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1. Title page

Acute effects of brexpiprazole on serotonin, dopamine, and norepinephrine systems: an *in vivo* electrophysiological characterization

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2. Running title page

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d) A list of non-standard abbreviations

5-HT Serotonin

8-OH-DPAT 2-dipropylamino-8-hydroxy-1,2,3,4- tetrahydronaphthalene

AP Anterior/posterior

Brex Brexpiprazole

CA3 Cornu Ammonis layer 3

Clo Clonidine

DA Dopamine

DOI 2,5-dimethoxy-4-iodoamphetamine

DOS Duration of silence

DRN Dorsal raphe nucleus

DV Dorsal/ventral

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i.v.	Intravenous
LC	Locus coeruleus
LGN	Lateral geniculate nucleus
ML	Mediolateral
NE	Norepinephrine
N.S.	Not significant
NET	Norepinephrine transporter
PCP	Phencyclidide
RT ₅₀	Recovery time to 50% (of baseline firing)
SERT	Serotonin transporter
VTA	Ventral tegmental area

e) Recommended section assignment: Neuropharmacology

3. Abstract

Brexipiprazole, a compound sharing structural molecular characteristics with aripiprazole, is currently under investigation for the treatment of schizophrenia and depression. Using electrophysiological techniques, the present study assessed the *in vivo* action of brexipiprazole on serotonin (5-HT)_{1A}, 5-HT_{1B}, 5-HT_{2A} receptor subtypes, dopamine (DA) D₂ autoreceptors, and α_1 - and α_2 -adrenergic receptors. In addition, the effects on 5-HT_{1A} autoreceptors in the dorsal raphe nucleus (DRN) and D₂ autoreceptors in the ventral tegmental area (VTA) were compared to those of aripiprazole, an agent in wide clinical use. In the DRN, brexipiprazole completely inhibited the firing of 5-HT neurons via 5-HT_{1A} agonism, and was more potent than aripiprazole (ED₅₀=230 and 700 μ g/kg, respectively). In the locus coeruleus, brexipiprazole reversed the inhibitory effect of the preferential 5-HT_{2A} receptor agonist DOI on norepinephrine neuronal firing (ED₅₀=110 μ g/kg), demonstrating 5-HT_{2A} antagonistic action. Brexipiprazole reversed the inhibitory effect of the DA agonist apomorphine on VTA DA neurons (ED₅₀=61 μ g/kg), whereas it was ineffective when administered alone, indicating partial agonistic action on D₂ receptors. Compared to aripiprazole, which significantly inhibited the firing activity of VTA DA neurons, brexipiprazole displayed less efficacy at D₂ receptors. In the hippocampus, brexipiprazole acted as a full agonist at 5-HT_{1A} receptors on pyramidal neurons. Furthermore, it increased 5-HT release by terminal α_2 -adrenergic heteroreceptor, but not 5-HT_{1B} autoreceptor antagonism. In the lateral geniculate nucleus, brexipiprazole displayed α_{1B} -adrenoceptor antagonistic action. Taken together, these results provide insight in the *in vivo* action of brexipiprazole on monoamine targets relevant in the treatment of depression and schizophrenia.

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4. Introduction

Brexipiprazole (OPC-34712) is a compound currently under the investigation for the treatment of depression and schizophrenia. Antipsychotic medications from the first and second generation are efficacious antagonists at dopamine (DA) D₂ receptors. Indeed, D₂ receptor antagonism is the effective strategy for treatment of positive symptoms in schizophrenia (Seeman and Lee, 1975; Rao and Remington, 2013). However, it is accompanied by unwanted motor side-effects leading to extrapyramidal symptoms, at least in part by decreasing dopaminergic transmission in the striatum (Glazer, 2000; Kapur *et al.*, 2000). These side-effects are dampened when combined with serotonin (5-HT)_{2A} receptors antagonism, a defining pharmacological characteristic of second generation antipsychotics (Stockmeier *et al.*, 1993) that may be of therapeutic benefit in the treatment of both schizophrenia and mood disorders (Blier and Szabo, 2005; Kuroki *et al.*, 2008).

Most second generation antipsychotics have higher *in vitro* affinity for 5-HT_{2A} than D₂ receptors; brexpiprazole and aripiprazole are different in this regard (5-HT_{2A} Ki=0.47 and 4.7 nM; D₂ Ki=0.30 and 0.87 nM, respectively; Maeda, Sugino, *et al.*, 2014). Whereas most other atypical antipsychotics are D₂ receptor antagonists, *in vitro* data indicates that brexpiprazole is a D₂ partial agonist with lower intrinsic activity at D₂ receptors than aripiprazole (Maeda, Sugino, *et al.*, 2014). Partial D₂ receptor agonism is thought to have a buffering action on DA neurotransmission by stimulating D₂ receptors under low DA conditions, while dampening their activation when DA levels are high (Burris, 2002). Indeed, *in vivo* systemic administration of aripiprazole has been shown to decrease the firing activity of ventral tegmental area (VTA) DA neurons to approximately 70% of baseline, whereas complete inhibition of these neurons by the DA agonist apomorphine was reversed by aripiprazole to a similar degree (Dahan *et al.*, 2009). In contrast, acute haloperidol and clozapine administration increases firing of VTA DA neurons by D₂ autoreceptors antagonism (Hand *et al.*, 1987). Despite a different action on D₂ autoreceptors, brexpiprazole improved behavioral measures predictive for antipsychotic efficacy, such as apomorphine-induced stereotypy and the conditioned avoidance response (Maeda, Lerdrup, *et al.*, 2014). Furthermore, brexpiprazole reduced

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head-twitches induced by the preferential 5-HT_{2A} receptor agonist DOI and restored phencyclidine (PCP)-induced cognitive impairments to greater extent than the 5-HT_{2A} receptor antagonist M100907, indicating *in vivo* antagonistic action at 5-HT_{2A} receptors (Maeda, Lerdrup, *et al.*, 2014).

