Georgia, VT) was mounted on the wall of the chamber in front of the monkey. Each press of a lever with a minimum downward force of approximately 0.25 N produced an audible click and was recorded as a response. Food pellets (Formula 0094, 1 g, Bioserve, Frenchtown, NJ) could be delivered to a tray located next to the lever. Mild electric shock (described in detail below) could be delivered to the bottom of the feet via brass electrodes that were fitted to shoes. Red and green lights mounted above the levers could be illuminated to serve as visual stimuli.

Monkeys were trained under a multiple schedule of food reinforcement. A daily session consisted of 4 cycles, each preceded by a 10-min time out period in which all lights in the chamber were off and responding had no programmed consequences. Each cycle consisted of two components: The first component (“Non-suppressed responding”) was signaled by red stimulus lights and consisted of an 18-response fixed-ratio (FR) schedule of food pellet delivery. The second component (“Suppressed responding”) signaled by green stimulus lights, followed immediately and consisted of the FR 18 schedule of food delivery combined with a 20-response, FR schedule of foot shock delivery (1.5 – 3.0 mA, adjusted for each monkey based on individual performance, 0.25 s duration). Delivery of a food pellet (FR 18 schedule) was followed by a 10-s time out in which responding had no programmed consequences. Both components were 5 min in duration.

Sessions were conducted 5 days per week at approximately the same time each day. On training sessions, monkeys received i.v. injections of saline in the 5th minute of each 10-min time out. For individual monkeys, performance was considered stable if the average rates of responding (responses/s) for non-suppressed responding and suppressed responding did not vary by more than $\pm 20\%$ over five consecutive sessions, with no upward or downward trends. Test sessions were initiated once performance was stable, and continued as long as the stability criteria were met on interceding training sessions.

On test sessions (conducted twice per week), i.v. injections of drug or vehicle were administered in the 5th minute of each time out, with drug or vehicle administered during the first time out and vehicle administered during the next 3 time outs. Thus, the intervals between Components corresponded to 5, 25, 45, and 65 minutes after injection. The effects of doses of each test compound were determined twice. Dose-response functions for the two drugs were determined in different orders for the 4 monkeys, although testing was usually completed for a compound prior to moving on to the next drug. During the course of the

study, if a monkey's performance did not meet the stability criteria or a catheter became dysfunctional and was repaired; training sessions were conducted until the stability criteria were met again.

Data analysis

Data were expressed as the mean responses/s ($\pm$ SEM) for each dose of test drug at the different time points. To evaluate the degree to which individual doses of drug increased rates of food-maintained responding suppressed by shock presentation ("suppressed responding") or decreased rates of food-maintained responding alone ("non-suppressed responding"), separate 2-within analyses of variance (ANOVAs) were calculated, with post-injection time and dose as factors. In addition, a priori Bonferroni t-tests were used to compare average rates of responding for each dose of drug to average rates of responding that occurred after administration of vehicle. For all tests, the family-wise error rate was constrained to $p \leq 0.05$.

Drugs

Zolpidem and triazolam were tested as the base form, and purchased from Sigma-Aldrich (St. Louis, MO). Both drugs were prepared in a vehicle of 50–80% propylene glycol, 10–40% saline, and 10% ethanol. The drugs were injected in a volume of 0.1–1.0 ml/kg, depending on the dose and solubility. All drugs were prepared the day of a test session.

Results

Zolpidem

Characteristic responding in the conflict procedure can be seen in the Figures represented by the points above vehicle (V). Average rates of responding in Suppressed Components were at or near zero, whereas average rates of responding in Non-Suppressed Components were at or near 2.5 responses/s. These characteristic rates of responding under Suppressed and Non-Suppressed conditions were stable throughout the study.

