

RESEARCH PAPER

Role of neurosteroids in the anticonvulsant activity of midazolam

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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 µg·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 µg·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 amnesic 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

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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; R  sch 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 (Lacap  re 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 3 β -hydroxysteroid dehydrogenase and then to neurosteroids by sequential A-ring reduction by 5 α -reductase and 3 α -hydroxysteroid oxidoreductase. 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 neuroster-

oidogenesis 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  mL^{−1}) was infused at a constant rate of 0.5 mL  min^{−1}. The infusion was stopped at 3 min or at the onset of tonic extension, whichever occurred first. The threshold value (mg  kg^{−1}) for tonic extension was determined according to the following formula: [infusion duration (s) $\times$ infusion rate (mL  min^{−1}) $\times$ PTZ concentration (mg  mL^{−1}) $\times$ 1000] / [60 (s) $\times$ weight of mouse (g)].

Treatment schedules

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

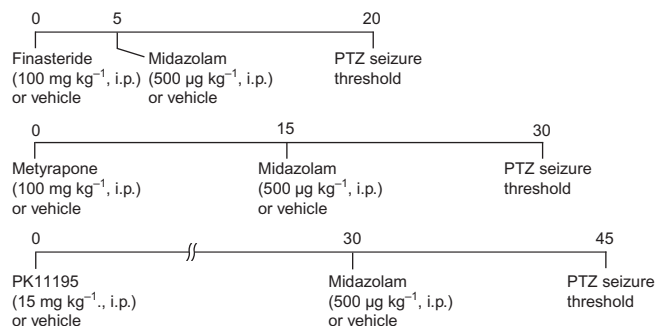


Figure 1

Drug treatment protocols. In some experiments, clonazepam (25 or 100 $\mu\text{g}\cdot\text{kg}^{-1}$, i.p.) was used instead of midazolam.

doses of 100 $\mu\text{g}\cdot\text{kg}^{-1}$ to 5 $\text{mg}\cdot\text{kg}^{-1}$ (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 $\text{mg}\cdot\text{kg}^{-1}$) that has previously been shown to partially inhibit neurosteroid synthesis (Kokate *et al.*, 1999). Metyrapone was used at a dose (100 $\text{mg}\cdot\text{kg}^{-1}$) 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 $\text{mg}\cdot\text{kg}^{-1}$) as in the study of Ugale *et al.* (2004). Clonazepam was administered at a dose of 100 $\mu\text{g}\cdot\text{kg}^{-1}$ (Akula *et al.*, 2009). The volume of all i.p. injections was 10 $\text{mL}\cdot\text{kg}^{-1}$.

Data analysis

Results are expressed as mean $\pm$ 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 $\text{mg}\cdot(10\text{ mL})^{-1}$; 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 compounds 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.

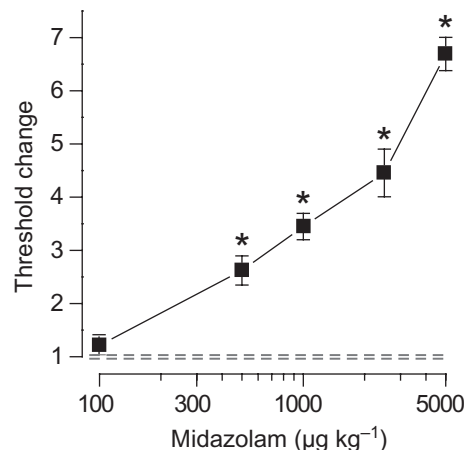


Figure 2

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 $\pm$ 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 $51.5 \pm 4.0\text{ mg}\cdot\text{kg}^{-1}$. Dashed lines indicate the limits of the SEM for the vehicle group. The mean threshold values for all groups other than the 100 $\mu\text{g}\cdot\text{kg}^{-1}$ group are significantly different from the value in the vehicle group. * $P < 0.001$; one-way ANOVA followed by Tukey's test.

Results

Midazolam causes a dose-dependent elevation in PTZ seizure threshold

I.v. infusion of PTZ (10 $\text{mg}\cdot\text{mL}^{-1}$) 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 $\mu\text{g}\cdot\text{kg}^{-1}$ but not 100 $\mu\text{g}\cdot\text{kg}^{-1}$.

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 $\text{mg}\cdot\text{kg}^{-1}$, i.p.) before giving a dose of midazolam (500 $\mu\text{g}\cdot\text{kg}^{-1}$, 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.

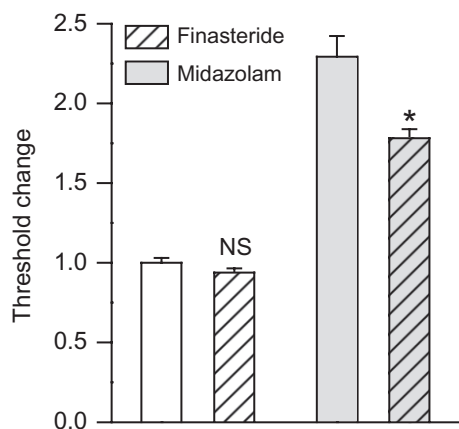


Figure 3

Finasteride pretreatment reduces the seizure threshold elevation induced by midazolam. Finasteride ($100 \text{ mg}\cdot\text{kg}^{-1}$, i.p.) or vehicle was administered 5 min before the treatment with midazolam ($500 \mu\text{g}\cdot\text{kg}^{-1}$, i.p.) or vehicle; 15 min after the second pretreatment, all animals were infused with PTZ. Bars indicate mean $\pm$ 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 $59.2 \pm 1.8 \text{ mg}\cdot\text{kg}^{-1}$. 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.