Aripiprazole and brexpiprazole are *in vitro* 5-HT_{1A} receptor agonists (K_i=1.3 and 0.12 nM, respectively; Maeda, Sugino, *et al.*, 2014), a relevant pharmacological characteristic in treatment of mood disorders and schizophrenia (Blier and Ward, 2003; Newman-Tancredi, 2010). For aripiprazole, *in vivo* electrophysiology studies showed acute agonistic action at 5-HT_{1A} autoreceptors in the dorsal raphe nucleus (DRN; Stark *et al.*, 2007; Dahan *et al.*, 2009). Interestingly, these autoreceptors were desensitized after only 2-day aripiprazole administration (Chernoloz *et al.*, 2009). Desensitization of 5-HT_{1A} receptors could increase 5-HT neurotransmission, a common effect of long-term antidepressant administration thought to be therapeutically beneficial in the treatment of mood disorders (Blier and El Mansari, 2013). The recent demonstration that the restorative effect of both acute and chronic brexpiprazole on impaired cognitive function was lost by 5-HT_{1A} receptor blockade further suggests *in vivo* 5-HT_{1A} receptor agonism by this compound (Maeda, Lerdrup, *et al.*, 2014; Yoshimi *et al.*, 2014).

Antagonism of α -adrenoceptors is a pharmacological feature that may have therapeutic implications for schizophrenia and depression (Fawcett and Barkin, 1998; Arnsten, 2004; Marcus *et al.*, 2010). *In vitro*, brexpiprazole has selective affinity for α_{1B} -adrenoceptors (K_i = 0.17 nM) over α_{1A} (K_i = 3.8 nM) and α_{1D} -subtypes (K_i = 2.6 nM; Maeda, Sugino, *et al.*, 2014). Furthermore, it may have antagonistic properties on α_2 -adrenoceptors (Maeda, Sugino, *et al.*, 2014), a pharmacological characteristic known to increase neurotransmission of NE (Ghanbari *et al.*, 2011; Chernoloz *et al.*, 2012) and 5-HT (Mongeau *et al.*, 1993; Haddjeri *et al.*, 1998) by blockade of terminal α_2 -adrenergic autoreceptors and -heteroreceptors, respectively.

Compared to aripiprazole, brexpiprazole has a 3-4 fold higher *in vitro* affinity for the serotonin transporter (SERT; IC₅₀=29 nM) and norepinephrine transporter (NET; IC₅₀=140 nM; Maeda, Sugino, *et al.*, 2014). Blockade of these transporters is known to enhance 5-HT and NE neurotransmission (El Mansari *et al.*, 2005; Chernoloz *et al.*, 2012). As agents that block these transporters are currently first-line in treatment of mood

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disorders, monoamine reuptake blocking properties could contribute to the clinical efficacy of brexpiprazole.

To complement and extend insight in its action on therapeutically relevant targets, the present study used electrophysiological techniques to determine the *in vivo* activity of brexpiprazole on 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and D₂ receptors, α_1 - and α_2 -adrenoreceptors, and on the SERT and NET.

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5. Methods

Animals

Experiments were carried out in male Sprague-Dawley rats (Charles River, St. Constant, QC, Canada) weighing 275-325 g housed under standard laboratory conditions (12:12 light-dark cycle with food and water ad libitum). *In vivo* extracellular unitary recordings were carried out in chloral hydrate anaesthetized rats (400 mg/kg; i.p.) that were mounted in a stereotaxic apparatus. Body temperature was maintained at 37 °C throughout the experiment utilizing a thermistor-controlled heating pad. If applicable, prior to the electrophysiological recordings a catheter was inserted in a lateral tail vein for systemic intravenous (i.v.) injection of pharmacologic agents. At the end of experiments, animals were euthanized by a lethal dose of chloral hydrate (4 % solution, i.p). All experiments were carried out in accordance with the Canadian Council on Animal Care and the local Animal Care Committee (University of Ottawa, Institute of Mental Health Research, Ottawa, Ontario, Canada).

Compounds

Brexpiprazole (Maeda, Sugino, *et al.*, 2014; 200 µg/kg), aripiprazole (200 µg/kg) and the DA agonist apomorphine (40 µg/kg) were dissolved in a 0.5 % lactic acid solution in distilled water; pH of the solution was adjusted to 4.5 by addition of NaOH. The preferential 5-HT_{2A} receptor agonist DOI (100 µg/kg), the α₂-adrenoreceptor agonist clonidine (10 and 400 µg/kg), and the 5-HT_{1A} receptor antagonist WAY 100.635 were dissolved in distilled water. The selective α_{1A}-adrenoreceptors antagonist (Wetzel *et al.*, 1995) SNAP 5089 (1 mg/kg) was dissolved in 20 % beta-cyclodextrin in distilled water. Brexpiprazole and aripiprazole were provided by Lundbeck A/S (Valby, Denmark); all other compounds were purchased from Sigma Aldrich (Oakville, ON, Canada).

