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Antiseizure Activity of Midazolam in Mice Lacking δ-Subunit Extrasynaptic $GABA_A$ Receptors

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Running Title Page

Running title: Mechanisms of antiseizure action of midazolam

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NON-STANDARD ABBREVIATIONS*:*

AD, afterdischarge; ADT, afterdischarge threshold; CC_{50}, median convulsant current; DKO, δ-subunit knockout; ED_{50}, median effective dose; GABA, γ-aminobutyric acid; SE, status epilepticus; TSPO, cholesterol transporter protein; PK11195, 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide

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ABSTRACT

Midazolam is a benzodiazepine anticonvulsant with rapid onset and short duration of action. Midazolam is the current drug of choice for acute seizures and status epilepticus, including those caused by organophosphate nerve agents. The antiseizure activity of midazolam is thought to result from its allosteric potentiation of synaptic $GABA_A$ receptors in the brain. However, there are indications that benzodiazepines promote neurosteroid synthesis via the 18kDa cholesterol transporter protein (TSPO). Therefore, we investigated the role of neurosteroids and their extrasynaptic $GABA_A$ receptor targets in the antiseizure activity of midazolam. Here, we used δ-subunit knockout (DKO) mice bearing a targeted deletion of the extrasynaptic receptors to investigate the contribution of the extrasynaptic receptors to the antiseizure activity of midazolam using the 6-Hz and hippocampus kindling seizure models. In both models, midazolam produced a rapid and dose-dependent protection against seizures (ED_{50},0.4 mg/kg). Moreover, the antiseizure potency of midazolam was undiminished in DKO mice compared with control mice. Pretreatment with PK11195, a TSPO blocker, or finasteride, a 5α-reductase neurosteroid inhibitor, did not affect the antiseizure effect of midazolam. The antiseizure activity of midazolam was significantly reversed by pretreatment with flumazenil, a benzodiazepine antagonist. Plasma and brain levels of the neurosteroid allopregnanolone were not significantly greater in midazolam-treated animals. These studies therefore provide strong evidence that neurosteroids and extrasynaptic $GABA_A$ receptors are not involved in the antiseizure activity of midazolam, which mainly occurs through synaptic $GABA_A$ receptors via direct binding to benzodiazepine sites. This study reaffirms midazolam's use for controlling acute seizures and status epilepticus.

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Introduction

Midazolam is a benzodiazepine antiseizure agent with rapid onset and short duration of action (Mandrioli et al., 2008). It is administered intravenously or intramuscularly to control acute seizures and status epilepticus (Galvin and Jelinek, 1987; Silbergleit et al., 2011; Reddy, 2014). Midazolam is considered as the drug of choice for persistent acute seizures and status epilepticus (SE), especially those caused by organophosphate nerve agents (McDonough et al., 2009). Due to superior pharmacokinetics, midazolam is being considered as a replacement for diazepam in the military antidote kit for nerve agents (Reddy and Reddy, 2015). The RAMPART study has confirmed the antiseizure efficacy of midazolam in SE to be equivalent or non-inferior to lorazepam in prehospital settings (Silbergleit et al., 2012). However, the molecular mechanisms underlying the midazolam action are not well understood.

The antiseizure activity of midazolam is thought to result from its allosteric potentiation of synaptic $GABA_A$ receptors. $GABA_A$ receptors are composed of five subunits from several classes. The major isoforms consist of 2α,2β, and 1γ or δ-subunits. The $GABA_A$ receptor mediates two types of inhibition, characterized as synaptic (phasic) and extrasynaptic (tonic) inhibition. Synaptic release of GABA results in the activation of low affinity γ-containing synaptic receptors, while high-affinity δ-containing extrasynaptic receptors are persistently activated by the ambient GABA levels. Benzodiazepines bind to γ-containing synaptic receptors leading to allosteric potentiation of GABA-gated hyperpolarization of the neuron (Mihic et al., 1994; Rovira and Ben-Ari, 1993; Kucken et al., 2003; Sarto-Jackson and Sieghart, 2008).

Additional mechanisms may also contribute to the pharmacological actions of midazolam. Benzodiazepines bind to other targets including TSPO, a 19kDa cholesterol transporter protein which was formerly called a peripheral-type benzodiazepine receptor (Gavish et al., 1999) or mitochondrial diazepam-binding receptor (McCauley et al., 1995; Reddy and Kulkarni, 1996; Papadopoulos et al.,

2006). Midazolam likely may interact with TSPO, a key protein in the biosynthesis of neurosteroids. There is evidence suggesting that midazolam can augment neurosteroidogenesis and, as a result, can increase the levels of endogenous neurosteroids (Matsumoto et al., 1994; So et al., 2010; Tokuda et al., 2010; Dhir and Rogawski, 2012). Allopregnanolone and related neurosteroids are powerful antiseizure agents (Reddy, 2011). However, it is unclear whether midazolam acts as a functional TSPO ligand to affect endogenous neurosteroid synthesis and produce pharmacological effects typical of neurosteroids. There is some evidence in support of functional TSPO ligand activity (Serra et al., 1999; So et al., 2010; Tokuda et al., 2010). Midazolam is shown to enhance the production of steroidogenesis in Leydig cells (So et al., 2010) and in hippocampal neurons in brain slices (Tokuda et al., 2010). These studies used cultured cells and steroidogenesis was only apparent at midazolam levels above 150 μM, which is three-fold higher than levels associated with antiseizure activity. Therefore, the extent to which neurosteroids contributes to midazolam actions remains unclear.

Neurosteroids are synthesized within the brain from cholesterol and steroid hormone precursors (Fig.1). Allopregnanolone, androstanediol, and allotetrahydrodeoxycorticosterone are the prototype endogenous neurosteroids (Carver and Reddy, 2013). The initial key step in neurosteroidogenesis is the conversion of cholesterol to pregnenolone by the mitochondrial enzyme P450scc (cytochrome P450 cholesterol side-chain cleavage enzyme) (Fig. 1). Access of cholesterol to P450scc requires steroidogenic acute regulatory protein (StAR), which functions to transfer cholesterol from the outer mitochondrial membrane to the inner membrane where P450scc is located (Stoffel-Wagner et al., 2003). TSPO likely functions as a complex with StAR and voltage-dependent anion channel at the outer mitochondrial membrane (Korneyev et al., 1993; Jefcoate 2002; Papadopoulos et al., 2006; Midzak et al., 2011ab). TSPO is highly expressed in tissue where steroidogenesis occurs, including various regions in the brain (Papadopoulos et al., 2006). Therefore, activation of TSPO by certain ligands (e.g. benzodiazepines) may facilitate increased production of neurosteroids that act on $GABA_A$ receptor targets (Kita and Furukawa 2008; Rupprecht et al., 2009, 2010; Nothdurfter et al. 2012).

Neurosteroids rapidly alter neuronal excitability through direct interaction with $GABA_A$ receptors (Hosie et al., 2007; Wu et al., 2013), thereby producing robust seizure protection (Reddy, 2011). Neurosteroids potentiate both synaptic and extrasynaptic $GABA_A$ receptors and appear to induce greater effects on extrasynaptic δ-subunit receptors that mediate tonic inhibition (Wohlfarth et al., 2002; Stell et al., 2003; Belelli et al., 2009; Brickley and Mody, 2012; Carver and Reddy, 2013; Carver et al., 2014). The net antiseizure action of midazolam could be due to a combination of its direct action on synaptic $GABA_A$ receptors along with the indirect actions mediated by neurosteroids that can activate both synaptic and extrasynaptic receptors. However, the precise role of TSPO and extrasynaptic receptors in midazolam activity remains unclear.

In the present study, we sought to investigate the role of endogenous neurosteroids and the extrasynaptic $GABA_A$ receptors in the antiseizure actions of midazolam using δ-subunit knockout (DKO) mice bearing a targeted null mutation of the δ-subunit gene that abrogates the expression and function of extrasynaptic $GABA_A$ receptors. Our results indicate that indirect actions mediated by neurosteroids and the extrasynaptic (δ-subunit) $GABA_A$ receptors are not involved in the antiseizure activity of midazolam. Instead, its direct activity occurs mainly at synaptic $GABA_A$ receptors.

Materials and Methods

Animals. Adult male and female C57BL/6 mice of 25 to 30g each were used in this study. Adult $GABA_A$ receptor δ-subunit knockout mice (DKO) were also used (Mihalek et al., 1999; Carver et al., 2014). All strains were maintained on a hybrid C57BL/6-129SV background. All mice were housed four to a cage with access to food and water *ad libitum*. The mice were housed in an environmentally controlled animal facility with a 12 h light/dark cycle. The animals were cared for in strict compliance with the guidelines outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All animal procedures were performed in a protocol approved by the university's Institutional Animal Care and Use Committee. Sex differences are apparent in seizure susceptibility

and protective effects of many antiseizure agents (Reddy, 2014). To identify the role of neurosteroid mechanisms in the antiseizure action of midazolam, we utilized male mice in the 6-Hz model. We utilized fully-kindled female mice to study the role of extrasynaptic $GABA_A$ receptors in the antiseizure action of midazolam.

6-Hz Model of Epilepsy. To study seizure protection activity of midazolam, we used the 6-Hz electrical stimulation model, which represents a psychomotor model for intractable complex partial seizures that are highly sensitive to anticonvulsants that positively modulate $GABA_A$ receptors (Barton et al., 2001; White et al., 2002; Kaminski et al., 2004). The 6-Hz model was carried out according to previously described protocol (Brown et al., 1953; Barton et al., 2001). Corneal stimulation (0.2 ms-duration monopolar rectangular pulses at 6 Hz for 3 s) was delivered by a constant-current device (ECT Corneal Electrode system, Ugo Basile, Comerio, Italy). For finding the CC_{99} value (stimulation current causing seizures in 99% of animals), different current intensities in the range of 5 to 40 mA were administered to separate groups of animals. A fixed current intensity of 32 mA (CC_{99} value) was used to subsequently allow direct comparison with the data obtained in the aforementioned studies. Ocular anesthetic (0.5% tetracaine) was applied to the corneas 15 min before stimulation, and the corneal electrodes were wetted with 0.9% saline just before stimulation. During stimulation, mice were briefly held and released into the observation cage (28×20×15 cm) immediately after current application. The following parameters were noted in each mouse: (a) brief intense motor agitation (<3 s) such as wild running, jumping and cage climbing; (b) stunned posture; (c) limbic seizure activity manifested as rearing (bipedal standing), forelimb automatic movements and clonus, twitching of the vibrissae, and straub tail; and (d) unilateral or bilateral clonic seizures. The cumulative duration of the seizure activity ranged from 13 to 65 s in untreated animals. At the end of a seizure, animals immediately resumed their normal exploratory behavior. The occurrence of seizures and behavioral seizure severity were utilized as indices of seizure susceptibility. A mouse was considered to be

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protected if it failed to display seizures as described above or resume its normal exploratory behavior within 10 s of the start of stimulation.

