BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01733.x
www.brjpharmacol.org

RESEARCH PAPER

Role of neurosteroids in the anticonvulsant activity of midazolam

Ashish Dhir[1] and Michael A Rogawski[1,2]

[1]*Department of Neurology, School of Medicine, University of California, Davis, Sacramento, CA, USA, and* [2]*Center for Neuroscience, University of California, Davis, Sacramento, CA, USA*

Correspondence
Michael A Rogawski, Department of Neurology, University of California, Davis, 4860 Y Street, Suite 3700, Sacramento, CA 95817, USA. E-mail: rogawski@ucdavis.edu

Keywords
midazolam; clonazepam; peripheral benzodiazepine receptor; neurosteroid; finasteride; PK 11195; metyrapone; pentylenetetrazol

Received
7 June 2011
Revised
1 September 2011
Accepted
16 September 2011

BACKGROUND AND PURPOSE
Midazolam is a short-acting benzodiazepine that is widely used as an i.v. sedative and anticonvulsant. Besides interacting with the benzodiazepine site associated with $GABA_A$ receptors, some benzodiazepines act as agonists of translocator protein (18 kDa) (TSPO) to enhance the synthesis of steroids, including neurosteroids with positive modulatory actions on $GABA_A$ receptors. We sought to determine if neurosteroidogenesis induced by midazolam contributes to its anticonvulsant action.

EXPERIMENTAL APPROACH
Mice were pretreated with neurosteroid synthesis inhibitors and potentiators followed by midazolam or clonazepam, a weak TSPO ligand. Anticonvulsant activity was assessed with the i.v. pentylenetetrazol (PTZ) threshold test.

KEY RESULTS
Midazolam (500–5000 $\mu g \cdot kg^{-1}$, i.p.) caused a dose-dependent increase in seizure threshold. Pretreatment with the neurosteroid synthesis inhibitors finasteride, a 5α-reductase inhibitor, and a functional TSPO antagonist PK 11195, reduced the anticonvulsant action of midazolam. The anticonvulsant action of midazolam was enhanced by the neurosteroidogenic drug metyrapone, an 11β-hydroxylase inhibitor. In contrast, the anticonvulsant action of clonazepam (100 $\mu g \cdot kg^{-1}$) was reduced by finasteride but not by PK 11195, indicating a possible contribution of neurosteroids unrelated to TSPO.

CONCLUSION AND IMPLICATIONS
Enhanced endogenous neurosteroid synthesis, possibly mediated by an interaction with TSPO, contributed to the anticonvulsant action of midazolam. Enhanced neurosteroidogenesis may also be a factor in the actions of other benzodiazepines, even those that only weakly interact with TSPO.

Abbreviations
PK11195, 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide; PTZ, pentylenetetrazol; TSPO, translocator protein (18 kDa)

Introduction

Midazolam is a rapid onset, short-acting water-soluble benzodiazepine that is administered parenterally as a sedative, anxiolytic, hypnotic and amnestic agent. The drug is also administered i.m., i.v. or intranasally to terminate acute seizures and status epilepticus (Galvin and Jelinek, 1987). Midazolam exhibits anticonvulsant activity in a variety of chemoconvulsant seizure models, including the pentylenetetrazol (PTZ) model in various species (Pieri, 1983; Jaimovich *et al.*, 1990; Raines *et al.*, 1990; Orebaugh and Bradford, 1994). As is the case for other benzodiazepines, the pharmacological actions of midazolam are mediated predominantly through an interaction with a high affinity binding site in brain representing an allosteric modulatory site on $GABA_A$ receptors (Kucken *et al.*, 2003; receptor nomenclature follows Alexander *et al.*, 2011). Binding of midazolam and other classic benzodiazepines, such as clonazepam, to a recognition site at the interface between extracellular domains of the $GABA_A$ receptor γ subunit and one of the α subunits allosterically modulates gating of the $GABA_A$ receptor chloride channel complex leading to enhanced channel current (Rovira and Ben-Ari, 1993; Rüsch and Forman, 2005; Eom *et al*., 2011). This allosteric modulation of $GABA_A$ receptors is believed to account for the principal pharmacological actions of benzodiazepines, including their anticonvulsant activity (Rogawski, 2002).

Some benzodiazepines also bind to a pharmacologically distinct and unrelated binding site in brain and peripheral tissues that was formerly described as the peripheral-type benzodiazepine receptor (Gavish *et al*., 1999) but is now referred to as translocator protein (18 kDa) (TSPO) (Papadopoulos *et al*., 2006). TSPO is expressed predominantly in mitochondria, where it is localized to the outer mitochondrial membrane. TSPO binds cholesterol with high affinity and transports it from the outer mitochondrial membrane to the inner mitochondrial membrane, where it is converted to pregnenolone by the cytochrome P450 side-chain cleavage enzyme. This sequence represents the initial and rate-limiting step in the biosynthesis of all steroids. TSPO agonist ligands, including benzodiazepines with TSPO binding activity (Mukhin *et al*., 1989), stimulate steroidogenesis by facilitating cholesterol delivery to the cytochrome P450 side-chain cleavage enzyme in the inner mitochondrial membrane (Lacapère and Papadopoulos, 2003).