In vivo electrophysiological recordings

Extracellular recordings of neurons in the VTA, DRN and locus coeruleus (LC) were carried out with a single-barrel glass micropipette (Stoelting, Spencerville, MD) preloaded with 2 M NaCl and with impedance between 2-6 MΩ. Neurons in the CA3

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region of the hippocampus and lateral geniculate nucleus (LGN) were recorded with a 5-barrel micropipette (impedances: central barrel 2-5 M Ω , side barrels 20-30 M Ω). The central barrel, used for unitary recordings, and one sidebarrel, used for automatic current balancing, were filled with 2 M NaCl; the other barrels were filled with brexpiprazole (1.2 mM in distilled water and 0.5% lactic acid, pH=4.5), 5-HT creatinine sulfate (10 mM in 0.2 M NaCl, pH=4), NE (10 mM in 0.2 M NaCl, pH=4) or quisqualic acid (1.5 mM in 0.2 M NaCl, pH=4). 5-HT and NE were ejected as cations and retained with a negative current; quisqualate and brexpiprazole were ejected as anions (-3 to +3 nA and -55 nA to -90 nA, respectively) and retained with a positive current.

Recording of 5-HT neurons

Putative 5-HT neurons were recorded by positioning single-barrel glass micropipettes at the following coordinates (in mm from lambda): anterior/posterior (AP) 1.0 to 1.2, mediolateral (ML) 0, dorsal/ventral (DV) 5.0 to 7.0. At these coordinates, only neurons with a bi- or triphasic extracellular waveform with a long-duration (0.8-1.2 ms) positive phase, and regular firing in the range of 0.8-2 Hz were recorded (Vandermaelen and Aghajanian, 1983).

Recording of LC NE neurons

NE neurons were recorded by positioning single-barrel glass micropipettes at the following coordinates (in mm from lambda): AP -1.0 to -1.2, ML 1.0 to 1.3, DV 5.0 to 7.0. NE neurons were identified using the following criteria: regular firing rate (1-3 Hz), a long duration (0.8-1.2 ms) of the rising phase of the action potential, and a brisk excitatory response followed by a short period of inhibition (~1 s) in reaction to a nociceptive pinch of the contralateral hind paw (Vandermaelen and Aghajanian, 1983). To test the effect of brexpiprazole on 5-HT_{2A} receptors, NE neurons were inhibited by the preferential 5-HT_{2A} receptor agonist DOI (100 μ g/kg, i.v; Szabo and Blier, 2001). Following a 60-second inhibition period, cumulative doses of brexpiprazole (50 and 100 μ g/kg, i.v.) were administered to reverse the inhibitory effect of DOI. The reversing effect of brexpiprazole was quantified relative to baseline firing activity.

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Recording of VTA DA neurons

Putative DA neurons were recorded by positioning single-barrel glass micropipettes at the following coordinates (in mm from lambda): AP 3.2 to 3.6, ML 0.6 to 1.0, DV 7.0 to 9.0. At these coordinates, neurons with a long-duration (3-5 ms) triphasic action potential with a marked negative deflection, an inflection or “notch” on the rising phase, irregular spontaneous single firing pattern (3-6 Hz), and slow bursting activity with decrementing action potential amplitude were recorded (Grace and Bunney, 1983). The start of a burst was defined as the occurrence of 2 spikes within 80 ms; end of a burst was defined as an ISI > 160 ms (Grace and Bunney, 1984). Firing and burst activity was quantified relative to baseline, and values were obtained from the second half of a 120 second period after injection of pharmacological agents. To test the effect of brexpiprazole on D₂ autoreceptors, DA neurons were inhibited by the DA agonist apomorphine (40 µg/kg, i.v.) which is known to produce a sustained suppression of firing on these neurons (Wang, 1981). Following a 60-second inhibition period, cumulative doses of brexpiprazole (25, 50 and 100 µg/kg, i.v.) were administered to reverse the inhibitory effect of apomorphine. The reversing effect of brexpiprazole on firing rate was quantified relative to baseline firing activity.

Recording of dorsal lateral geniculate nucleus (LGN) neurons

LGN neurons were recorded by positioning multibarrel micropipettes at the following coordinates (in mm from lambda): AP 3.8-4.2, ML 4.0-4.2, DV 4.5-5.5. They were characterized by flashing light in the eye of the rat, which causes a brisk excitatory response in these neurons (Curet and de Montigny, 1988). LGN neurons are known to be excited by iontophoretic application of NE via activation α_1 -adrenergic receptors (Rogawski and Aghajanian, 1980, 1982; Menkes *et al.*, 1981). Since adrenoceptors of the 1B subtype are densely expressed in the dorsal LGN, whereas α_{1D} -adrenoceptors are absent and there is minimal presence of α_{1B} -adrenoceptors (Day *et al.*, 1997), the LGN was chosen to assess the effect of brexpiprazole on α_{1B} -adrenoceptors. Neuronal responsiveness to the iontophoretic application of NE before and after administration of brexpiprazole was assessed by determining the total number of spikes produced, corrected for baseline activity. Baseline activity was defined as the average firing rate 30-

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seconds pre- and post ejection. For all data points, the average number of spikes excited was obtained by averaging the neural response to at least two consecutive ejections of NE separated by an interval of at least 100 seconds.