Hippocampus Kindling Model of Epilepsy. To study seizure protection activity of midazolam, we also used the hippocampus kindling model, which is the best model of human temporal lobe epilepsy characterized by progressive complex partial seizures with secondary generalization (Goddard et la., 1969; Albright and Burnham, 1980). A mild focal, nonconvulsant electrical stimulus to the hippocampus on a daily basis leads to the development of a kindled state exhibiting electrographic and behavioral seizures. In mouse kindling, the focal electroencephalogram afterdischarge (AD) models complex partial seizures, whereas the behavioral motor seizure stages 4/5 models generalized seizures. Electrode implantation and stimulation procedures for mouse hippocampus kindling were performed as described previously (Reddy and Mohan, 2011; Reddy et al., 2012). Mice were anesthetized by an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). A twisted bipolar stainless-steel wire electrode (model MS303/1; Plastic Products, Roanoke, VA) was stereotaxically implanted in the right hippocampus (2.9 mm posterior and 3.0 mm lateral to bregma and 3.0 mm below the dorsal surface of the skull) using the Franklin and Paxinos atlas (Franklin and Paxinos, 1997) and anchored with dental acrylic to four jeweler's screws placed in the skull. A period of 7 to 10 days was allowed for recovery. The stimulation paradigm consisted of 1-ms duration, bipolar, square current pulses delivered at 60 Hz for 1s using a kindling stimulator (A-M Systems, Sequim, WA). The afterdischarge threshold (ADT) was determined by stimulating at 5-min intervals beginning with an intensity of 25 μA and increasing in steps of 25 μA until an AD of at least 5s was obtained. Stimulation on subsequent days used a stimulation intensity of 125% threshold value. The afterdischarge was recorded from the hippocampus electrode with a Grass CP511 preamplifier (Astro-Med, West Warwick, RI) and stored in digital form using Axoscope 8.1 software (Axon Instruments, Foster City, CA). AD duration was the total duration of hippocampal electrographic spike activity (amplitude >2x baseline) occurring in a rhythmic pattern at a frequency of 1 Hz. The day of

ADT determination was considered day 0 of kindling. Stimulation was continued on a 5 days/week schedule each afternoon. Seizure activity after each stimulation was rated according to the criterion of Racine (1972) as modified for the mouse: stage 0, no response or behavior arrest; stage 1, chewing or head nodding; stage 2, chewing and head nodding; stage 3, forelimb clonus; stage 4, bilateral forelimb clonus and rearing; and stage 5, falling. Kindling stimulation was delivered daily until stage 5 seizures were elicited on 3 consecutive days. Mice were used for drug testing when they consistently exhibited stage 5 seizures after stimulation, which is considered the "fully kindled" state.

Determination of Plasma and Brain Neurosteroid Levels. To determine plasma and brain neurosteroid levels attained at midazolam doses that protect against seizures, allopregnanolone concentrations were determined 15 min after administration of 2.5x ED_{50} doses of midazolam (1 mg/kg, i.p.) in mice. Animals were anesthetized with an injection of ketamine (100 mg/kg)-xylazine (10 mg/kg) solution and ~0.5 ml carotid blood was collected in heparinized tubes. The plasma was separated by centrifugation at 12,000× *g* for 10 min and stored at -20 °C. Brains were rapidly dissected and cortex and hippocampus samples were isolated by microdisection under a microscope. Brain tissue samples were homogenized in phosphate buffer solution. The concentration of allopregnanolone was analyzed by liquid chromatography-mass spectrometry (LC-Ms/MS) as previously described (Reddy et al., 2004). Plasma and brain levels of allopregnanolone were estimated using 3β-methyl- allopregnanolone as internal standard. The steroid and internal standard were extracted with 0.9 ml of hexane. Each sample was analyzed using the atmospheric pressure chemical ionization technique under acidic conditions in a triple Quad system (Applied Biosystems) under the multiple reaction monitoring detection modes.

Test Drugs and Treatment Protocols. Midazolam (Akorn, Inc., Lake Forest, IL) was diluted in sterile saline. Finasteride (Steraloids Inc., Newport, RI) and other drugs for injection were made in 20% β-cyclodextrin in saline. Drug solutions were administered subcutaneously or intraperitonially in a

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volume equaling 1% of the animal's body weight. To examine the ability of midazolam to suppress the expression of seizures, the drug was given 15 min prior to 6-Hz stimulation in naïve mice or kindling stimulations in fully-kindled mice. Midazolam is a benzodiazepine-site agonist at synaptic receptors containing the α1/2/3/5-subunits in combination with any of the β-subunits and the γ2-subunit (Whiting et al., 2000; Mohler et al., 2002; Kucken et al., 2003). Three different inhibitors, PK11195, finasteride, and flumazenil, were selected for pharmacological intervention of midazolam response in both 6-Hz and kindling models (Fig.1). PK11195 [1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide] is a specific TSPO ligand used to verify the TSPO mediated neurosteroid production (Auta et al., 1993; Reddy and Kulkarni, 1996; Kita et al., 2004; Rupprecht et al., 2009). Finasteride is a 5α-reductase inhibitor that blocks the bioconversion of dihydroprogesterone into allopregnanolone and related precursors into neurosteroids (see Fig.1). Finasteride blocks the synthesis of neurosteroids such as allopregnanolone and related 5α-reduced pregnane and androstane analogs that modulate $GABA_A$ receptor function (Finn et al., 2006; Mukai et al., 2008; Gangisetty and Reddy, 2010). Flumazenil is a benzodiazepine site antagonist at $GABA_A$ receptors and is used to reverse benzodiazepine overdose (Haefely, 1998; Hoffman and Warren, 1993; Khan et al., 2000).

Data Analysis. Group data are expressed as the mean ± standard error of the mean (SEM). To construct dose-response curves, midazolam and test drugs were tested at several doses spanning the dose producing 50% protection (ED_{50}). ED_{50} values and their corresponding 95% confidence limits were determined by log-probit analysis using the Litchfield and Wilcoxon method (PHARM/PCS Version 4.2; Micro-Computer Specialists, Philadelphia, PA, USA). Differences in kindling seizure stages between groups were compared with the nonparametric Kruskal-Wallis test followed by the Mann-Whitney *U*-test. Comparison of means of the AD duration between groups was made with a one-way analysis of variance, followed by an unpaired two-tailed Student's *t*-test. Comparison of the mean percentage inhibition of seizure stage and AD duration in fully kindled animals was made by Wilcoxon

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signed ranks test and paired two-tailed Student's *t*-test, respectively. ED_{50} values of test drugs in the kindling model were determined by non-linear curve fitting using the Levenberg–Marquardt algorithm to a logistic equation where the maximum inhibition was assumed to be 100%. In all statistical tests, the criterion for statistical significance was $p < 0.05$.

Results

Seizure Expression in 6-Hz Model in Mice. We used the 6-Hz test to assess the antiseizure effect of midazolam. In control or naïve mice, corneal stimulation with currents in the range of 5 to 40 mA resulted in a current-dependent increase in the fraction of mice reaching the designated seizure end point (Fig. 2A). The CC_{50} value, the current to produce seizure occurrence in 50% animals was 10 (95% confidence limits, 9–12) mA. The CC_{99} value, the current to produce seizure occurrence in 99% of animals, is 32 mA. Thus, the pharmacological studies were carried out with 32-mA stimulation, which is 3.1 times the CC_{50} value and always resulted in seizure behavior.

Antiseizure Activity of Midazolam in the 6-Hz Seizure Model. Pretreatment with midazolam produced dose-dependent protection in the 6-Hz test (Fig. 2B). The ED_{50} value derived from the experiment of Fig. 2B is presented in Table 1. The estimated ED_{50} value for reduction of seizures is 0.4 mg/kg. We additionally assessed the time-course of action of midazolam in the 6-Hz model using a 2.5× ED_{50} dose. As shown in Fig. 2C, midazolam (1 mg/kg, i.p.) showed a maximal protective effect within 15 min of injection, and significant protection up to 2 h; minimal protection was evident at 4 h. Midazolam at 3 mg/kg and higher doses produced significant motor impairment. The pharmacological inhibitor studies were carried out with a dose of 1 mg/kg, which did not cause motor impairment, but always resulted in 100% seizure protection in the 6-Hz model.

Antiseizure Activity of Midazolam in the 6-Hz Model Occurs via a Neurosteroid-Independent Pathway. There are two major mechanisms by which midazolam can possibly produce seizure

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protection: (i) direct potentiation of synaptic $GABA_A$ receptor-mediated inhibition; and (ii) indirect potentiation via interaction with TSPO cholesterol transporter and elevation of neurosteroid biosynthesis (Fig.1). To directly test the TSPO-linked neurosteroid pathway, the antiseizure activity of midazolam was examined in the presence of PK11195, a functional TSPO inhibitor (Le Fur et al., 1983; Auta et al., 1993; Romeo et al., 1993; Bitran et al., 2000; Kita et al., 2004). Pretreatment with PK11195 (15 mg/kg, i.p.) produced little or no effect on the antiseizure activity of midazolam, as reflected by the similar dose-protection curves (Fig.2D). The ED_{50} values are listed in Table 1. The potency ratio of PK11195+midazolam (ED_{50}, 0.36; CF, 0.2-0.52 mg/kg) versus midazolam alone (ED_{50}, 0.4; CF, 0.11-0.69 mg/kg) was not significant as calculated by the Litchfield & Wilcoxon chi-square test ($p>0.05$). To further test whether 5α-reduced neurosteroid synthesis is involved in the antiseizure activity of midazolam, we utilized the 5α-reductase inhibitor and neurosteroid synthesis inhibitor finasteride (Finn et al., 2006). Pretreatment with finasteride (50 mg/kg, i.p.) did not affect the antiseizure activity of midazolam, demonstrated by similar dose-protection curves to the control (Fig.2D). The potency ratio of finasteride+midazolam (ED_{50}, 0.36; CF, 0.17-0.55 mg/kg) versus midazolam (ED_{50}, 0.4; CF, 0.11-0.69 mg/kg) was also not significant as per the Litchfield & Wilcoxon chi-square analysis. By themselves, PK1195 (15 mg/kg, i.p.) and finasteride (50 mg/kg, i.p.) did not affect the seizure occurrence and seizure severity in the 6-Hz test (Fig.2F). These results indicate that midazolam does not appear to protect against 6-Hz seizures through the TSPO-linked neurosteroid pathway.