As is the case for hormonal steroids, a variety of evidence supports the concept that TSPO agonist ligands can also enhance the synthesis of endogenous neurosteroids, including allopregnanolone, that lack hormonal activity but serve as positive allosteric modulators of $GABA_A$ receptors (Romeo *et al*., 1993; Serra *et al*., 1999; Bitran *et al*., 2000; Verleye *et al*., 2005). In accordance with their effects on $GABA_A$ receptors, such neurosteroids exhibit anticonvulsant activity in various seizure models, including PTZ-induced seizures in mice (Kokate *et al*., 1994). The effect of TSPO agonist ligands on neurosteroidogenesis is believed to occur through enhanced mitochondrial synthesis of pregnenolone, which is converted to progesterone by microsomal 3β-hydroxysteroid dehydrogenase and then to neurosteroids by sequential A-ring reduction by 5α-reductase and 3α-hydroxysteroidoxidoreductase. Enhancement of endogenous neurosteroid synthesis, induced by TSPO agonist ligands, can produce pharmacological effects typical of neurosteroids, including anxiolytic actions (Kita and Furukawa, 2008; Rupprecht *et al*., 2009). In some experimental situations, the enhanced neurosteroid synthesis (Serra *et al*., 1999; Frye *et al*., 2009) and behavioural effects (Bitran *et al*., 2000; Kita *et al*., 2004; Rupprecht *et al*., 2009) of TSPO agonists can be reduced by the isoquinoline carboxamide TSPO antagonist, PK 11195.

Although there is some evidence that midazolam interacts with TSPO (Schoemaker *et al*., 1981; Bender and Hertz, 1987; Mak and Barnes, 1990; Matsumoto *et al*., 1994), until recently, it was not known if midazolam acts as a functional TSPO ligand to affect steroidogenesis. However, So *et al*. (2010) have now reported that midazolam enhances Leydig cell production of progesterone and testosterone. In addition, Tokuda *et al*. (2010) observed that midazolam enhances the synthesis of neurosteroids in hippocampal neurons in brain slices. These latter authors presented evidence that neurosteroidogenesis in addition to an interaction with the benzodiazepine binding site on $GABA_A$ receptors is required for the actions of midazolam on synaptic inhibition. We therefore hypothesized that the *in vivo* actions of midazolam are also due to a combination of direct effects on $GABA_A$ receptors (through the intrinsic benzodiazepine site) as well its indirect actions on neurosteroidogenesis mediated by TSPO. In the present study, we investigated this possibility with the use of various agents that modify endogenous neurosteroid synthesis.

Experimental procedures

Animals

All animal care and experimental protocols were approved by the Animal Care and Use Committee of the University of California, Davis in strict compliance with the *Guide for the Care and Use of Laboratory Animals* of the National Research Council (National Academy Press, Washington, D.C.; http://www.nap.edu/readingroom/books/labrats/). The animal facilities were fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Male NIH Swiss mice (22–30 g) were kept in a vivarium under controlled environmental conditions (temperature, 22–26°C; humidity, 40–50%) with an artificial 12 h light/dark cycle. Wood chips were used in all cages. Experiments were performed during the light phase of the light/dark cycle after a minimum 30 min period of acclimation to the experimental room.

PTZ seizure threshold test

PTZ seizure threshold was determined according to a protocol used previously in our laboratory (Dhir *et al*., 2011). Given i.v., PTZ elicits a sequence of seizure signs beginning with twitch and progressing to clonus and tonic limb extension. In the present study, tonic extension was used as the endpoint. In preliminary experiments, tonic extension was found to be more sensitive to midazolam than the twitch or clonus phases. It is noteworthy that this is the endpoint originally used in studies characterizing the actions of midazolam against PTZ seizures in mice (Pieri, 1983). A 27-gauge, $^3/_4$ inch butterfly needle was inserted into the lateral tail vein, and the needle was secured with a narrow piece of adhesive tape. The animal was placed inside a 2 L glass beaker with free aeration from the top and allowed to move freely. The needle was connected by polyethylene tubing to a Beckton, Dickinson 1 mL syringe mounted on an infusion pump (Model '11' plus syringe pump; Harvard Apparatus, Holliston, MA). PTZ solution ($10\ mg\cdot mL^{-1}$) was infused at a constant rate of $0.5\ mL\cdot min^{-1}$. The infusion was stopped at 3 min or at the onset of tonic extension, whichever occurred first. The threshold value ($mg\cdot kg^{-1}$) for tonic extension was determined according to the following formula: [infusion duration (s) × infusion rate ($mL\cdot min^{-1}$) × PTZ concentration ($mg\cdot mL^{-1}$) × 1000] / [60 (s) × weight of mouse (g)].

Treatment schedules

In an initial experiment, the dose–response relationship for midazolam elevation of seizure threshold was assessed at

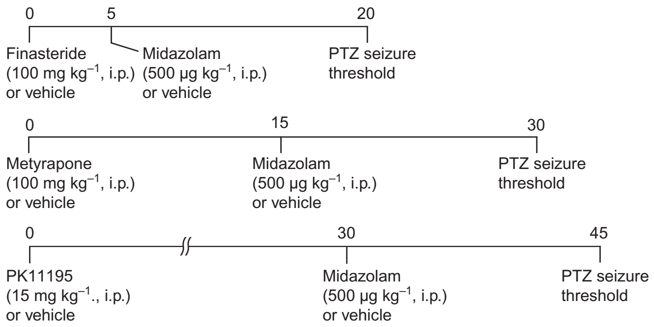

Figure 1

Drug treatment protocols. In some experiments, clonazepam (25 or100 μg·kg^{-1}, i.p.) was used instead of midazolam.