Recording of pyramidal neurons in the CA3 region of the hippocampus

CA3 pyramidal neurons were recorded by positioning multibarrel micropipettes at the following coordinates (in mm from lambda): AP: 3.8-4.2, L: 4.0-4.2, D 3.5-4.5. Since most CA3 pyramidal neurons are not spontaneously active in chloral hydrate anesthetized rats, a small ejection current (-1 to +1 nA) was applied to the quisqualate barrel in order to activate them within their physiological firing range (10 to 15 Hz; Ranck 1973). The current and duration of 5-HT and NE ejection was kept constant before and after each i.v. injection of brexpiprazole. Neuronal responsiveness to the iontophoretic application NE was assessed by determining the total number of spikes suppressed from start of ejection to recovery to 80% of baseline firing rate. Activity of the NET was assessed by determining the recovery time to 50% of the maximal inhibitory effect following a high current ejection of NE (10-20 nA), and was expressed as RT₅₀ (De Montigny *et al.*, 1980). Neural responsiveness to 5-HT was assessed by determining the total number spikes inhibited during a 50-second ejection divided by the ejection current of 5-HT (2-5 nA). Activity of the SERT was assessed by determining the recovery time (in seconds) from the end of a 50-second ejection of 5-HT (20 nA) that fully inhibited the neuron, to 50% recovery of baseline firing and was expressed as RT₅₀ (Piñeyro *et al.*, 1994). Partial or full agonism of brexpiprazole on 5-HT_{1A} receptors was assessed by comparing the inhibitory effect of ejection 5-HT alone to the inhibitory effect of concomitant ejection of 5-HT and brexpiprazole, following restoration of the firing rate to the same level as before ejecting brexpiprazole by increasing quisqualate ejection. In this paradigm, co-application of partial agonist reduces the inhibitory effect of 5-HT, whereas co-application of a full agonist does not change the inhibitory effect of 5-HT (Dong *et al.*, 1998; Ghanbari *et al.*, 2010). To test whether the inhibitory effect of 5-HT and brexpiprazole was mediated by 5-HT_{1A} receptors, the inhibitory effect of iontophoretic 5-HT and brexpiprazole application was compared before and after administration of the selective 5-HT_{1A} receptor antagonist WAY 100.635 (100 µg/kg, i.v.).

Electrical stimulation of afferent 5-HT projections to hippocampus

A bipolar electrode (NE-110, David Kopf, Tujanga, CA) was inserted at the following coordinates (in mm from lambda): AP +1.0, L0.0, D: 7.8-8.2, in order to electrically stimulate 5-HT afferents while recording a CA3 pyramidal neuron using a multibarrel pipette (see above). A stimulator (S8800, Grass instruments, Quincey, MA) was used to deliver 200 square pulses (0.5 ms, 1 or 5 Hz) at 300 μ A. Duration of inhibition per stimulation was plotted in a peristimulus time histogram with a 2 ms bin size. The inhibitory effect of 5-HT fiber stimulation on CA3 neurons was expressed as duration of silence (DOS, in ms), defined as the period from the first bin showing a 50% reduction in the number of events per bin from the prestimulus value and the first subsequent bin attaining a 90% recovery of the number of events per bin from prestimulus values (Chaput *et al.*, 1986). Electrical stimulation of 5-HT afferents causes endogenous release of 5-HT, and briefly suppresses firing of CA3 pyramidal neurons by activating postsynaptic 5-HT_{1A} receptors, an effect previously shown to be independent of 5-HT reuptake inhibition (Chaput *et al.*, 1986) To determine the action of brexpiprazole on α_2 -adrenoceptors on 5-HT terminals, we determined whether brexpiprazole (500 μ g/kg, i.v.) could prevent and, in a subsequent experiment, reverse the decreasing effect on DOS of a high dose of the α_2 -adrenoceptor agonist clonidine (400 μ g/kg i.v.). To validate this paradigm, a low dose of clonidine (10 μ g/kg, i.v.) was administered before the high dose of clonidine. Indeed, it is well established that a low dose of clonidine primarily activates α_2 -adrenergic autoreceptors on NE terminals and so decreases NE tone on α_2 -adrenoceptors located on 5-HT terminals, resulting in an increased DOS. A subsequent high dose of clonidine activates α_2 -adrenoceptors on 5-HT terminals, thereby producing a decrease in DOS (Mongeau *et al.*, 1994).

To determine the effect on the activity of terminal 5-HT_{1B} autoreceptors, the effect of 3 doses of brexpiprazole (500 μ g/kg, i.v.) on the DOS following low and high frequency (1 and 5 Hz, respectively) stimulations in the same neuron was compared. Previous *in vivo* and *in vitro* studies showed that increasing the stimulation from 1 to 5 Hz results in greater activation of terminal 5-HT_{1B} receptors and consequently, decreased 5-HT release. Therefore, a longer DOS following 1 Hz compared to 5 Hz stimulations is

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indicative for functional terminal 5-HT_{1B} autoreceptors (Chaput *et al.*, 1986; Blier *et al.*, 1989).