Benzodiazepine Antagonist Flumazenil Inhibits the Antiseizure Activity of Midazolam in the 6-Hz Model. To provide direct evidence for the involvement of synaptic $GABA_A$ receptors in the antiseizure activity of midazolam, the benzodiazepine site antagonist flumazenil, which competitively blocks midazolam binding to γ2-containing synaptic $GABA_A$ receptors (Haefely, 1988; Hoffman and Warren, 1993; Khan et al., 2000; Mohler et al., 2002; Kucken et al., 2003), was utilized as a definitive pharmacological blocker. Pretreatment with flumazenil (10 mg/kg, i.p.) significantly reduced the

antiseizure effect of midazolam, reflected by reduced maximal protective responses even at higher doses of midazolam (Fig.2E). Flumazenil caused ~70% inhibition of maximal protection of 1 mg/kg diazepam. The potency ratio of flumazenil+midazolam (ED_{50}, <3 mg/kg) versus midazolam (ED_{50}, 0.4; CF, 0.11-0.69 mg/kg) differ significantly as calculated by the Litchfield & Wilcoxon chi-square test ($p<0.05$). Flumazenil alone (10 mg/kg, i.p.) did not significantly affect the seizure occurrence or seizure severity in the 6-Hz test (Fig.2F). These results indicate that midazolam protects against 6-Hz seizures via synaptic $GABA_A$ receptors by binding to specific benzodiazepines sites on the receptor channel complex.

Antiseizure Activity of Midazolam in the Hippocampus Kindling Model in DKO Mice. To determine the role of extrasynaptic $GABA_A$ receptors, which are also the main target of neurosteroids, in the antiseizure activity of midazolam, we utilized δ-subunit knockout mice (DKO) which lack extrasynaptic receptors in the brain. Benzodiazepines with $GABA_A$ receptor modulatory activity have protective effects in the kindling model of epilepsy (Cleton et al., 1999; Reddy and Rogawski, 2010). Therefore, we selected the kindling model for characterization of the antiseizure activity of midazolam. To evaluate the systemic activity of midazolam in protecting against kindled seizures, we tested midazolam in fully-kindled WT and DKO mice. Mice were subjected to once-daily kindling via an implanted electrode in the dentate gyrus region at 125% AD threshold until they exhibited stage 5 seizures for 3 consecutive days, which is considered the “fully kindled” state. Mice reached the fully kindled state with consistent stage 5 seizures after 12 to 16 stimulations. Daily kindling stimulation was associated with a steady progression of behavioral seizures and AD duration, which remained consistent once they reached the fully-kindled state. The overall mean value of the initial AD threshold in WT and DKOs were 50 ± 6 μA (n=13) and 37 ± 4 μA (n=11), respectively. Three parameters were assessed in fully-kindled mice as indices of midazolam efficacy: (a) stimulation-induced electrographic AD duration, (b) behavioral seizure intensity measured as per the Racine scale, and (c) duration of generalized seizures. As shown in Fig. 3, midazolam produced a dose-dependent suppression of

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behavioral seizure activity (Fig. 3A) and AD duration (Fig. 3B), with significant effects at 0.1, 0.5, and 1 mg/kg doses in WT mice. At the highest dose tested, behavioral seizures were nearly completely suppressed. The estimated ED_{50} values for suppression of seizure stage and AD duration were 0.55 and 0.76 mg/kg, respectively. The antiseizure potency of midazolam was undiminished in DKO mice compared with WT mice (Fig.3AB). Although there was a modest difference in behavioral response at a lower dose (0.1 mg/kg) in DKO mice, the overall dose-response profiles of midazolam were similar between WT and DKO mice (Fig.3AB). There was no significant difference in the ED_{50} value of midazolam in WT and DKO mice (Table 1). The percent inhibition of seizures and AD duration was also similar between the genotypes (Fig.4AB), except for a modest inhibition of seizures in WT group at the 0.1 mg/kg dose. The electrographic events are illustrated in Fig.4. Midazolam pretreatment markedly reduced the AD duration in a dose-dependent manner in fully-kindled DKO mice. There were no significant differences in the overall amplitude or extent of AD duration between midazolam-treated WT and DKO mice. Thus, these studies provide strong evidence that the extrasynaptic $GABA_A$ receptors are not required for the antiseizure effects of midazolam, which may occur through its binding to the synaptic receptors.

Time Course for Seizure Protection by Midazolam in Fully Kindled DKO Mice. The time courses for seizure protection after a 2× ED_{50} dose of midazolam are shown in Fig. 3CD. Midazolam (1 mg/kg, i.p.) exhibited a rapid onset of seizure protection. In WT and DKOs, the protection was maximal at 15 min and diminished during the 240-min period after the injection as evident by its time-dependent decrease in behavioral seizures (Fig. 3C) and AD duration (Fig.3D). On the day after midazolam treatment, all DKO mice exhibited full-blown stage 5 seizures with normal AD duration, indicating that midazolam treatment suppresses the expression of kindled seizures but does not alter the kindled state. These results are consistent with the possibility that midazolam protection is due to synaptic $GABA_A$ receptor inhibition that occurs rapidly within a few minutes.

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Antiseizure Activity of Midazolam in the Hippocampus Kindling Model Occurs via a Neurosteroid-Independent Pathway in DKO Mice. As in the 6-Hz model, we assessed the two potential mechanisms by which midazolam can suppress seizures in the kindling model in DKO mice: direct potentiation of synaptic $GABA_A$ receptors and TSPO-mediated neurosteroid biosynthesis. We examined the effects of midazolam in fully-kindled WT and DKO mice with and without pretreatments of PK11195, a functional TSPO antagonist, and finasteride, a 5α-reductase inhibitor known to block the synthesis of allopregnanolone and related 5α-reduced neurosteroids. Kindled mice were treated with midazolam (1 mg/kg, i.p.) with or without PK11195 pretreatment (15 mg/kg, i.p.) and finasteride (50 mg/kg, i.p.), and the time-course for seizure protection following kindling stimulations was determined in WT (Fig.5AB) and DKO (Fig.5CD) mice. Pretreatment with PK11195 did not influence the protective effects of midazolam, evident in similar time-protection curves for behavioral seizure stage (Fig.5AC) and AD duration (Fig.5BD) between genotypes. Pretreatment with finasteride did not diminish the antiseizure effects of midazolam in DKO mice (Fig.5CD) compared with control WT mice (Fig.5AB). In both genotypes, PK11195 and finasteride alone had no significant effect on either kindling seizure expression (Fig.6AC) or the AD duration (Fig.6BD). Together, these results suggest that midazolam protection against kindling seizures is not mediated through TSPO-linked production of 5α-reduced neurosteroids.

Flumazenil Inhibits the Antiseizure Activity of Midazolam in the Hippocampus Kindling Model in DKO Mice. To confirm whether the protective effects of midazolam against kindled seizures in DKO mice occur via the drug's ability to bind to the benzodiazepine site of synaptic $GABA_A$ receptors, pharmacological studies were conducted with the benzodiazepine site antagonist flumazenil. In kindled DKO mice, flumazenil (10 mg/kg, i.p.) alone did not affect the behavioral seizures (Fig.6C) and AD duration (Fig.6D) compared to the control WT group (Fig.6AB). Like WT mice, DKO animals pretreated with flumazenil displayed a significant ($p<0.01$), time-dependent blockade of midazolam's antiseizure effects and a greater AD duration, leading to drastically worsened seizure

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protection. Together, these results indicate that midazolam's seizure protection results from synaptic $GABA_A$ receptor inhibition via specific, flumazenil-sensitive benzodiazepine sites. These results are consistent with earlier reports of flumazenil-sensitive antiseizure action of diazepam in the kindling model (Gangisetty and Reddy, 2010). Like midazolam, the protective effect of diazepam (1 mg/kg, i.p.) against kindled behavioral seizure stage (0.16±0.12) and electrographic AD duration (8 ± 2 s) was significantly reduced by pretreatment with flumazenil (seizure stage, 4.5±0.34; AD duration 43 ± 7 s).

Effect of Midazolam on Neurosteroid Levels in Plasma and Brain. To provide direct support for the lack of involvement of neurosteroids in the antiseizure action of midazolam, plasma and brain levels of the neurosteroid allopregnanolone were determined by LC-MS/MS in animals pretreated with midazolam. Allopregnanolone levels are commonly measured by mass spectrometry assay (Reddy et al., 2004; Porcu et al., 2009; Snelling et al., 2014). Allopregnanolone levels were determined 30 min after injection with an antiseizure dose of midazolam (1 mg/kg, i.p.) in mice. As shown in Fig. 7, midazolam treatment was not associated with a significant increase in plasma allopregnanolone levels (4.3 ± 0.96 ng/ml) compared with the control (3.3 ± 0.34 ng/ml). Allopregnanolone levels in the hippocampus were not significantly higher in the midazolam-treated group (102 ± 10 ng/g) relative to the untreated control group (79 ± 11 ng/g). There were no significant differences in the cortex allopregnanolone levels achieved with anticonvulsant dose of midazolam in mice (Fig.7). Thus, antiseizure doses of midazolam are not associated with significant change in plasma and brain neurosteroid levels.

Discussion

The principal findings of the study are that neurosteroid synthesis is not greatly affected by midazolam and the drug's antiseizure activity was unaffected by the neurosteroid synthesis inhibitors PK11195 and finasteride. This is shown for the first time using two distinct seizure models: the 6-Hz

test triggering generalized psychomotor seizures, and hippocampus kindling specifically eliciting limbic seizures. In the latter approach, we demonstrated that the antiseizure activity of midazolam is undiminished in DKO mice, which lack extrasynaptic forms of the $GABA_A$ receptors, thus definitively proving that the δ-containing extrasynaptic $GABA_A$ receptors are not required for the antiseizure activity of midazolam. Moreover, the seizure protection of midazolam was eliminated by pretreatment of animals with the benzodiazepine site antagonist flumazenil. Taken together, these results indicate that the antiseizure action of midazolam occurs through a neurosteroid-independent, non-extrasynaptic receptor pathway and is mediated by the drug's direct action on the benzodiazepine site of synaptic $GABA_A$ receptors. However, there are certain aspects that must be considered in regards to the interpretation of the findings. Since the relationship of animal seizure models to human epilepsy or SE is not well defined, it cannot be directly concluded that extrasynaptic receptors do not participate in the midazolam's ability to control seizures in the clinical setting. Neurosteroids are powerful endogenous antiseizure agents, most likely due to their direct ability to potentiate GABA neurotransmission in the brain via $GABA_A$ receptors. This study also cannot rule out the neurosteroid involvement in the etiology of seizures in human as it was intended to unravel the extent of such mechanisms in the antiseizure actions of midazolam. Neurosteroids are more relevant in seizures related to hormonal aspects such as catamenial epilepsy and stress (Reddy, 2014).