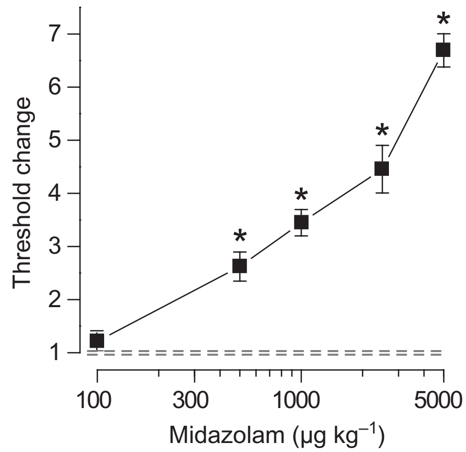

Figure 2

Dose–response relationship for midazolam protection against tonic extension in response to i.v. PTZ infusion in mice. Midazolam was administered i.p. 15 min before the beginning of the PTZ infusion. Data points indicate mean ± SEM of threshold values from six to eight mice normalized with respect to the mean threshold value in the vehicle-treated control group, which was 51.5 ± 4.0 mg·kg^{-1}. Dashed lines indicate the limits of the SEM for the vehicle group. The mean threshold values for all groups other than the 100 μg·kg^{-1} group are significantly different from the value in the vehicle group. $^*P < 0.001$; one-way ANOVA followed by Tukey's test.

doses of 100 μg·kg^{-1} to 5 mg·kg^{-1} (15 min pretreatment time). The doses and time of administration of test substances in the remaining experiments with midazolam are shown in Figure 1. In the first protocol, the 5α-reductase inhibitor finasteride was used at a dose (100 mg·kg^{-1}) that has previously been shown to partially inhibit neurosteroid synthesis (Kokate *et al*., 1999). Metyrapone was used at a dose (100 mg·kg^{-1}) that has previously been shown to elevate seizure threshold through enhanced endogenous neurosteroid synthesis (Kaminski and Rogawski, 2011). PK 11195 was used at a dose (15 mg·kg^{-1}) as in the study of Ugale *et al*. (2004). Clonazepam was administered at a dose of 100 μg·kg^{-1} (Akula *et al*., 2009). The volume of all i.p. injections was 10 mL·kg^{-1}.

Data analysis

Results are expressed as mean ± SEM; the significance of the difference in the responses of treatment groups with respect to control is based on one-way ANOVA followed by specific *post hoc* comparisons using Tukey's test. Differences were considered statistically significant when the probability of type I error was less than 0.05.

Materials

Midazolam was administered as a commercially available injectable solution [midazolam hydrochloride, 10 mg·(10 mL)$^{-1}$; APP Pharmaceuticals, Schaumburg, IL] containing sodium chloride (0.8 % w/v), disodium edetate (0.01% w/v), benzyl alcohol as preservative (1% v/v) with HCl to adjust the pH 3–3.6. Pentylenetetrazol (PTZ) (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% w/v saline. The remaining compunds were all obtained from Tocris Bioscience (Ellisville, MO) except for clonazepam, which was from Sigma-Aldrich. Finasteride was dissolved in 25% hydroxypropyl-β-cyclodextrin (Trappsol; Cyclodextrin Technologies Development, High Springs, FL). Metyrapone was dissolved in double distilled water, and PK 11195 and clonazepam were suspended in 1% Tween 80 and adjusted to volume with saline.

Results

Midazolam causes a dose-dependent elevation in PTZ seizure threshold

I.v. infusion of PTZ (10 mg·mL^{-1}) led to a sequence of seizure signs consisting of myoclonic jerk, clonus and tonic extension, followed by death. In the present study, cumulative dose to the onset of tonic extension was the measure of seizure threshold. Animals protected from tonic extension invariably survived, whereas those that experienced tonic extension expired immediately after the seizure. Figure 2 plots the fractional change in mean threshold for groups of animals that had been treated 15 min before the onset of the PTZ infusion with various doses of midazolam. There was a dose-dependent elevation in threshold with increasing midazolam dose that was significant for midazolam doses of 500 to 5000 μg·kg^{-1} but not 100 μg·kg^{-1}.

Finasteride pretreatment reduces the seizure threshold elevation induced by midazolam

To assess whether endogenous neurosteroid production contributes to the seizure threshold elevation induced by midazolam, mice were pretreated with the neurosteroid synthesis inhibitor finasteride (100 mg·kg^{-1}, i.p.) before giving a dose of midazolam (500 μg·kg^{-1}, i.p.) that in the experiment of Figure 2 caused a significant increase in threshold. As shown in Figure 3, finasteride pretreatment by itself did not alter the PTZ seizure threshold. However, finasteride did partially reduce the threshold elevation caused by midazolam.

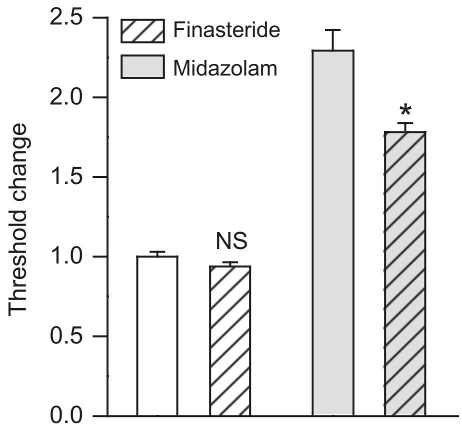

Figure 3

Finasteride pretreatment reduces the seizure threshold elevation induced by midazolam. Finasteride (100 mg·kg^{-1}, i.p.) or vehicle was administered 5 min before the treatment with midazolam (500 µg·kg^{-1}, i.p.) or vehicle; 15 min after the second pretreatment, all animals were infused with PTZ. Bars indicate mean ± SEM of fractional threshold change values for tonic extension from 6–14 mice normalized with respect to the mean threshold value in the vehicle only control group, which was 59.2 ± 1.8 mg·kg^{-1}. In the absence of finasteride, midazolam caused a 2.3-fold increase in threshold (P < 0.001). Finasteride did not reduce the threshold significantly in the absence of midazolam (NS) but did reduce the threshold with midazolam pretreatment. *P < 0.001; one-way ANOVA followed by Tukey's test.