Data analysis / Statistics

Electrophysiological recordings made, and filtered from artifacts by wavemark analysis, using Spike2 software version 6.17 (Cambridge Electronic Design, Cambridge, United Kingdom). Quantification of firing activity was performed using Spike2, with the exception of firing and burst analysis of VTA DA neurons for which burstiDAator (Oosterhof and Oosterhof, 2013) software was used. In experiments when no competitive exogenous ligand was present, linear regression analysis was used to determine ED₅₀ values, and to compare the slope and intercept when comparing the effect of aripiprazole and brexpiprazole. In presence of a competitive exogenous ligand, non-linear curve fitting was used to obtain ED₅₀ values. Experiments with less than 3 observations within the same subject were analyzed with a paired t-test; experiments with more than 3 observations within the same subject were analyzed with repeated measurements analysis of variance followed by a Tukey *post-hoc* test. All data were analyzed with Graphpad Prism (Version 5.01, Graphpad software, La Jolla, CA). Data is presented as mean ± S.E.M.; a p-value <0.05 was considered significant.

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6. Results

Effect of brexpiprazole and aripiprazole on VTA DA neurons

Brexpiprazole at cumulative doses of 200, 400 and 800 $\mu\text{g}/\text{kg}$ did not significantly alter firing rate ($F_{1,39}=3.15$, $n=11$, $p=0.083$, figure 1C) or bursting activity ($F_{1,29}=1.61$, $n=10$, $p=0.21$ figures 1D) of VTA DA neurons from baseline activity. Aripiprazole, administered at these same doses, significantly decreased firing activity ($F_{1,22}=11.93$, $n=6$, $p=0.0023$, figures 1C) and bursting activity of VTA DA neurons ($F_{1,18}=7.58$, $n=5$, $p=0.013$, figures 1D). For an illustrative trace of the effect of brexpiprazole and aripiprazole see figures 1A and 1B, respectively.

Effect of brexpiprazole on D_2 autoreceptors on VTA DA neurons

Following a 60-second inhibition period of putative DA neurons in the VTA by the DA receptor agonist apomorphine (40 $\mu\text{g}/\text{kg}$, i.v.), brexpiprazole administration (25-100 $\mu\text{g}/\text{kg}$), reversed the effect of apomorphine to approximately 65% of baseline firing. Sigmoidal curve fitting ($n=9$) yielded an ED_{50} value of 61 $\mu\text{g}/\text{kg}$ for brexpiprazole on this effect (figure 2B; for an illustrative trace see figure 2A).

Effect of brexpiprazole and aripiprazole on 5-HT_{1A} autoreceptors

In the DRN, brexpiprazole at cumulative doses of 100 and 200 $\mu\text{g}/\text{kg}$ inhibited the firing of 5-HT neurons ($n=11$), an effect not reversed by administration of the NE reuptake inhibitor desipramine (5 mg/kg) whereas the selective 5-HT_{1A} antagonist WAY 100.635 reversed this inhibition ($n=3$, for an illustrative trace see figure 3A). Similarly, an inhibitory effect of aripiprazole was observed on 5-HT neurons ($n=15$) that was reversed by the selective 5-HT_{1A} antagonist WAY 100.635 but not desipramine ($n=3$). For aripiprazole, the experiment was initially conducted by administering cumulative doses of 200 $\mu\text{g}/\text{kg}$ ($n=6$) based on a previous study (Dahan *et al.*, 2009). As the inhibitory effect of these injections did not fully inhibit 5-HT neurons up to a cumulative dose of 600 $\mu\text{g}/\text{kg}$, aripiprazole was administered at 500 $\mu\text{g}/\text{kg}$ ($n=9$) in a subsequent experiment; data of these experiment were pooled, as statistical analysis demonstrated that the dose-responsiveness of DRN neurons to aripiprazole at increments of 200 $\mu\text{g}/\text{kg}$

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and 500 $\mu\text{g}/\text{kg}$ were not different (slope: $F_{1,45}=0.04$, $p>0.05$, intercept: $F_{1,46}=0.08$, $p>0.05$). Statistical analysis demonstrated a significantly lower ED_{50} value for brexpiprazole than for aripiprazole ($\text{ED}_{50} = 230$ and $700 \mu\text{g}/\text{kg}$, respectively, $F_{1,78}=31.51$, $p<0.0001$, figure 3B).

Effect of brexpiprazole on postsynaptic 5-HT_{1A} receptors

In the hippocampus, systemic administration of brexpiprazole up to a dose of 1500 $\mu\text{g}/\text{kg}$ did not change the number of spikes suppressed/nA following iontophoretic application of 5-HT on CA3 pyramidal neurons ($F_{1,30}=2.72$, $p>0.05$; data not shown).