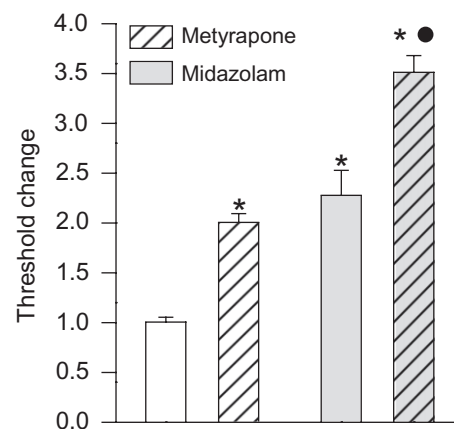


Figure 4

Metyrapone elevates seizure threshold in the absence and presence of midazolam. Metyrapone ($100 \text{ mg}\cdot\text{kg}^{-1}$, 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 $\pm$ 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 $51.0 \pm 4.4 \text{ mg}\cdot\text{kg}^{-1}$. $*P < 0.001$, significantly different from vehicle only control group; $\bullet P < 0.001$, 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 ($100 \text{ mg}\cdot\text{kg}^{-1}$, 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 ($500 \mu\text{g}\cdot\text{kg}^{-1}$, 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 ($15 \text{ mg}\cdot\text{kg}^{-1}$, i.p.) did not affect the seizure threshold. However, pretreatment with PK 11195 did significantly reduce the elevation in threshold produced by midazolam ($500 \mu\text{g}\cdot\text{kg}^{-1}$, i.p.) (Figure 5).

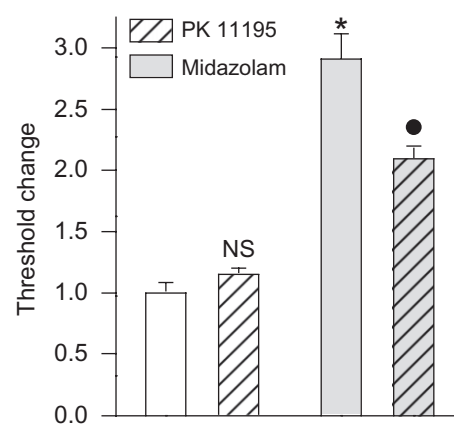


Figure 5

PK 11195 pretreatment reduces the seizure threshold elevation induced by midazolam. PK11195 ($15 \text{ mg}\cdot\text{kg}^{-1}$, i.p.) was administered 30 min before the treatment with midazolam ($500 \mu\text{g}\cdot\text{kg}^{-1}$, i.p.) or vehicle; 15 min after the second pretreatment, all animals were infused with PTZ. Bars indicate mean $\pm$ 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; $\bullet P < 0.001$, 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 $\mu\text{g}\cdot\text{kg}^{-1}$ clonazepam. In a second experiment with a lower dose of clonazepam (25 $\mu\text{g}\cdot\text{kg}^{-1}$), 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 5 α -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 11 β -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

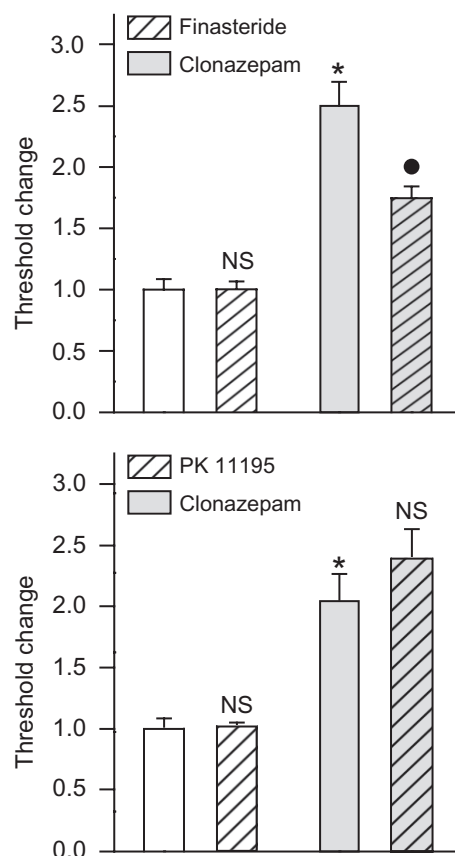


Figure 6

Finasteride (upper panel) but not PK 11195 (lower panel) pretreatment reduces the seizure threshold elevation induced by clonazepam. Finasteride (100 $\text{mg}\cdot\text{kg}^{-1}$, i.p.) or vehicle was administered 5 min before the treatment with clonazepam (100 $\mu\text{g}\cdot\text{kg}^{-1}$, 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 $\text{mg}\cdot\text{kg}^{-1}$, 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 $\pm$ 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 $46.9 \pm 3.6 \text{ mg}\cdot\text{kg}^{-1}$ in the experiment with finasteride and $51.9 \pm 2.7 \text{ mg}\cdot\text{kg}^{-1}$ 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.

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

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.

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