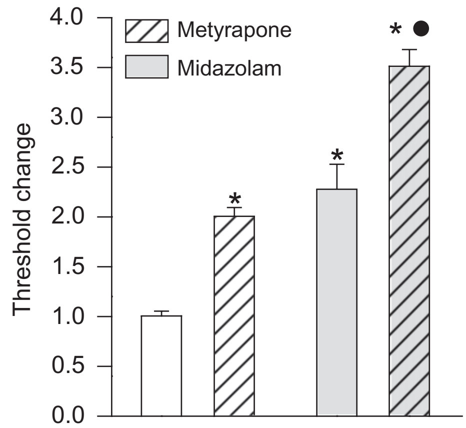

Figure 4

Metyrapone elevates seizure threshold in the absence and presence of midazolam. Metyrapone (100 mg·kg^{-1}, i.p.) or vehicle was administered 15 min before treatment with midazolam or vehicle; 15 min after the second pretreatment, all animals were infused with PTZ. Bars indicate mean ± SEM of fractional change values in tonic extension threshold from six to nine mice normalized with respect to the mean threshold value in the vehicle only control group, which was 51.0 ± 4.4 mg·kg^{-1}. *P < 0.001, significantly different from vehicle only control group; ●P < 0.001, significantly different from midazolam only group; one-way ANOVA followed by Tukey's test.

Metyrapone enhances the seizure threshold elevation induced by midazolam

To provide further support for the involvement of neurosteroids in the anticonvulsant action of midazolam, the 11β-hydroxylase inhibitor metyrapone, which enhances endogenous neurosteroid synthesis (Rupprecht *et al.*, 1998; Kaminski and Rogawski, 2011), was given before midazolam. By itself, metyrapone (100 mg·kg^{-1}, i.p.) caused a significant elevation in the threshold (Figure 4), confirming our previous report (Kaminski and Rogawski, 2011). When metyrapone was administered before midazolam (500 µg·kg^{-1}, i.p.), there was a further increment in threshold.

PK 11195 inhibits the seizure threshold elevation induced by midazolam

As an additional approach to assessing the role of neurosteroidogenesis in the action of midazolam we used PK 11195, a high-affinity ligand of TSPO that acts as an antagonist in some situations (Le Fur *et al.*, 1983) and inhibits the behavioural effects of TSPO ligands that stimulate neurosteroidogenesis (Auta *et al.*, 1993; Romeo *et al.*, 1993; Tsuda *et al.*, 1998; Frye *et al.*, 2009). By itself, PK 11195 (15 mg·kg^{-1}, i.p.) did not affect the seizure threshold. However, pretreatment with PK 11195 did significantly reduce the elevation in threshold produced by midazolam (500 µg·kg^{-1}, i.p.) (Figure 5).

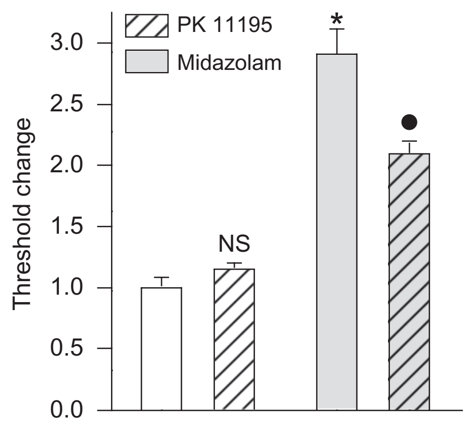

Figure 5

PK 11195 pretreatment reduces the seizure threshold elevation induced by midazolam. PK11195 (15 mg·kg^{-1}, i.p.) was administered 30 min before the treatment with midazolam (500 µg·kg^{-1}, i.p.) or vehicle; 15 min after the second pretreatment, all animals were infused with PTZ. Bars indicate mean ± SEM of fractional threshold change values for tonic extension from 6–12 mice normalized with respect to the mean threshold value in the vehicle only control group (same as Figure 4). *P < 0.001, significantly different from vehicle only control group; ●P < 0.001, significantly different from midazolam only group; one-way ANOVA followed by Tukey's test.

Effects of finasteride and PK 11195 on the seizure threshold elevation induced by clonazepam

To assess the specificity of the effects of finasteride and PK 11195, a series of experiments were conducted with clonazepam, a benzodiazepine that binds only weakly to TSPO (Schoemaker *et al.*, 1981; Marangos *et al.*, 1982; Bender and Hertz, 1987; Guarneri *et al.*, 1995; McCauley *et al.*, 1995) and does not enhance neurosteroid synthesis in some *in vitro* preparations (Mukhin *et al.*, 1989; Papadopoulos *et al.*, 1992; Tokuda *et al.*, 2010). As shown in Figure 6 (*upper panel*), finasteride pretreatment did reduce the seizure threshold elevation produced by 100 μg·kg^{-1} clonazepam. In a second experiment with a lower does of clonazepam (25 μg·kg^{-1}), there was a trend towards an effect of finasteride, although it did not reach statistical significance (data not shown). However, unlike the situation with midazolam, PK 11195 did not reduce the seizure threshold elevation produced by clonazepam (Figure 6, *lower panel*).