Iontophoretic application of both brexpiprazole and 5-HT had an inhibitory effect on CA3 pyramidal neurons ($n=12$, for an illustrative trace see figure 4A). When brexpiprazole and 5-HT were ejected concomitantly, the neuronal inhibition of CA pyramidal neurons did not differ from when 5-HT was ejected alone ($p>0.05$, $n=12$, figure 4D). This inhibitory effect of both agents was significantly dampened after systemic administration of the 5-HT_{1A} receptor antagonist WAY 100.635 ($p<0.05$ and $p<0.01$, respectively, figure 4B and 4C).

Effect of brexpiprazole on the SERT

In the hippocampus, the RT_{50} value (a measure for SERT activity) following iontophoretic application of 5-HT on CA3 pyramidal neurons was not changed by administrations of brexpiprazole up to a dose of 1500 $\mu\text{g}/\text{kg}$ ($F_{1,32}=0.47$, $p>0.05$; data not shown).

Effect of brexpiprazole on terminal 5-HT_{1B} autoreceptors

The DOS produced on the same neuron by electrical stimulation of 5-HT afferents with stimulation frequencies of 1 and 5 Hz was increased by brexpiprazole at doses of 500, 1000 and 1500 $\mu\text{g}/\text{kg}$ (figure 7B). For 5 Hz stimulations, the corresponding DOS at these doses were 27 ± 2 , 38 ± 3 , 48 ± 5 and 49 ± 4 . Statistical analysis demonstrated that under control conditions and after cumulative doses of brexpiprazole, the DOS after 1 Hz stimulation remained significantly greater than the DOS after 5 Hz (slope: $F_{1,36}=0.91$, $p>0.05$, intercept: $F_{1,37}=13.96$, $p=0.0006$).

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Effect of brexpiprazole on 5-HT_{2A} receptors in the LC

Following a 60-second inhibition period of NE neurons in the LC by the 5-HT_{2A} agonist DOI (100 µg/kg, i.v.), brexpiprazole administration (50-400 µg/kg), reversed NE neural firing to approximately 80% of baseline firing with an ED₅₀ value estimated at 110 µg/kg (figure 5B; for an illustrative trace see figure 5A).

Effect of brexpiprazole on postsynaptic α_{1B}-adrenoceptors in the LGN

In the LGN, acute administrations of brexpiprazole at a dose of 500 and 1000 µg/kg significantly decreased the excitatory action of exogenous NE on LGN neurons by 44 % and 77 %, respectively (F_{2,5}=20.10, p=0.0034, p<0.05 and p<0.001 for these respective doses, figure 6B) with an ED₅₀ value estimated at 630 µg/kg. The excitatory effect of NE ejection was not changed by prior administration of the α_{1A}-adrenoceptor antagonist SNAP 5089 (1 mg/kg, i.v), whereas brexpiprazole administration still decreased neuronal excitability in the LGN after SNAP 5089 administration (n=2, for illustration see figure 6A).

Effect of brexpiprazole on postsynaptic α₂-adrenoceptors

In the hippocampus CA3 region, systemic administration of brexpiprazole up to a dose of 1500 µg/kg did not change the number of spikes inhibited/nA following iontophoretic application of NE (F_{1,28}=0.95, p>0.05; data not shown).

Effect of brexpiprazole on the NET

In the hippocampus CA3 region, the RT₅₀ value following iontophoretic application of NE on CA3 pyramidal neurons was not changed by administration of brexpiprazole up to a dose of 1500 µg/kg (F_{1,30}=0.01, p>0.05; data not shown).

Effect of brexpiprazole on terminal α₂-adrenoceptors on 5-HT terminals

Clonidine at a dose of 10 µg/kg significantly increased the DOS value following 1 Hz stimulation compared to baseline, an effect completely reversed by a subsequent injection of clonidine at a dose of 400 µg/kg (F_{3,5}=13.63, n=6, p<0.0001, figure 7A). Following these doses of clonidine, the DOS increased with cumulative administrations

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of brexpiprazole (500, 1000 and 1500 $\mu\text{g}/\text{kg}$, i.v.) in a dose-dependent fashion (Tukey *post-hoc*, $p < 0.01$, $p < 0.001$ and $p < 0.001$ for these doses, respectively).

Administration of brexpiprazole alone also increased the DOS value in a dose-dependent manner ($F_{3,4} = 4.16$, $n = 5$, $p = 0.02$, figure 7B). Tukey *post-hoc* testing revealed a significant effect of brexpiprazole on DOS at a dose of 1000 and 1500 $\mu\text{g}/\text{kg}$ ($p < 0.01$ and $p < 0.01$, respectively). Clonidine administration (10 and 400 $\mu\text{g}/\text{kg}$) had no altering effect on the DOS when it was administered after brexpiprazole ($p > 0.05$, figure 7B).