In the present study, midazolam displayed dose-dependent protection against seizures in both 6-Hz and kindling models; the potency of the benzodiazepine in the 6-Hz test and kindling model is similar and largely corresponds with its potencies as $GABA_A$ receptor modulator. In the 6-Hz model, pretreatment with PK11195 or finasteride (ED_{50} of 0.36 mg/kg each) did not elicit any significant change in seizure protection compared to midazolam alone (ED_{50} of 0.4 mg/kg). Animals pretreated with flumazenil, however, displayed a dose-dependent blockade of midazolam's anticonvulsant effects, leading to drastically worsened seizure protection (ED_{50} >3 mg/kg). When given alone, the inhibitors did not affect seizures. The data from the kindling model displayed a similar trend, showing negligible

difference in the antiseizure responses between the control midazolam group and groups pretreated with PK11195 or finasteride. However, flumazenil pretreatment significantly inhibited the antiseizure effect of midazolam. Inhibitors alone also yielded no protection in the kindling model. Additionally, plasma and brain levels of allopregnanolone were similar between vehicle and midazolam groups. These results demonstrate a lack of enhanced endogenous neurosteroid synthesis, either mediated by a TSPO or 5α-reductase pathway, following antiseizure doses of midazolam. The extrasynaptic δ-containing $GABA_A$ receptors do not contribute to the antiseizure action of midazolam, which is possibly mediated by its direct binding to benzodiazepine sites on the γ-containing postsynaptic $GABA_A$ receptors.

Our data suggest that there is little difference in the antiseizure response of midazolam between WT and DKO and that δ-subunit extrasynaptic receptors are not major contributors to the antiseizure effects of midazolam. However, antiseizure effects of midazolam are slightly reduced in DKO mice at few low doses. This subtle difference may not account for the particularly effective antiseizure action of midazolam in DKO mice. Such minor differences could be due to compensational or other developmental limitations apparent in transgenic mice, such as the DKO mouse model that exhibit subunit plasticity due to germline deletion of δ-subunit (Spigelman et al., 2002).

Synaptic $GABA_A$ receptors mediate the antiseizure activity of benzodiazepines. $GABA_A$ receptors are pentameric in structure, with five subunits that form a central Cl^- ion channel (Fig.1). There are 19 subunits (α1–6, β1–3, γ1–3, δ,ε,θ, ρ1–2). Each subunit has four transmembrane segments, with both the amino and carboxy termininals located extracellularly. These extracellular domains form the primary recognition sites for GABA and allosteric recognition sites for benzodiazepines and neurosteroids (Hosie et al., 2007'; Carver and Reddy, 2013). Subunit composition and receptor location determines sensitivity to benzodiazepines. A typical benzodiazepine-sensitive, postsynaptic $GABA_A$ receptor consists of two α1, 2, 3, or 5 subunits, two β2 or β3 subunits (or one each), and a γ2 subunit (Mihic et al., 1994; Kucken et al., 2003). Midazolam binds on these receptors and acts as a

positive allosteric modulator in augmenting the inhibition in neurons. The benzodiazepine-sensitive receptors mediate phasic inhibition through the generation of fast, rapidly desensitizing inhibitory postsynaptic currents in neurons. However, benzodiazepines do not bind to extrasynaptic $GABA_A$ receptors that mediate tonic inhibition. These extrasynaptic receptors contain the δ-subunit, rather than the γ-subunit characteristic of synaptic $GABA_A$ receptors (Farrant and Nusser, 2005; Carver and Reddy, 2013). Receptors containing α4,α5, or α6 are commonly located at perisynaptic or extrasynaptic sites. Receptor isoforms with α4, α6, or δ subunits are insensitive to benzodiazepines such as diazepam and midazolam, whereas those with α1, α2, α3, α5, or γ2 subunits are benzodiazepine sensitive. Due to their synaptic location, these isoforms undergo dynamic plasticity (such as internalization or subunit switching) in response to prolonged seizures or SE, resulting in benzodiazepine resistance that becomes a refractory condition as time elapses.

Presently, three benzodiazepines are primarily used in the management of acute seizures and SE: diazepam, lorazepam, and midazolam. Like diazepam and lorazepam, midazolam is an effective anticonvulsant for the treatment of SE (Alldredge et al., 2001; Shorvon and Ferlisti, 2011; Silbergleit et al., 2012). SE is a neurological emergency characterized by continuous seizure activity for 5 to 30 min or more and these seizures respond to benzodiazepines if given within 5 to 20 min. A meta-analysis has confirmed that midazolam by any route is superior to diazepam (McMullan et al., 2010). Midazolam offers several unique advantages over other benzodiazepines, including its rapid onset of action, short duration, water solubility, and extended shelf life (Mandrioli et al., 2008). Midazolam has a maximum shelf life of 2-3 years at room temperature. Midazolam is administered intravenously or intramuscularly to terminate acute seizures and SE, including those caused by nerve agents (Galvin and Jelinek, 1987; Silbergleit et al., 2011; 2012). Midazolam is likely to replace diazepam in the nerve agent treatment regimen because of its superior pharmacokinetic features. (Reddy and Reddy, 2015). It is available in a water-soluble form for formulation of aqueous injectable products. At pH<5, midazolam is deprotonated and converts to an open ring configuration due to acid-catalyzed hydrolysis

of the 4,5-double bond of the diazepine ring (Orive et al., 1989). These physicochemical features are attractive to formulate midazolam into aqueous injectable products with longer shelf-lives. Midazolam also has some of the significant limitations found in other benzodiazepines. Chiefly, it must be administered within 10 to 20 minutes, after which there is little or no protection against seizures and progressive neurological damage occurs (Goodkin et al., 2007; Apland et al., 2014). This timeline is often not practical in many cases because it can take a minimum of 30-60 min to get medical intervention. Midazolam has an extremely short half-life (1.5-2.5 h), and therefore needs frequent boluses or continuous infusion. Repeated high doses of midazolam may result in sedation, respiratory depression, and tolerance. Seizures lasting longer than 5 to 20 minutes may not respond to midazolam due to internalization of $GABA_A$ receptors (Naylor et al., 2005; Goodkin et al., 2005).

In conclusion, the results from this study indicate that the antiseizure effects of midazolam are not mediated by endogenous neurosteroids such as allopregnanolone. Our results with transgenic DKO mice provide strong evidence that the extrasynaptic (δ-subunit) $GABA_A$ receptors are not required for the antiseizure effects of midazolam. The antiseizure activity of midazolam is directly mediated by synaptic (γ2-subunit) $GABA_A$ receptors through specific benzodiazepine binding sites. These studies further reaffirm that midazolam is an effective antiseizure agent for controlling acute seizures and SE.

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Authorship Contribution

Participated in research design: SDR, DSR
Conducted experiments: SDR, IY, BLC, DSR
Performed data analysis: SDR, DSR
Wrote or contributed to the writing of the manuscript: SDR, DSR

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Footnote to title page

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FIGURE LEGENDS

Fig. 1: Neurosteroid biosynthetic pathways and potential sites of modulation by midazolam. The upper panel shows blockade sites of PK11195 and finasteride in the neurosteroid biosynthesis pathway in the brain. The prototype endogenous neurosteroids allopregnanolone (3α-hydroxy-5α-pregnane-20-one), androstanediol (5α-androstan-3α,17β-diol), and allotetrahydrodeoxy-corticosterone (THDOC) are synthesized from cholesterol and steroid hormone precursors. Activation TSPO by appropriate ligands (e.g. benzodiazepines) facilitates the intramitochondrial flux of cholesterol and thereby increases the availability of cholesterol to the cytochrome P-450$_{SCC}$, an enzyme located in the inner mitochondrial membrane that catalyzes cholesterol side-chain cleavage to yield pregnenolone. Pregnenolone is the first key intermediate for neurosteroid biosynthesis. PK11195 inhibits the TSPO transport protein, prohibiting cholesterol from entering the mitochondrial membrane. The steroids progesterone, deoxycorticosterone, and testosterone are further reduced into neurosteroids by the common 5α-reductase enzymatic pathway. Finasteride blocks the 5α-reductase, thereby preventing the synthesis of neurosteroids allopregnanolone, androstanediol, and THDOC. The bottom panel shows the potential GABA$_A$ receptor mechanisms for midazolam. Midazolam binds to the benzodiazepine site located on the γ-containing synaptic GABA$_A$ receptors. The extent of the drug to increase the rate of δ-extrasynaptic GABA$_A$ receptor opening remains unclear.

Fig. 2: Antiseizure activity of midazolam in the 6-Hz model in mice: influence of neurosteroidogenesis blockers PK11195 and finasteride, and the benzodiazepine antagonist flumazenil. (A) Current-seizure occurrence curve at various current intensities in 6-Hz stimulation model. The EC$_{99}$ value of 32-mA was derived from this curve. (B) Dose-response relationship for protective activity of midazolam in the 6-Hz seizure test. (C) Time-course for protection by midazolam in the 6-Hz seizure test. Midazolam was administered 15 min before electrical stimulation. Points signify percentage of animals protected from seizures within a group of 6-8 at a given dose or time. (D) Effect of neurosteroidogenic inhibitors PK11195 and finasteride on the protective effect of midazolam in the 6-Hz seizure test. PK11195 (15 mg/kg) and finasteride (50 mg/kg) were administered i.p. 30 and 60 min prior to midazolam injection, respectively. Points signify percentage of animals without seizures within groups of 6-8. ED$_{50}$ values are listed in Table 1. (E) Effect of benzodiazepine antagonist flumazenil on the antiseizure effect of midazolam in the 6-Hz seizure test. Flumazenil (10 mg/kg) was given i.p. 30 min prior to midazolam injection. Points signify percentage of animals without seizures

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within groups of 6-8. Curves are arbitrary fits to the data. ED_{50} values are listed in Table 1. (F) Effect of test inhibitors alone on seizure susceptibility in the 6-Hz test. Percent seizure occurrence considers visible symptoms of any length or magnitude as a full seizure. Seizure duration measures mean seizure length ± S.E.M. of data from 6-8 animals.

Fig.3. Inhibition of hippocampus kindled seizures by midazolam in a dose-dependent *(A,B)* and time-course fashion (C,D) in fully kindled WT and DKO mice. Dose-response curves for behavioral seizure stage (A) and AD duration (B) with varying doses of midazolam (0.1–3 mg/kg, ip). Time-course curves for behavioral seizure stage (C) and AD duration (D) at various time points after midazolam (1 mg/kg, ip). Mice that were fully kindled as described in Methods section were injected intraperitoneally with vehicle or midazolam 15 min before stimulation. Each point represents the mean ± S.E.M. of data from 6 to 9 animals. **P* < 0.05 vs. WT control. AD, afterdischarge; DKO, δ-knockout mice.