Discussion

This study for the first time provides evidence for the involvement of neurosteroids in the anticonvulsant activity of midazolam. As expected, midazolam caused a dose-dependent anticonvulsant action in the i.v. PTZ threshold model. The anticonvulsant activity of midazolam was significantly reduced by finasteride, a 5α-reductase inhibitor that is well recognized to suppress neurosteroidogenesis in mice (Kokate *et al.*, 1999; Finn *et al.*, 2006). For example, at the dose used in the present study, finasteride eliminates the rise in plasma allopregnanolone induced by elevation of its precursor progesterone (Reddy *et al.*, 2001) and also inhibits local neurosteroid synthesis in the brain (Tokuda *et al.*, 2010). By itself, finasteride did not affect the seizure threshold, indicating that the effect on midazolam is not due to an enhancement of seizure susceptibility, unrelated to the action on neurosteroidogenesis. Moreover, our result is consistent with several other studies demonstrating that finasteride does not influence basal seizure susceptibility (Reddy and Rogawski, 2002; Lawrence *et al.*, 2010), which lead to the conclusion that basal (unstimulated) neurosteroid levels do not have a tonic influence on seizure susceptibility.

In contrast to the effect of finasteride, metyrapone, an 11β-hydroxylase inhibitor, has been shown to increase neurosteroidogenesis by causing a buildup of neurosteroid precursors such as progesterone and 11-deoxycorticosterone that ordinarily flow to glucocorticoid synthesis (Kaminski and Rogawski, 2011). In the present study, metyrapone by itself elevated the seizure threshold consistent with our previous report (Kaminski and Rogawski, 2011). Midazolam caused a further and largely additive increment in threshold confirming that enhanced neurosteroidogenesis can augment the action of midazolam.

The TSPO ligand PK 11195 by itself did not affect PTZ seizure threshold. PK 11195 in some but not all situations acts as a TSPO antagonist (Le Fur *et al.*, 1983; Mizoule *et al.*, 1985; Matsumoto *et al.*, 1994). As such, it inhibits TSPO agonist-induced steroidogenesis (Cavallaro *et al.*, 1992). PK 11195 by itself has variable effects on basal steroidogenesis. In some situations it has no effect consistent with a role as a TSPO antagonist (Cavallaro *et al.*, 1992), whereas in other cases, it may increase (Mukhin *et al.*, 1989; McCauley *et al.*, 1995) or decrease (Frye *et al.*, 2009) steroidogenesis. These latter actions could be due to weak intrinsic (partial agonist) activity or to effects on endogenous TSPO ligands. Even though PK 11195 may reduce endogenous neurosteroid levels in some circumstances, the lack of effect of PK 11195 on basal seizure threshold is consistent with the results of several previous studies with finasteride discussed above that have concluded

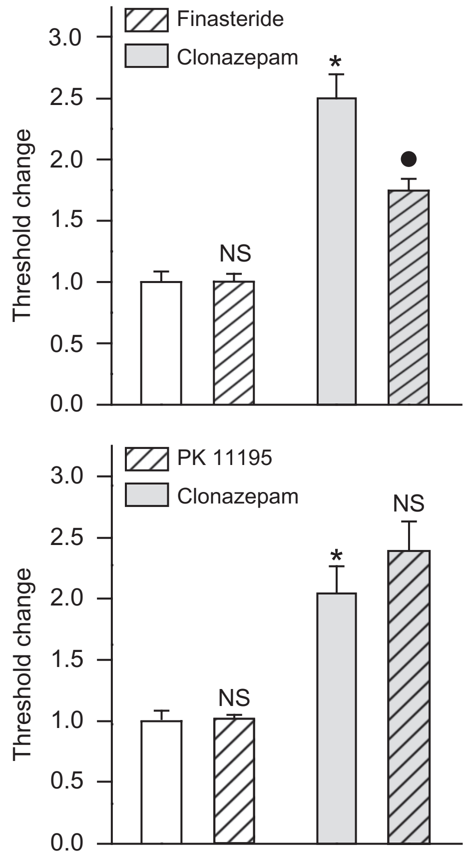

Figure 6

Finasteride (upper panel) but not PK 11195 (lower panel) pretreatment reduces the seizure threshold elevation induced by clonazepam. Finasteride (100 mg·kg^{-1}, i.p.) or vehicle was administered 5 min before the treatment with clonazepam (100 μg·kg^{-1}, i.p.) or vehicle; 15 min after the second pretreatment, all animals were infused with PTZ. In the absence of finasteride, clonazepam caused a 2.8-fold increase in threshold ($P < 0.001$). Finasteride did not reduce the threshold significantly in the absence of midazolam (NS) but did reduce the threshold with clonazepam pretreatment ($P < 0.001$). PK11195 (15 mg·kg^{-1}, i.p.) was administered 30 min before the treatment with clonazepam or vehicle; 15 min after the second pretreatment, all animals were infused with PTZ. Bars indicate mean ± SEM of fractional threshold change values for tonic extension from 6–13 mice normalized with respect to the mean threshold value in the vehicle only control group, which was 46.9 ± 3.6 mg·kg^{-1} in the experiment with finasteride and 51.9 ± 2.7 mg·kg^{-1} in the experiment with PK 11195. *$P < 0.001$, significantly different from vehicle only control group; ●$P < 0.001$, significantly different from clonazepam only group; one-way ANOVA followed by Tukey's test.

that basal neurosteroid levels do not influence seizure susceptibility. Here we took advantage of the ability of PK 11195 to antagonize neurosteroidogenesis activated by TSPO ligands. We observed that PK 11195 caused a significant inhibition of the seizure threshold increase produced by midazolam. This provides evidence that the anticonvulsant action of midazolam depends in part on its ability to interact with TSPO as an agonist.