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7. Discussion

Brexipiprazole: effect on VTA DA neurons

Brexipiprazole reversed the inhibitory action of the DA agonist apomorphine on neural firing of VTA DA neurons, thus demonstrating antagonistic action at D₂ autoreceptors (figure 2). Interestingly, brexipiprazole by itself did not significantly change the firing rate or bursting activity of DA neurons (figure 1). In contrast, the classical D₂ receptor antagonist haloperidol is known to increase firing and burst activity of VTA DA neurons when administered acutely, an effect attributable to blockade of endogenous DA inhibitory tone on D₂ autoreceptors (Pucak and Grace, 1994). Since brexipiprazole did not alter firing and bursting activity of VTA DA neurons, this result demonstrates that *in vivo*, it neither acts as a pure antagonist, nor as an agonist with high intrinsic activity on D₂ autoreceptors. Aripiprazole, similar to brexipiprazole, was previously shown to reverse neuronal inhibition of VTA DA neurons by apomorphine, although less potently (Dahan *et al.*, 2009). In the present study, aripiprazole significantly reduced the firing and bursting activity of VTA DA neurons to a similar degree as reported previously (figure 1; Dahan *et al.* 2009). The difference in effects on DA neurons of aripiprazole and brexipiprazole is likely due to a lower intrinsic activity of the latter agent at D₂ receptors, in line with their *in vitro* profiles (Maeda, Sugino, *et al.*, 2014). Notably, the present study also demonstrated more potent *in vivo* agonism of brexipiprazole on 5-HT_{1A} receptors than aripiprazole (discussed below). Since activation of prefrontal 5-HT_{1A} receptors is known to excite VTA DA neurons (Arborelius *et al.*, 1993; Díaz-Mataix *et al.*, 2005; Gronier, 2008), a difference in degree of 5-HT_{1A} receptor agonism could contribute to the distinct effects of brexipiprazole and aripiprazole on the firing activity of DA neurons.

Brexipiprazole: effect on the 5-HT system

Brexipiprazole dose-dependently inhibited the firing rate of 5-HT neurons in the DRN (figure 3). This inhibitory effect was due to agonism at 5-HT_{1A} receptors on these neurons, as the selective 5-HT_{1A} receptor antagonist WAY 100.635, but not the NE reuptake inhibitor desipramine reversed the inhibitory action of brexipiprazole (figure

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3A). Compared to aripiprazole, brexpiprazole was significantly more potent at activating 5-HT_{1A} autoreceptors (figure 3B), a finding in line with the *in vitro* affinity for 5-HT_{1A} receptors of these agents (Maeda, Sugino, *et al.*, 2014). To the best of our knowledge, two previous studies assessed the dose-dependent effect of aripiprazole to inhibit DRN 5-HT neurons; Dahan *et al.* (2009) reported an ED₅₀ value of 540 µg/kg, in close similarity to the present findings, while the ED₅₀ for aripiprazole in the work by Stark *et al.* (2007) is estimated at 200-250 µg/kg. The reason for this difference remains to be established, although linear vs. logarithmic dose-response estimation could play a role.

In the hippocampus, the activity of the SERT, assessed using the RT₅₀ index, remained unaltered following brexpiprazole administration up to a dose of 1500 µg/kg, whereas drugs that occupy SERT to a significant extent *in vivo* are known to prolong the RT₅₀ index (Piñeyro *et al.*, 1994; Ghanbari *et al.*, 2010). Microiontophoretic application of brexpiprazole and 5-HT both inhibited CA3 pyramidal neurons (figure 4A,C), an effect attributable to 5-HT_{1A} activation. Indeed, the inhibitory effect of these agents was blocked after systemic administration of the 5-HT_{1A} antagonist WAY 100.635 (figure 4B,C). Importantly, the inhibitory effect of 5-HT alone was unaltered when brexpiprazole was co-ejected (figure 4D). This absence of competitive action on 5-HT_{1A} receptors of brexpiprazole with 5-HT indicates that brexpiprazole acted as a full 5-HT_{1A} receptor agonist *in vivo*. Indeed, previous studies using the same paradigm showed full agonistic action of BAY 3702 (Dong *et al.*, 1998) and trazodone (Ghanbari *et al.*, 2010), whereas 8-OHDPAT (2-dipropylamino-8-hydroxy-1,2,3,4- tetrahydronaphthalene; Hadrava *et al.* 1996), gepirone (Hadrava *et al.*, 1995), and asenapine (Ghanbari *et al.*, 2009) acted as partial agonists at 5-HT_{1A} receptors in the hippocampus.

Brexpiprazole administration dose-dependently increased the DOS following electrical stimulations of 5-HT afferents at 1 Hz (figure 7B), an effect that could theoretically be attributable to terminal 5-HT_{1B} autoreceptors and/or α₂-adrenergic heteroreceptor antagonism (Chaput *et al.*, 1986; Mongeau *et al.*, 1993). However, brexpiprazole administration did not change the difference in DOS following 1 and 5 Hz stimulations, indicating that terminal 5-HT_{1B} autoreceptors functionality was unaffected by brexpiprazole. In contrast, brexpiprazole acted as a potent α₂-adrenergic heteroreceptor antagonist, as shown in two complementary experiments where brexpiprazole reversed

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(figure 7A) and prevented (figure 7B) the typical decrease in DOS caused by a high dose of the α_2 -adrenoceptor agonist clonidine (400 $\mu\text{g}/\text{kg}$, i.v.). Such effects have previously been observed following acute administration of the antidepressants mianserin and mirtazapine (Mongeau *et al.*, 1993; Haddjeri *et al.*, 1996).

Brexpiprazole: effect on the NE system.