Fig. 4. Antiseizure activity of midazolam in fully-kindled WT and DKO mice. (A,B) Percent inhibition of fully kindled seizures and AD duration in WT (A) and DKO mice (B) by midazolam. Percent inhibition of seizure stage was calculated as $100 \times (1–S/5)$, where *S* is the seizure stage following drug treatment. Percentage inhibition of AD was calculated as $100 \times (1–D/D_c)$, where *D* is the AD duration after drug treatment and D_c is the average control AD duration without any drug treatment. The overall mean control AD duration in WT and DKO was 43 ± 2.0 s and 38 ±2.1, respectively. Each bar represents the mean ± S.E.M. of values from 6 to 8 animals. **P* < 0.05 vs. control. (C,D) Representative traces illustrating inhibition of AD in a fully kindled WT (C) and DKO (D) mouse by midazolam. Traces show depth EEG recordings from a stimulating electrode in the hippocampus. Arrows indicate onset of the 1 s kindling stimulus, which is followed by the stimulus artifact. Midazolam was administered i.p. 15-min prior to the stimulation; control trace was obtained without drug treatment.

Fig. 5: Effect of neurosteroid synthesis inhibitors and flumazenil on the antiseizure activity of midazolam in fully-kindled WT and DKO mice. The extent of inhibition of midazolam-induced time-dependent protection of seizure activity by PK11195, finasteride and flumazenil in WT *(A,B)* and DKO (*C,D*) mice. Test inhibitors PK11195 (15 mg/kg), finasteride (50 mg/kg) and flumazenil (10 mg/kg) were administered i.p. 30 or 60 min prior to midazolam injection. The interval between midazolam injection and the kindling stimulus is plotted on the abscissa and the seizure stage of animals is plotted on the

ordinate. Each point represents the mean ± S.E.M. of data from 6 to 9 animals. *p<0.05 vs. vehicle or midazolam control group.

Fig. 6: Effect of pharmacological inhibitors PK11195, finasteride, and flumazenil alone on seizure susceptibility in fully-kindled WT and DKO mice. Test inhibitors PK11195 (15 mg/kg), finasteride (50 mg/kg) and flumazenil (10 mg/kg) were administered i.p. without midazolam 30 or 60 min prior to stimulation. Each point represents the mean ± S.E.M. of data from 6 to 9 animals.

Fig. 7: Allopregnanolone levels in midazolam-treated mice. Brain and plasma levels of allopregnanolone were measured using LC-MS/MS assay. Plasma and brain samples were collected 30 min after midazolam (1 mg/kg, ip) injection. Vehicle signifies drug-free animals. Values represent mean concentrations ± S.E.M. of data from 6-8 animals.

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Table 1

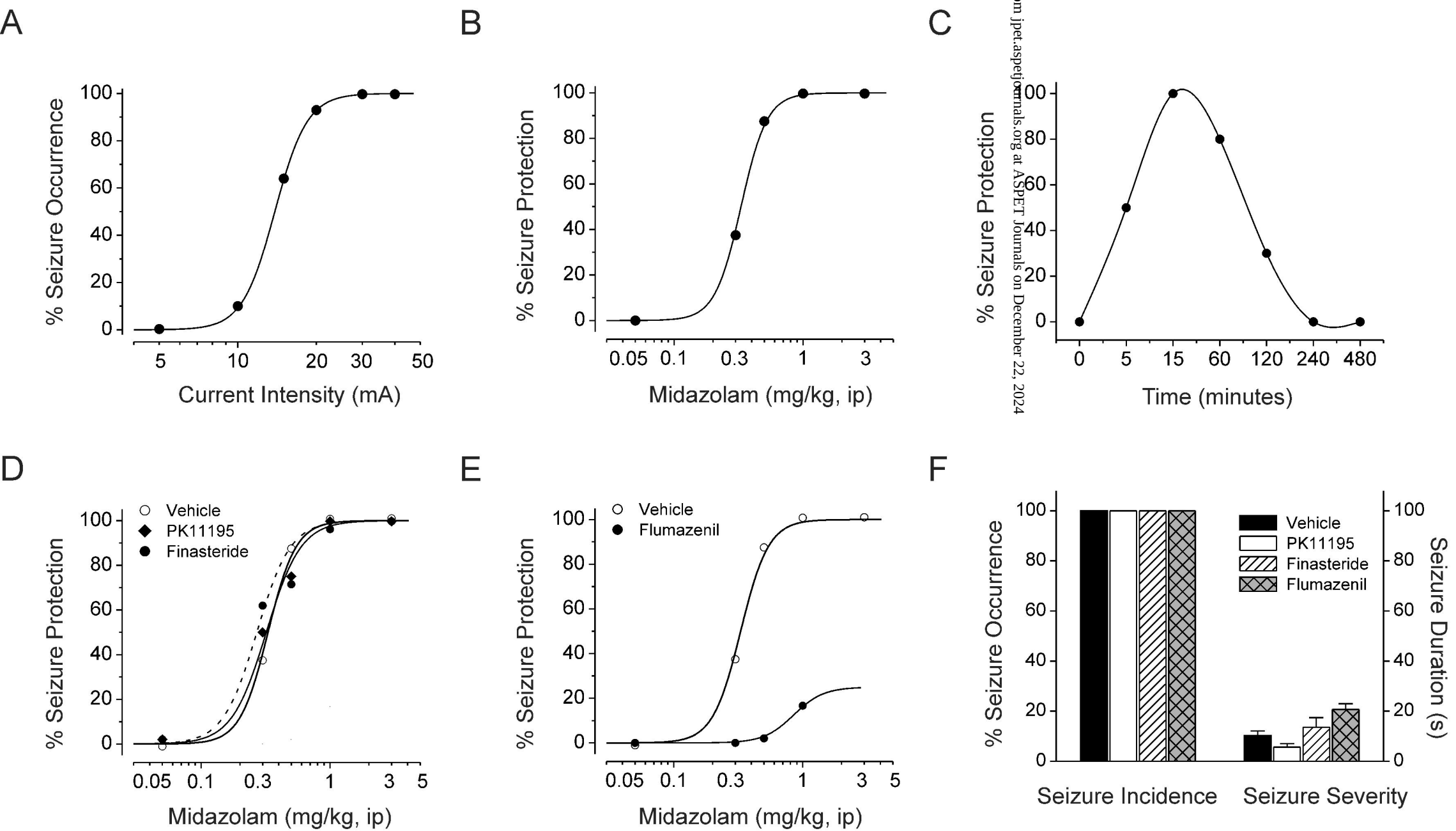
Antiseizure ED_{50} values (mg/kg) of midazolam in the 6-Hz and kindling models of epilepsy in WT and DKO mice.

Drug	6-Hz model WT mice	Kindling model WT mice	Kindling model DKO mice
Midazolam	0.40 (0.11–0.69)	0.55 (0.36-0.91)	0.6 (0.4-0.97)
Midazolam + PK11195	0.36 (0.21–0.52)	ND	ND
Midazolam + Finasteride	0.36 (0.17–0.55)	ND	ND
Midazolam + Flumazenil	>2.0 (ND)*	ND	ND

Numbers in parentheses are 95% confidence intervals. ND, not determined (blockers were tested by time-course profiles, see Fig.5); *p<0.01 vs. midazolam alone.

Figure 1

Figure 2



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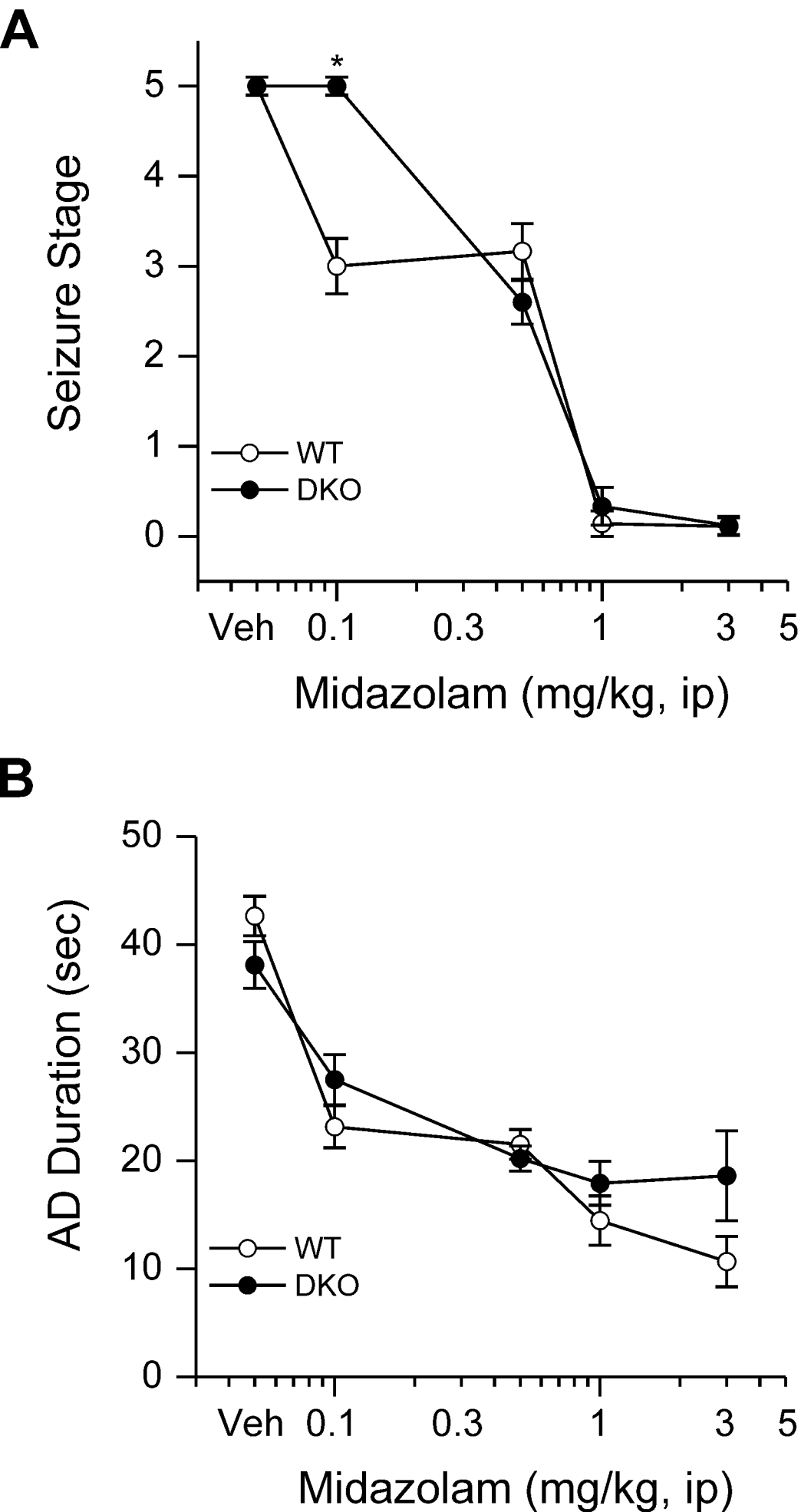