To further explore the role of TSPO, we conducted experiments with clonazepam, a benzodiazepine that is a very weak TSPO ligand. Surprisingly, we observed that the seizure threshold increase produced by clonazepam was reduced by finasteride. This occurred at a higher dose of clonazepam but not at a lower dose. Given the weak affinity of clonazepam for TSPO, the concentrations achieved in brain with either dose are unlikely to produce substantial occupancy of TSPO. Accordingly, PK 11195 failed to reduce the effect of clonazepam on seizure threshold demonstrating that the activity of this benzodiazepine was not mediated by TSPO. At present, how finasteride inhibits the response to clonazepam is uncertain. There are no known pharmacokinetic interactions between finasteride and benzodiazepines, including clonazepam and midazolam. While clonazepam does not stimulate neurosteroid synthesis in mitochondria (Papadopoulos *et al*., 1992), isolated cell systems (Mukhin *et al*., 1989) or some brain regions (Tokuda *et al*., 2010), in at least one region of the CNS (retina), it can potently and rapidly (within minutes) enhance neurosteroid synthesis (Guarneri *et al*., 1995). This effect of clonazepam, which occurs in a PK 11195-independent fashion, appears to be mediated by a direct interaction with $GABA_A$ receptors. Whether a similar action occurs in brain regions relevant to the anticonvulsant activity of clonazepam remains to be determined.

In conclusion, our results demonstrate a role for neurosteroids in the anticonvulsant action of midazolam. We propose that in addition to directly activating $GABA_A$ receptors through an agonist interaction with the intrinsic benzodiazepine recognition site, midazolam enhanced neurosteroid synthesis through an agonist interaction with TSPO, although we cannot exclude the possibility that this occurred in part through a direct interaction with $GABA_A$ receptors, as is likely to be the case for clonazepam. Whether the enhanced neurosteroidogenesis occurred peripherally or directly in the brain was not defined in the present study. Although there is evidence that midazolam can influence neurosteroid synthesis locally in the brain (Tokuda *et al*., 2010), neurosteroids synthesized peripherally can readily enter the brain to influence seizure susceptibility. Therefore, enhanced peripheral neurosteroid synthesis could contribute to the neurosteroid-related component of the effect of midazolam on seizure threshold noted in the present study. Neurosteroids are known to bind to distinct sites on $GABA_A$ receptors through which they cause positive allosteric modulation of GABA responses (at low concentrations) and direct activation of the receptor (at higher concentrations) (Hosie *et al*., 2007). Unlike neurosteroids, agonists that act at the benzodiazepine recognition site do not directly activate $GABA_A$ receptors in the absence of GABA. Moreover, neurosteroids cause markedly greater maximal potentiation of GABA responses than agonists at the benzodiazepine recognition site (Kokate *et al*., 1994). The capacity of neurosteroids to cause large magnitude positive modulation of GABA responses and also to directly activate $GABA_A$ receptors confers potent anticonvulsant properties on neurosteroids (Rogawski and Reddy, 2004). The combination of the direct action of midazolam on synaptic $GABA_A$ receptors along with the indirect actions mediated by neurosteroids could account for the particularly effective anticonvulsant action of midazolam (Raines *et al*., 1990). Benzodiazepines only act on a restricted subset of $GABA_A$ receptor isoforms (Olsen and Sieghart, 2008). Neurosteroids, in contrast, act on all $GABA_A$ receptors subunit combinations and produce a particularly large augmentation in the activity of certain non-synaptic forms, such as those containing δ subunits, that mediate tonic inhibition (Stell *et al*., 2003; Farrant and Nusser, 2005). It is reasonable to speculate that an effect on non-synaptic, benzodiazepine-insensitive $GABA_A$ receptors mediated indirectly by neurosteroids also contributes to the powerful anticonvulsant action of midazolam. Neurosteroids may also contribute to the anticonvulsant actions of other benzodiazepines with TSPO binding activity, and there are benzodiazepines, most notably clonazepam, that may influence neurosteroids through mechanisms that do not involve TSPO.

Conflicts of interest

None.

References

Akula KK, Dhir A, Kulkarni SK (2009). Effect of various antiepileptic drugs in a pentylenetetrazol-induced seizure model in mice. Methods Find Exp Clin Pharmacol 31: 423–432.

Alexander SPH, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th Edition. Br J Pharmacol 164 (Suppl. 1): S1–S324.

Auta J, Romeo E, Kozikowski A, Ma D, Costa E, Guidotti A (1993). Participation of mitochondrial diazepam binding inhibitor receptors in the anticonflict, antineophobic and anticonvulsant action of 2-aryl-3-indoleacetamide and imidazopyridine derivatives. J Pharmacol Exp Ther 265: 649–656.

Bender AS, Hertz L (1987). Pharmacological characteristics of diazepam receptors in neurons and astrocytes in primary cultures. J Neurosci Res 18: 366–372.

Bitran D, Foley M, Audette D, Leslie N, Frye CA (2000). Activation of peripheral mitochondrial benzodiazepine receptors in the hippocampus stimulates allopregnanolone synthesis and produces anxiolytic-like effects in the rat. Psychopharmacology (Berl) 151: 64–71.

Cavallaro S, Korneyev A, Guidotti A, Costa E (1992). Diazepam-binding inhibitor (DBI)-processing products, acting at the mitochondrial DBI receptor, mediate adrenocorticotropic hormone-induced steroidogenesis in rat adrenal gland. Proc Natl Acad Sci U S A 89: 10598–10602.