Separate 2-within ANOVAs were performed on rates of responding in the Suppressed and Non-Suppressed Components. For Suppressed responding following zolpidem injections, significant main effects of dose [$F(4,12) = 8.527$, $p < 0.05$] and post-injection time [$F(3,9) = 5.891$, $p < 0.05$] were observed, as well as a significant dose $\times$ post-injection time interaction [$F(12,36) = 3.918$, $p < 0.05$]. At the 5 and 25 min post-injection times (Figure 1, left panels), zolpidem did not alter rates of suppressed responding (Bonferroni t-tests, $p > 0.05$). At the 45 min post-injection time, zolpidem engendered a dose-dependent increase in average rates of suppressed responding, with the 1.0 mg/kg dose significantly above vehicle levels (Bonferroni t-test, $p < 0.05$). At the 65 min post-injection time, zolpidem engendered a significant increase in rates of suppressed responding, with 0.3 and 1.0 mg/kg significantly different from vehicle (Bonferroni t-test, $p < 0.05$).

The 2-within ANOVA for Non-Suppressed responding revealed only a significant dose $\times$ post-injection time interaction [$F(12,36) = 2.145$, $p < 0.05$]. At the 5 min post-injection time (Figure 1, right panels), zolpidem treatment resulted in significant decreases in rates of non-suppressed responding, with the 1.0 mg/kg dose significantly different from vehicle levels (Bonferroni t-test, $p < 0.05$). At the 25, 45, and 65 min post-injection time, no significant effects of zolpidem on suppressed or non-suppressed responding were evident.

Triazolam

For triazolam, the ANOVA for Suppressed responding revealed a significant main effect of dose [$F(4,12) = 9.260$, $p < 0.05$] and a dose $\times$ post-injection time interaction [$F(12,36) = 2.658$,

$p < 0.05$]. In contrast to the pattern of effects seen with zolpidem, when tested at a 5-min post-injection time (Figure 2, left panels), triazolam engendered significant increases in average rates of suppressed responding, with the rates of responding at 0.01 mg/kg significantly above vehicle levels (Bonferroni t-test, $p < 0.05$), whereas at the 0.03 mg/kg dose, rates of responding decreased to vehicle-like levels. At the 25 min post-injection time, the potency of the increase in Suppressed responding appeared to change, with 0.003 mg/kg significantly different from vehicle ($p < 0.05$). At the 45 min post-injection time, the dose of 0.01 mg/kg of triazolam resulted in an increase in rates of suppressed responding compared with vehicle ($p < 0.05$); and at the 65 min post-injection time, both the 0.01 and 0.03 mg/kg doses of triazolam engendered significant increases in rates of suppressed responding.

For Non-Suppressed responding, the ANOVA revealed only a main effect of dose [$F(4,12) = 15.088$, $p < 0.05$], likely reflecting the very similar dose-response functions across post-injection times. However, the *a priori* Bonferroni t-tests revealed some differences across post-injection times (Figure 2, right panels). In this respect, the average rates of non-suppressed responding at 0.03 mg/kg were significantly different from vehicle at both the 5 min and 25 min post-injection times, whereas no significant effects of dose vs. vehicle were observed at the 45 min and 65 min post-injection times.

Discussion

In an effort to determine the duration of action of the effects of zolpidem, this study was designed to determine the effects of this drug on responding maintained by food, with and without a response-contingent noxious stimulus, over an approximately 1-hour period. As reported previously with conflict tests employing cumulative dosing procedures (Rowlett et al. 2005; 2006), after a 5-min post-injection interval zolpidem did not increase rates of suppressed responding, i.e., it lacked an anti-conflict effect. This finding is consistent with other acute tests of anxiolysis with rodents, as well as human and non-human primates (Sanger and Zivkovic 1988; Griebel et al. 1996; Bailey et al. 2009). Unexpectedly, over three 20-min post-injection intervals, an anti-conflict effect of zolpidem emerged. At the same time, the pronounced attenuation of non-suppressed responding observed at 5 min dissipated, such that by 65 min post-injection the rate-decreasing effects of zolpidem were absent entirely.