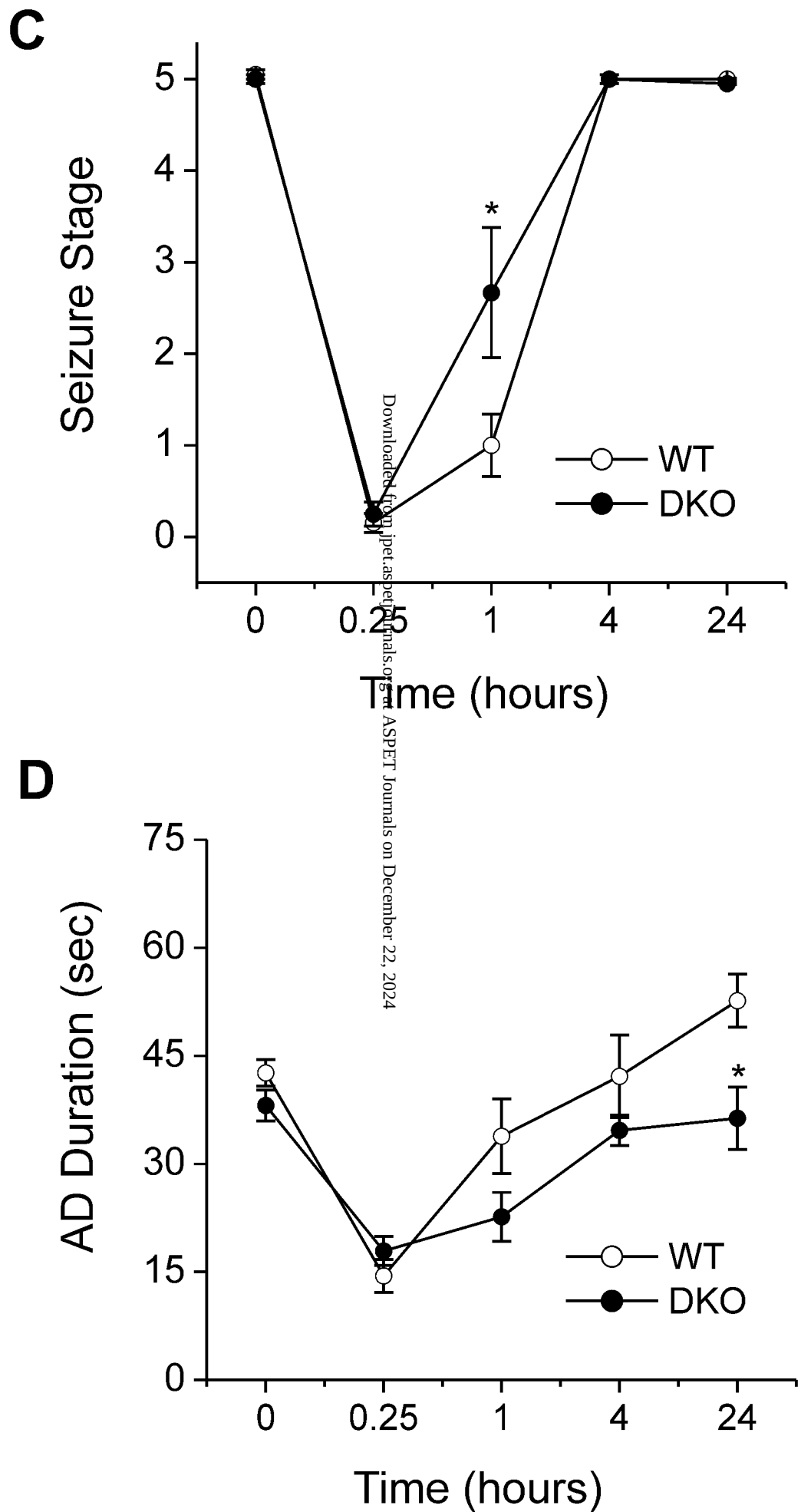
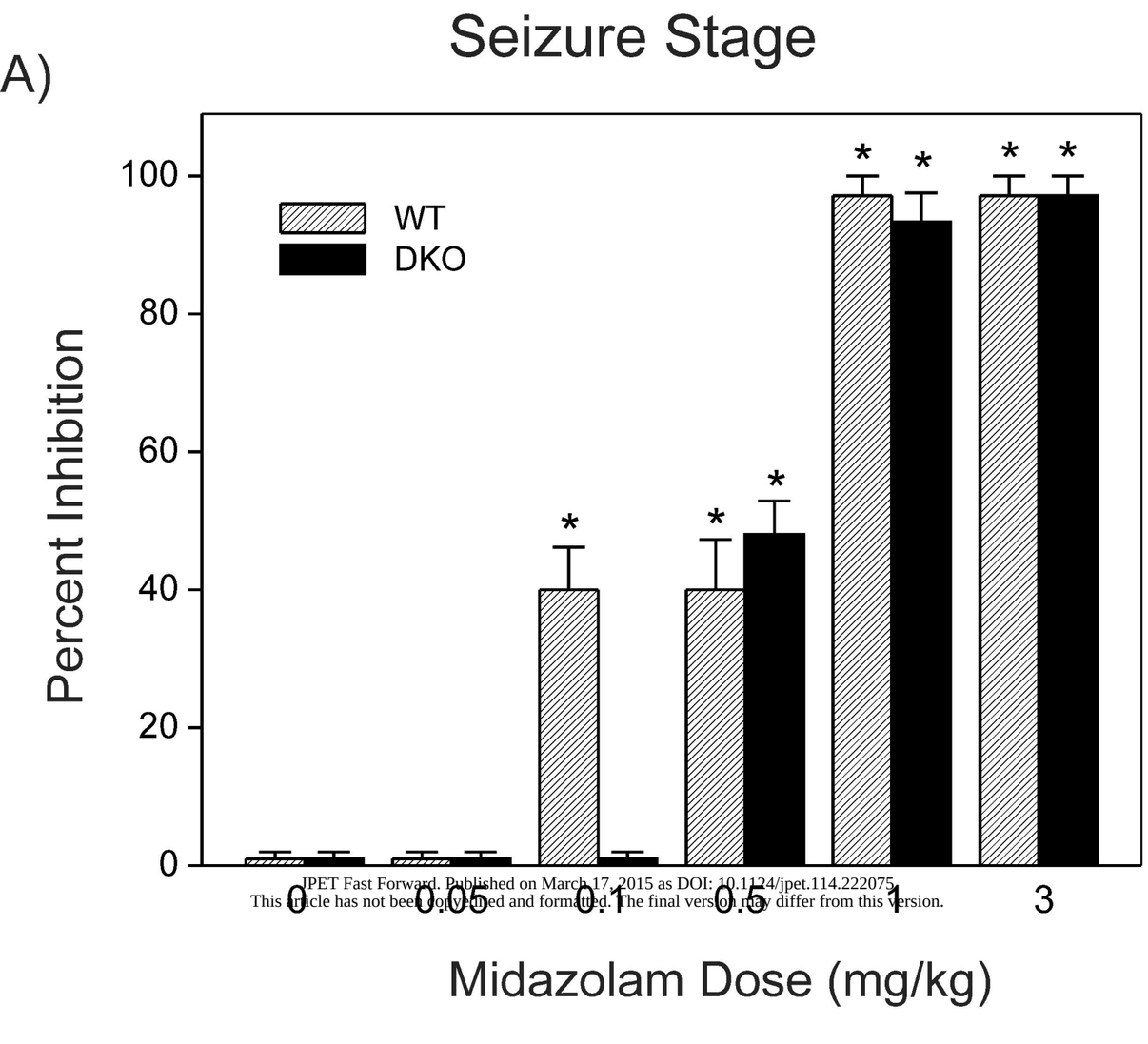
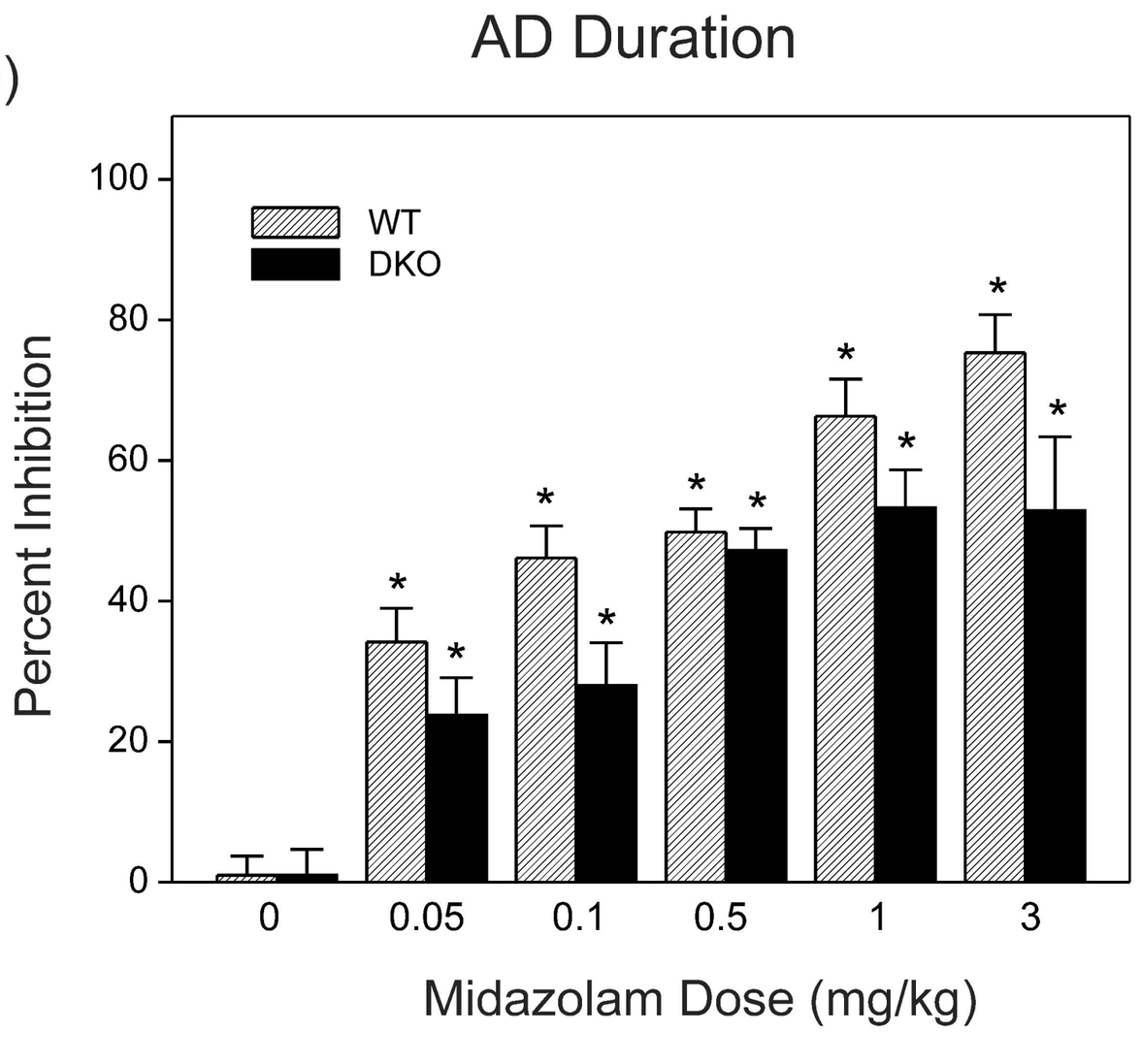
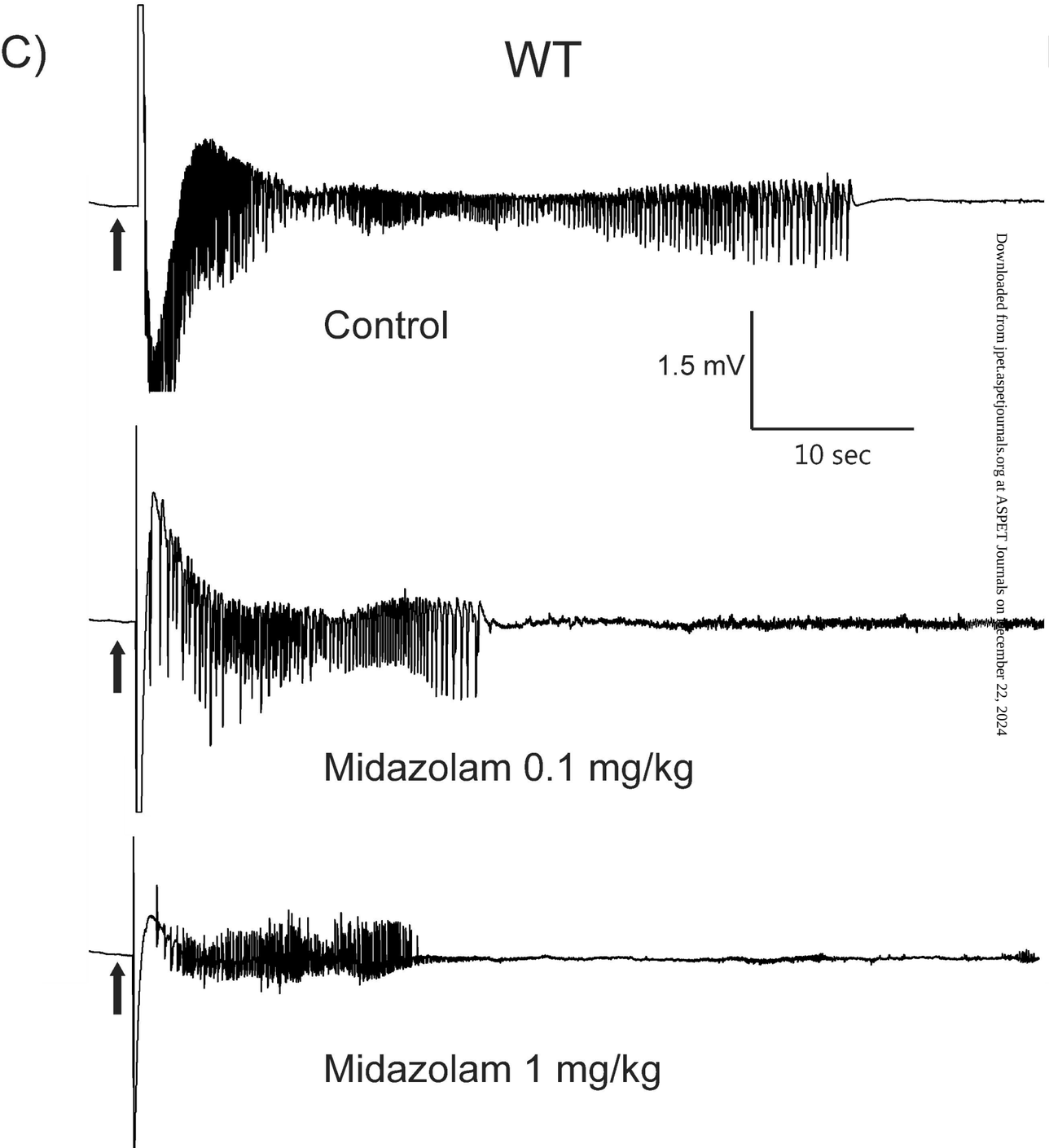