Dhir A, Zolkowska D, Murphy RB, Rogawski MA (2011). Seizure protection by intrapulmonary delivery of propofol hemisuccinate. J Pharmacol Exp Ther 336: 215–222.

Eom W, Lee JM, Park J, Choi K, Jung SJ, Kim HS (2011). The effects of midazolam and sevoflurane on the $GABA_A$ receptors with alternatively spliced variants of the γ2 subunit. Korean J Anesthesiol 60: 109–118.

Farrant M, Nusser Z (2005). Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. Nat Rev Neurosci 6: 215–229.

Finn DA, Beadles-Bohling AS, Beckley EH, Ford MM, Gililland KR, Gorin-Meyer RE *et al*. (2006). A new look at the 5α-reductase inhibitor finasteride. CNS Drug Rev 12: 53–76.

Frye CA, Paris JJ, Rhodes ME (2009). Increasing 3α,5α-THP following inhibition of neurosteroid biosynthesis in the ventral tegmental area reinstates anti-anxiety, social, and sexual behavior of naturally receptive rats. Reproduction 137: 119–128.

Galvin GM, Jelinek GA (1987). Midazolam: an effective intravenous agent for seizure control. Arch Emerg Med 4: 169–172.

Gavish M, Bachman I, Shoukrun R, Katz Y, Veenman L, Weisinger G *et al*. (1999). Enigma of the peripheral benzodiazepine receptor. Pharmacol Rev 51: 629–650.

Guarneri P, Guarneri R, Cascio C, Piccoli F, Papadopoulos V (1995). γ-Aminobutyric acid type A/benzodiazepine receptors regulate rat retina neurosteroidogenesis. Brain Res 683: 65–72.

Hosie AM, Wilkins ME, Smart TG (2007). Neurosteroid binding sites on $GABA_A$ receptors. Pharmacol Ther 116: 7–19.

Jaimovich DG, Shabino CL, Noorani PA, Bittle BK, Osborne JS (1990). Intravenous midazolam suppression on pentylenetetrazol-induced epileptogenic activity in a porcine model. Crit Care Med 18: 313–316.

Kaminski RM, Rogawski MA (2011). 11β-Hydroxylase inhibitors protect against seizures in mice by increasing endogenous neurosteroid synthesis. Neuropharmacology 2011: 133–137.

Kita A, Furukawa K (2008). Involvement of neurosteroids in the anxiolytic-like effects of AC-5216 in mice. Pharmacol Biochem Behav 89: 171–178.

Kita A, Kohayakawa H, Kinoshita T, Ochi Y, Nakamichi K, Kurumiya S *et al*. (2004). Antianxiety and antidepressant-like effects of AC-5216, a novel mitochondrial benzodiazepine receptor ligand. Br J Pharmacol 142: 1059–1072.

Kokate TG, Svensson BE, Rogawski MA (1994). Anticonvulsant activity of neurosteroids: correlation with γ-aminobutyric acid-evoked chloride current potentiation. J Pharmacol Exp Ther 270: 1223–1229.

Kokate TG, Banks MK, Magee T, Yamaguchi S, Rogawski MA (1999). Finasteride, a 5α-reductase inhibitor, blocks the anticonvulsant activity of progesterone in mice. J Pharmacol Exp Ther 288: 679–684.

Kucken AM, Teissére JA, Seffinga-Clark J, Wagner DA, Czajkowski C (2003). Structural requirements for imidazobenzodiazepine binding to GABA(A) receptors. Mol Pharmacol 63: 289–296.

Lacapère JJ, Papadopoulos V (2003). Peripheral-type benzodiazepine receptor: structure and function of a cholesterol-binding protein in steroid and bile acid biosynthesis. Steroids 68: 569–585.

Lawrence C, Martin BS, Sun C, Williamson J, Kapur J (2010). Endogenous neurosteroid synthesis modulates seizure frequency. Ann Neurol 67: 689–693.

Le Fur G, Vaucher N, Perrier ML, Flamier A, Benavides J, Renault C *et al*. (1983). Differentiation between two ligands for peripheral benzodiazepine binding sites, [3H]RO5-4864 and [3H]PK 11195, by thermodynamic studies. Life Sci 33: 449–457.

Mak JC, Barnes PJ (1990). Peripheral type benzodiazepine receptors in human and guinea pig lung: characterization and autoradiographic mapping. J Pharmacol Exp Ther 252: 880–885.

Marangos PJ, Patel J, Boulenger JP, Clark-Rosenberg R (1982). Characterization of peripheral-type benzodiazepine binding sites in brain using [^{3}H]Ro 5-4864. Mol Pharmacol 22: 26–32.

Matsumoto T, Ogata M, Koga K, Shigematsu A (1994). Effect of peripheral benzodiazepine receptor ligands on lipopolysaccharide-induced tumor necrosis factor activity in thioglycolate-treated mice. Antimicrob Agents Chemother 38: 812–816.

McCauley LD, Park CH, Lan NC, Tomich JM, Shively JE, Gee KW (1995). Benzodiazepines and peptides stimulate pregnenolone synthesis in brain mitochondria. Eur J Pharmacol 276: 145–153.

Mizoule J, Gauthier A, Uzan A, Renault C, Dubroeucq MC, Guérémy C *et al*. (1985). Opposite effects of two ligands for peripheral type benzodiazepine binding sites, PK 11195 and RO5-4864, in a conflict situation in the rat. Life Sci 36: 1059–1068.

Mukhin AG, Papadopoulos V, Costa E, Krueger KE (1989). Mitochondrial benzodiazepine receptors regulate steroid biosynthesis. Proc Natl Acad Sci U S A 86: 9813–9816.