In order to see if these time-dependent effects of zolpidem were unique to this drug, we conducted a similar study with the triazolobenzodiazepine, triazolam. Triazolam also has a relatively short duration of action (e.g., Smith et al. 1987) but has a pharmacological profile different from that of zolpidem: Triazolam is non-selective for the 4 benzodiazepine-sensitive GABA_A receptor subtypes, whereas zolpidem binds with highest affinity to $\alpha 1$ subunit-containing GABA_A receptors but does not bind to $\alpha 5$ subunit-containing receptors (for review, see Sanger 2004). Furthermore, triazolam has anti-conflict effects (anxiolytic-like effects) following 5-min time interval and cumulative dosing in our rhesus monkey conflict model (Licata et al. 2005). When tested for time-dependent effects using a single-dosing procedure, triazolam showed anti-conflict effects at all time points. In fact, the dose-response function for increasing rates of suppressed responding shifted slightly to the left (~3-fold) from the 5-min to the 25-min post-injection interval, suggesting that triazolam might have a delayed peak of action when administered i.v. Consistent with this idea, i.v. triazolam pharmacokinetics in healthy human volunteers fits best to a two-compartment model, with slight delays in peak performance effects following peak plasma exposures, presumably reflecting a delay due to drug distributing to an effect compartment (Smith et al. 1987).

Similar to zolpidem, the effects of triazolam on non-suppressed responding dissipated over the post-injection times, with no significant effects at 45 and 65 min post injection. This decrease in the ability of triazolam to attenuate response rates in the non-suppressed components was less robust than the similar decrease induced by zolpidem, for which the rate-suppressing effects dissipated completely. Regardless, for both drugs the anxiolytic-like effects either emerged or remained intact, whereas the rate-suppressing effects of both drugs decreased over a relatively short time period.

The findings from this study were unexpected, particularly the results with zolpidem; and the precise mechanism(s) of action is (are) unclear. When administered orally, zolpidem is rapidly absorbed and has a relatively short duration of action (e.g., Patat et al. 1994). However, it seems unlikely that the effects observed in the present study simply reflect declining brain and plasma levels of zolpidem, because while the response rate-suppressing effects of zolpidem declined, the anti-conflict effects emerged rather than declined. Triazolam also is a short-acting drug (e.g., Smith et al. 1987), and in general, the anti-conflict effects of triazolam remained relatively stable, whereas the suppression of response rates declined over the experimental session. Again, this pattern of effects is not consistent with a simple decline in drug levels due to metabolism and/or re-distribution. One possible factor that may be important is route of administration: The use of the i.v. route might play a key role in the time-dependent effects of zolpidem in the conflict procedure, although it again is difficult to explain the differences between zolpidem and triazolam based solely on route of administration.

Perhaps the most parsimonious explanation of these findings is based on the phenomenon of acute tolerance. In this regard, even with steady-state brain levels the response rate-suppressing effects of the drugs decline due to pharmacodynamic adaptation(s). In contrast, the anti-conflict effects of the drugs are not subject to pharmacodynamic adaptation(s), and therefore would be expected to decline as brain and plasma levels decline. The key difference between zolpidem and triazolam is that the response rate-suppressing effects of zolpidem predominate early in the session, thereby “masking” any anti-conflict effect induced by zolpidem. That is, as acute tolerance occurs to the rate-decreasing effects, the anti-conflict effects are expressed. An important assumption of this idea is that tolerance to the anti-conflict effects of benzodiazepine-type drugs either does not occur or develops at a rate slower than tolerance to the rate-decreasing effects. Lack of tolerance to the anxiolytic effects of benzodiazepines has been reported experimentally, and lack of tolerance with anxiolytic measures is a hallmark finding in long-term clinical trials (e.g., Margules and Stein 1968; Verster and Volkerts 2004), although the extent to which anxiolytic tolerance may or may not occur remains unclear (e.g., Hutchinson et al. 1996).

Our working hypothesis of acute tolerance can be couched readily in terms of the pharmacology of GABA_A receptor subtypes. Thus, findings using both pharmacological approaches and transgenic mouse techniques suggest that the ataxic effects of benzodiazepines and related drugs are attributable to the $\alpha 1$ subunit-containing GABA_A receptor (McKernan et al. 2000; Rudolph et al. 2001; Platt et al. 2002; Rowlett et al. 2005). Anxiolytic effects, in contrast, likely involve GABA_A receptors containing $\alpha 2$ and/or $\alpha 3$ subunits (Löw et al. 2000; Dias et al.; Rowlett et al. 2005; Dixon et al. 2005). Assuming ataxic effects contribute significantly to the response rate-suppressing effects of zolpidem and triazolam, the findings in the present study suggest that the pharmacodynamic adaptation(s) that result in acute tolerance occur via the $\alpha 1$ subunit-containing receptor. In contrast, effects mediated via $\alpha 2/\alpha 3$ subunit-containing receptors either do not show acute tolerance or tolerance occurs at a considerably slower rate than those mediated via $\alpha 1$ receptors.