Figure 4







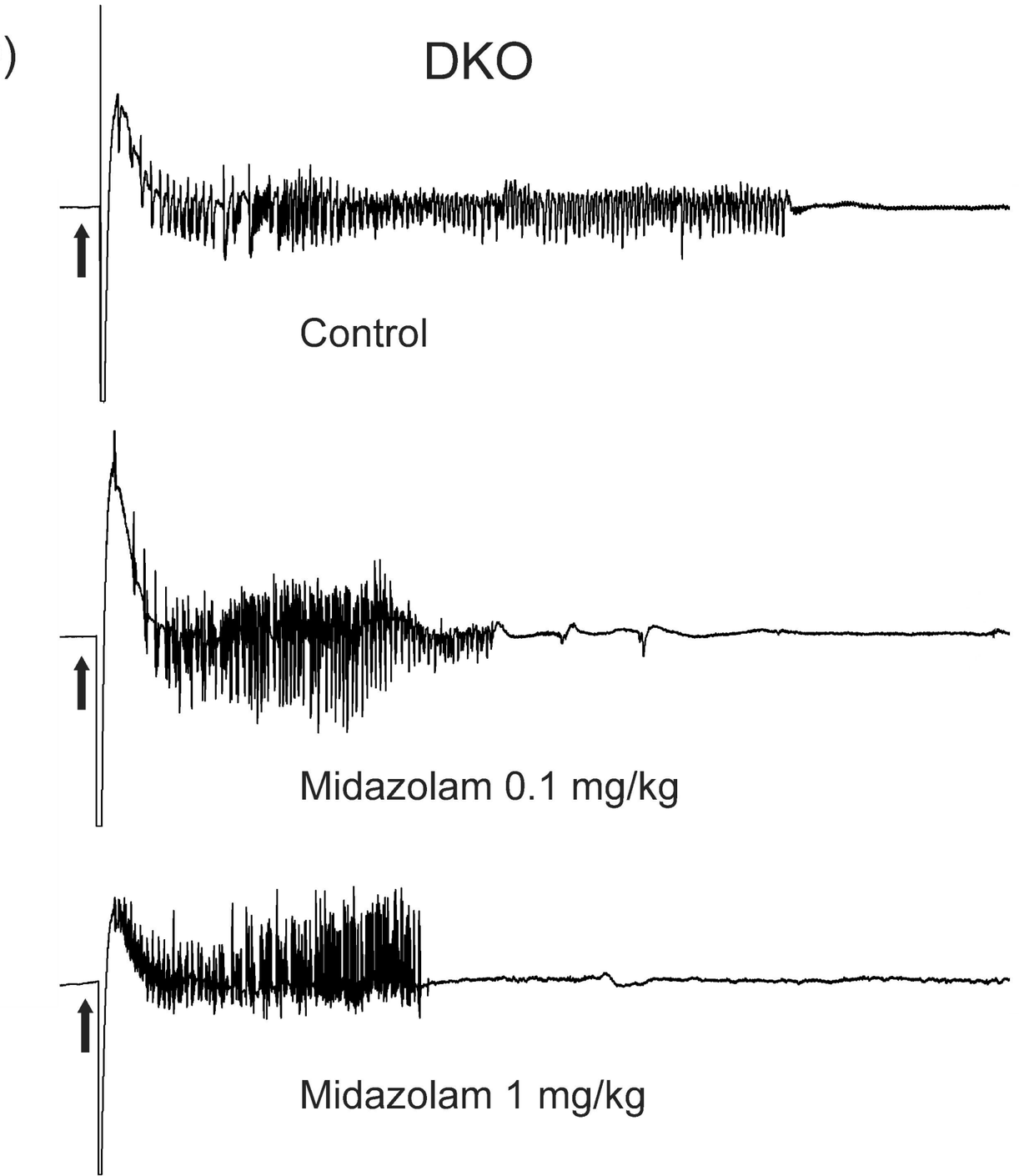


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Figure 5

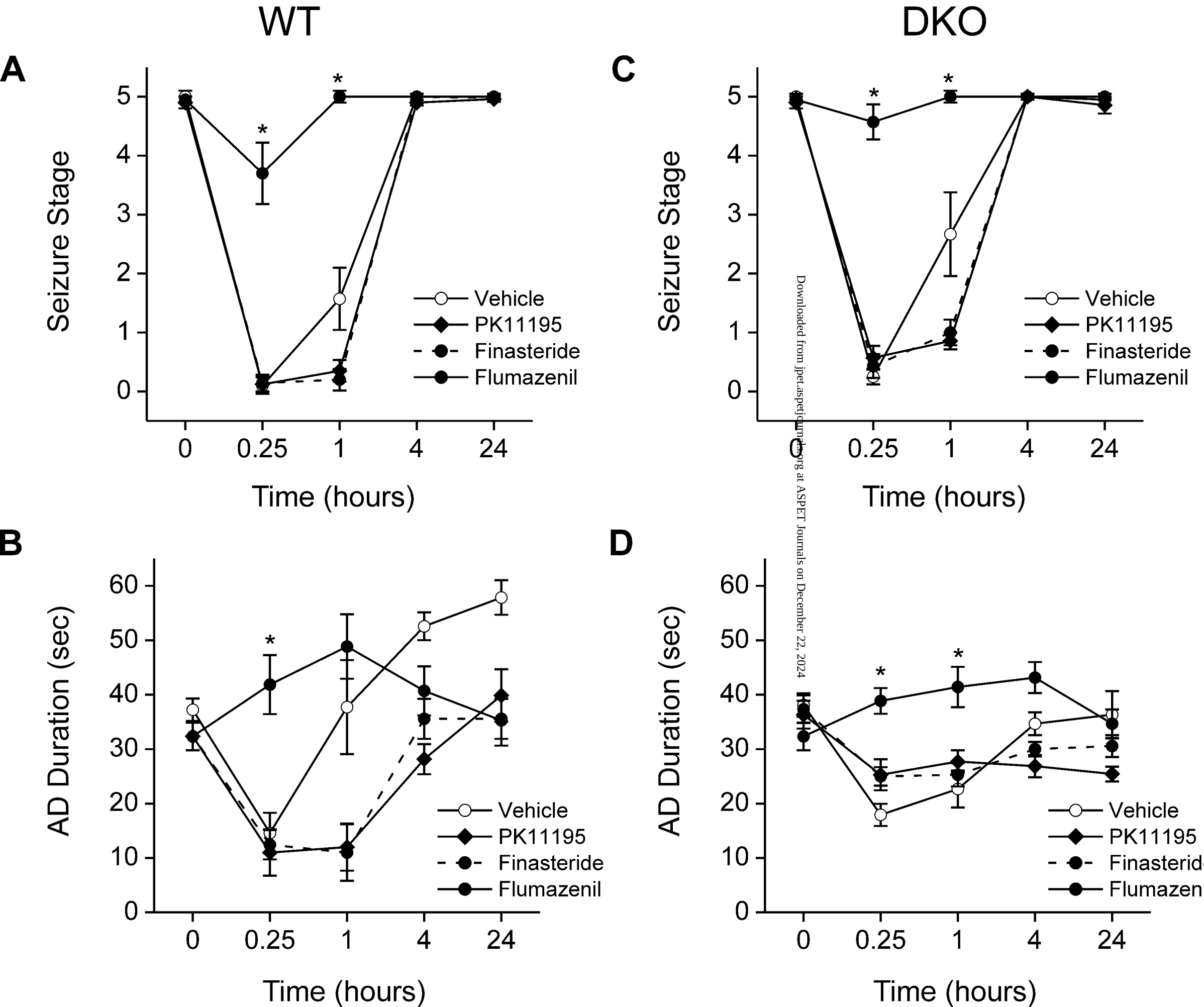


Figure 6

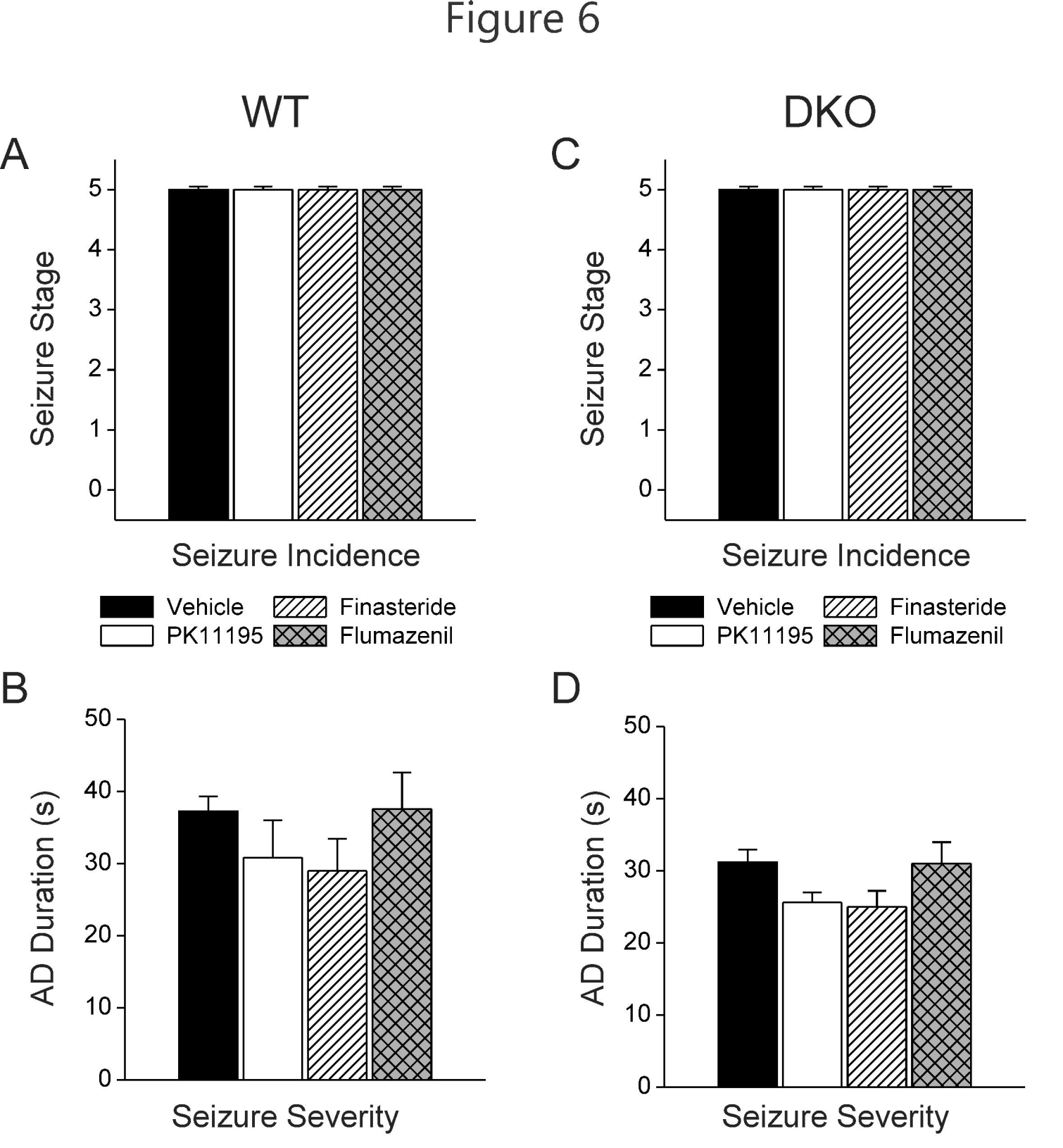
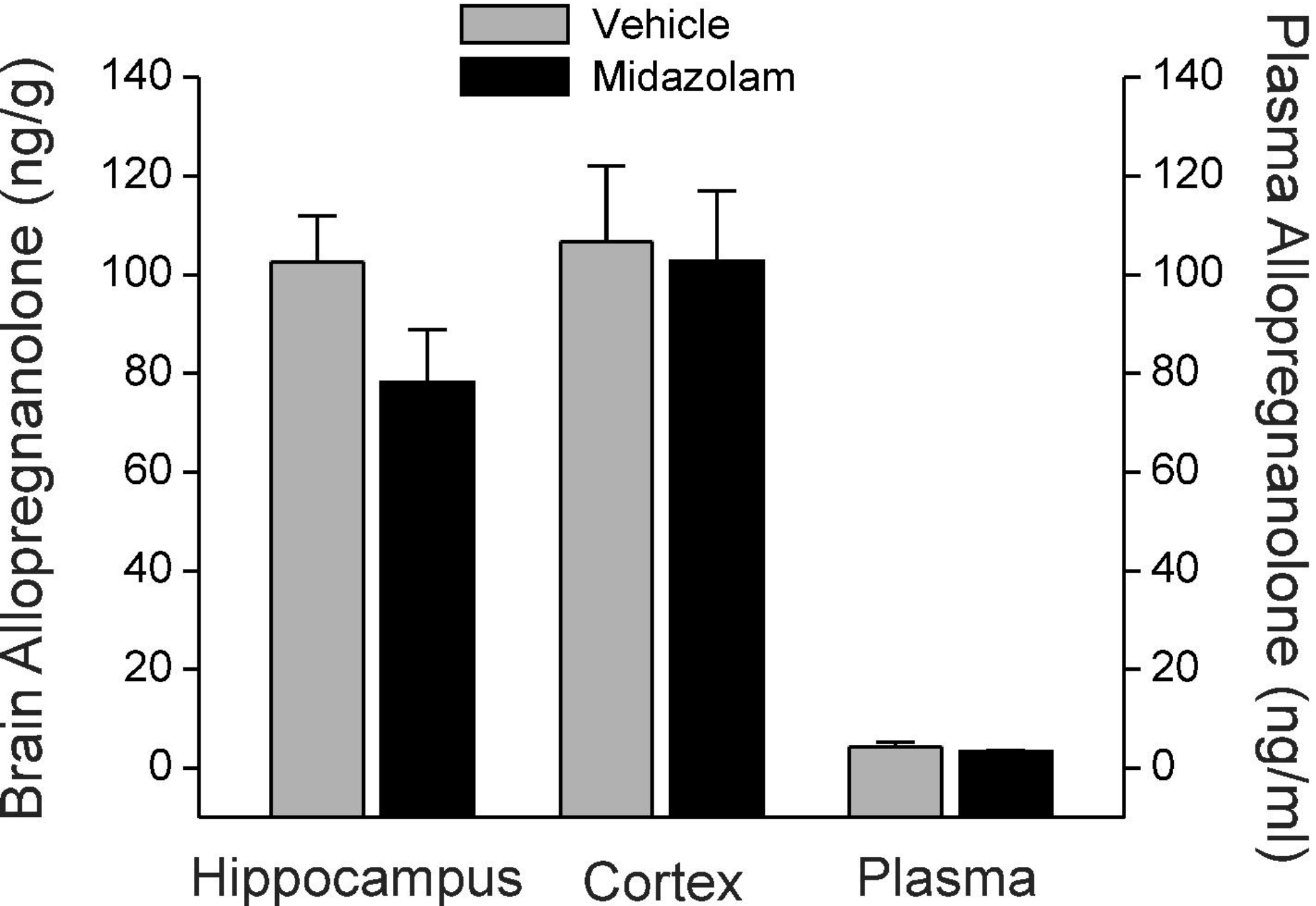

WT
DKO
A
C
Seizure Stage
0
1
2
3
4
5
Seizure Incidence
Vehicle
PK11195
Finasteride
Flumazenil
B
D
AD Duration (s)
0
10
20
30
40
50
Seizure Severity

Figure 7


Vehicle
Midazolam
Brain Allopregnanolone (ng/g)
Plasma Allopregnanolone (ng/ml)
140
120
100
80
60
40
20
0
Hippocampus
Cortex
Plasma