Olsen RW, Sieghart W (2008). International Union of Pharmacology. LXX. Subtypes of γ-aminobutyric $acid_A$ receptors: classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacol Rev 60: 243–260.

Orebaugh SL, Bradford SM (1994). Intravenous versus intramuscular midazolam in treatment of chemically induced generalized seizures in swine. Am J Emerg Med 12: 284–287.

Papadopoulos V, Guarneri P, Kreuger KE, Guidotti A, Costa E (1992). Pregnenolone biosynthesis in C6-2B glioma cell mitochondria: regulation by a mitochondrial diazepam binding inhibitor receptor. Proc Natl Acad Sci U S A 89: 5113–5117.

Papadopoulos V, Baraldi M, Guilarte TR, Knudsen TB, Lacapère JJ, Lindemann P *et al*. (2006). Translocator protein (18 kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol Sci 27: 402–409.

Pieri L (1983). Preclinical pharmacology of midazolam. Br J Clin Pharmacol 16 (Suppl. 1): 17S–27S.

Raines A, Henderson TR, Swinyard EA, Dretchen KL (1990). Comparison of midazolam and diazepam by the intramuscular route for the control of seizures in a mouse model of status epilepticus. Epilepsia 31: 313–317.

Reddy DS, Rogawski MA (2002). Stress-induced deoxycorticosterone-derived neurosteroids modulate $GABA_A$ receptor function and seizure susceptibility. J Neurosci 22: 3795–3805.

Reddy DS, Kim HY, Rogawski MA (2001). Neurosteroid withdrawal model of perimenstrual catamenial epilepsy. Epilepsia 42: 328–336.

Rogawski MA (2002). Principles of antiepileptic drug action. In: Levy RH, Mattson RH, Meldrum BS, Perucca E (eds). Antiepileptic Drugs, 5th edn. Lippincott Williams & Wilkins: Philadelphia, PA, pp. 3–22.

Rogawski MA, Reddy DS (2004). Neurosteroids: endogenous modulators of seizure susceptibility. In: Rho JM, Sankar R, Cavazos J (eds). Epilepsy: Scientific Foundations of Clinical Practice. Marcel Dekker: New York, pp. 319–355.

Romeo E, Cavallaro S, Korneyev A, Kozikowski AP, Ma D, Polo A *et al*. (1993). Stimulation of brain steroidogenesis by 2-aryl-indole-3-acetamide derivatives acting at the mitochondrial diazepam-binding inhibitor receptor complex. J Pharmacol Exp Ther 267: 462–471.

Rovira C, Ben-Ari Y (1993). Developmental study of benzodiazepine effects on monosynaptic $GABA_A$-mediated IPSPs of rat hippocampal neurons. J Neurophysiol 70: 1076–1085.

Rupprecht R, Ströhle A, Hermann B, di Michele F, Spalletta G, Pasini A *et al*. (1998). Neuroactive steroid concentrations following metyrapone administration in depressed patients and healthy volunteers. Biol Psychiatry 44: 912–914.

Rupprecht R, Rammes G, Eser D, Baghai TC, Schüle C, Nothdurfter C *et al*. (2009). Translocator protein (18 kD) as target for anxiolytics without benzodiazepine-like side effects. Science 325: 490–493.

Rüsch D, Forman SA (2005). Classic benzodiazepines modulate the open-close equilibrium in $\alpha_1\beta_2\gamma_{2L}$ γ-aminobutyric acid type A receptors. Anesthesiology 102: 783–792.

Schoemaker H, Bliss M, Yamamura HI (1981). Specific high-affinity saturable binding of $[^3H]$ Ro5-4864 to benzodiazepine binding sites in the rat cerebral cortex. Eur J Pharmacol 71: 173–175.

Serra M, Madau P, Chessa MF, Caddeo M, Sanna E, Trapani G *et al*. (1999). 2-Phenyl-imidazo[1,2-a]pyridine derivatives as ligands for peripheral benzodiazepine receptors: stimulation of neurosteroid synthesis and anticonflict action in rats. Br J Pharmacol 127: 177–187.

So EC, Chang YT, Hsing CH, Poon PW, Leu SF, Huang BM (2010). The effect of midazolam on mouse Leydig cell steroidogenesis and apoptosis. Toxicol Lett 192: 169–178.

Stell BM, Brickley SG, Tang CY, Farrant M, Mody I (2003). Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by δ subunit-containing $GABA_A$ receptors. Proc Natl Acad Sci U S A 100: 14439–14444.

Tokuda K, O'Dell KA, Izumi Y, Zorumski CF (2010). Midazolam inhibits hippocampal long-term potentiation and learning through dual central and peripheral benzodiazepine receptor activation and neurosteroidogenesis. J Neurosci 30: 16788–16795.

Tsuda M, Suzuki T, Misawa M (1998). Subsensitivity to mitochondrial diazepam binding inhibitor receptor agonist FGIN-1-27-induced antiseizure effect in diazepam-withdrawn mice. Life Sci 62: PL213–PL217.

Ugale RR, Mittal N, Hirani K, Chopde CT (2004). Essentiality of central GABAergic neuroactive steroid allopregnanolone for anticonvulsant action of fluoxetine against pentylenetetrazole-induced seizures in mice. Brain Res 1023: 102–111.

Verleye M, Akwa Y, Liere P, Ladurelle N, Pianos A, Eychenne B *et al*. (2005). The anxiolytic etifoxine activates the peripheral benzodiazepine receptor and increases the neurosteroid levels in rat brain. Pharmacol Biochem Behav 82: 712–720.