Several reports exist in the literature of acute tolerance to the behavioral effects of benzodiazepines and related drugs. Using a drug discrimination procedure, Gerak et al (2008) trained monkeys to discriminate midazolam from saline, and a single pretreatment with the benzodiazepine chlordiazepoxide resulted in a shift to the right in the midazolam dose-response function, consistent with an acute tolerance interpretation. In other studies, acute tolerance was observed in healthy volunteers on measures such as sedation, attention, and benzodiazepine-associated electroencephalogram (EEG) signals (e.g., Ingum et al. 1994; Ihrnsen et al. 2004; Barbanoj et al. 2007), and, most importantly, at least one study with healthy volunteers has shown results consistent with acute tolerance with zolpidem (de Haas et al. 2009). Therefore, the idea that acute tolerance occurs with benzodiazepine-type compounds is not without precedent.

Although acute tolerance appears to readily account for our findings, other potential mechanisms of action warrant consideration. For example, rather than tolerance mediated by neuroadaptations, the differences in anxiolytic-like and rate-decreasing effects may reflect distinct rates of distribution/elimination associated with different brain regions. At present, this hypothesis is difficult to test due to lack of information on the site(s) of action that mediate anxiolytic vs. motor effects, although a starting point likely is the amygdala (Davis et al. 2010).

Regardless of the mechanisms of action, the finding of anti-conflict effects of zolpidem following longer post-injection intervals is noteworthy, especially since considerable evidence suggests that this drug lacks anxiolytic effects or induces relatively weak anxiolysis (e.g., Sanger and Zivkovic 1988; Griebel et al., 1996; Mitchell et al. 2004; Bailey et al. 2009). Interestingly, some studies have shown evidence for anxiolysis with zolpidem, particularly those using the elevated plus maze procedure (e.g., Auta et al. 1993; Davies et al. 1994; Griebel et al. 1996; Vanover et al. 1999). In most cases, however, the effects of zolpidem were relatively weak compared with non-selective benzodiazepines and tend to occur at sedative-motor doses. Moreover, other effects of zolpidem (e.g., hyperphagia) apparently can confound interpretation of behavioral outcomes as anxiolysis (Mitchell et al. 2004). The extent to which acute tolerance to the behavioral-suppressing effects of zolpidem plays a role in the mixed results with zolpidem and anxiolysis is unknown. It is interesting to speculate that if such a phenomenon occurs generally, then zolpidem in people may switch from inducing a hypnotic effect to an anxiolytic effect at later time points (e.g., later in the night after the individual has gone to bed). Given the relatively high co-morbidity of insomnia and anxiety disorders (Uhde et al. 2009), such a characteristic of zolpidem certainly would be considered clinically beneficial.

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Figure 1.

Effects of intravenous injections of zolpidem in rhesus monkeys trained under a conflict procedure. A single injection of zolpidem was administered, and rates of suppressed responding (left panels) and non-suppressed responding (right panels) were measured at different time intervals following the injection. In the figure legend, *Suppressed* refers to a signaled component of the schedule in which food-maintained lever pressing and response-contingent shock were programmed concomitantly; and *Non-Suppressed* refers to a signaled component of food-maintained lever pressing. Data are expressed as mean rates of responding $\pm$ SEM for N=4 monkeys. Note that * $p < 0.05$ compared with vehicle (V) under Non-Suppressed conditions, and ** $P < 0.05$ compared with vehicle under Suppressed conditions.

Figure 2.
Effects of intravenous injections of triazolam in rhesus monkeys trained under a conflict procedure. All details are as in Figure 1.