In the LC, NE neurons are innervated by inhibitory GABA neurons that express excitatory 5-HT_{2A} receptors; activation of 5-HT_{2A} receptors enhances GABA neurotransmission and inhibits NE neurons (Szabo and Blier, 2001). Indeed, systemic administration of the preferential 5-HT_{2A} receptor agonist DOI strongly inhibited NE neurons. Since this effect was potently reversed by brexpiprazole, antagonistic action of this agent on 5-HT_{2A} receptors was clearly demonstrated (figure 5). In the present study, the ED₅₀ value of brexpiprazole for 5-HT_{2A} receptors antagonism was approximately 2-fold higher than for D₂ receptor antagonism (110 vs. 61 $\mu\text{g}/\text{kg}$, respectively), which is qualitatively in line with its *in vitro* profile (Maeda, Sugino, *et al.*, 2014). Notably, caution should be taken when comparing *in vitro* and *in vivo* data of agents in their effectiveness to reverse the action of receptor agonists, since ED₅₀ values are relative to the affinity, intrinsic activity, and dose of the used agonist. Additionally, the multiple receptor activity of brexpiprazole could affect more than one neuronal substrate *in vivo*. To illustrate this point, the *in vitro* affinity of asenapine is one order of magnitude greater for 5-HT_{2A} than D₂ receptors (Shahid *et al.*, 2009), whereas the *in vivo* ED₅₀ value for D₂ receptors type was found to be approximately two-fold higher than for 5-HT_{2A} receptors (Ghanbari *et al.*, 2009).

In the hippocampus CA3 region, brexpiprazole did not modify the inhibitory response to iontophoretic application of NE or the RT₅₀ index, indicating no action on postsynaptic α_2 -adrenoceptors and the NET, respectively (De Montigny *et al.*, 1980; Curet and de Montigny, 1988). In the LGN, neurons are known to almost exclusively express α_1 -adrenergic receptors of the 1B subtype (Day *et al.*, 1997). In this brain region, the antagonistic effect of brexpiprazole on these receptors was assessed by quantifying neuronal excitation following iontophoretically applied NE, a response known to be mediated by α_1 -adrenoceptors (Rogawski and Aghajanian, 1980, 1982; Menkes *et al.*,

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1981). Neuronal excitation decreased with brexpiprazole administration, strongly suggesting antagonistic action at α_1 -adrenergic receptors (figure 6B). Since there is no expression of α_{1B} -adrenoreceptors yet minimal expression of α_{1A} -adrenoreceptors, (Day *et al.*, 1997), the excitatory effect of NE after administration of the selective α_{1A} -adrenoreceptor antagonist SNAP 5089 was assessed (Wetzel *et al.*, 1995). The excitatory effect of iontophoretic application of NE on LGN neurons was not altered by SNAP 5089 administration, whereas consecutive administrations of brexpiprazole largely blocked this excitatory effect (figure 6A). Taken together, these results suggest antagonistic properties of brexpiprazole predominantly at α_{1B} -adrenoreceptors, consistent with its 20-fold higher affinity for this receptor subtype over α_{1A} - and α_{1D} -adrenoreceptors (Maeda, Sugino, *et al.*, 2014).

Conclusion

The present results show acute *in vivo* action of brexpiprazole at all three monoamine (5-HT, NE and DA) systems. Similar to aripiprazole, brexpiprazole acted as a partial D_2 receptor agonist *in vivo*, but is relatively less efficacious at this receptor type. Clinically, D_2 receptor partial agonism is thought to buffer fluctuations in DA transmission (Burris, 2002; Shapiro *et al.*, 2003), in line with the behavioural effects of brexpiprazole in animal models for schizophrenia (Maeda, Lerdrup, *et al.*, 2014). Furthermore, the potent *in vivo* agonistic action on 5-HT_{1A} receptors of brexpiprazole could be a relevant pharmacological feature in treatment of both mood disorders and schizophrenia (Blier and Ward, 2003; Newman-Tancredi, 2010). Acute brexpiprazole reduced inhibition on two important interaction nodes between the 5-HT and NE system; first it blocked 5-HT_{2A} receptors, a receptor type that dampens LC NE firing when 5-HT neurotransmission is enhanced (Dremencov *et al.*, 2007; Chernoloz *et al.*, 2009). Second, brexpiprazole blocked α_2 -adrenoreceptors on 5-HT terminals, a receptor type that dampens 5-HT release when NE neurotransmission is elevated (Mongeau *et al.*, 1993). As the therapeutic effect of potent 5-HT_{2A} receptor antagonism in combination with 5-HT reuptake inhibitors is well recognized (Nelson and Papakostas, 2009), the present data support the use of brexpiprazole as an augmentation strategy. This notion is strengthened by the recent demonstration of clinically efficaciousness of brexpiprazole as adjunct to

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antidepressants in major depressive disorder (Thase *et al.*, 2014). In addition, combined but not separate administration of brexpiprazole and NE or 5-HT reuptake inhibitors had an antidepressant-like effect in rodents (Hirose *et al.*, 2014). Following this *in vivo* pharmacological characterization and since brexpiprazole will be administered on long-term basis in the clinic, it will be crucial to investigate the effect of sustained brexpiprazole administration on therapeutically relevant monoamine targets.

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9. Authorship Contributions.

Participated in research design: C.A. Oosterhof, M. El Mansari, P. Blier.

Conducted experiments: C.A.Oosterhof.

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Wrote or contributed to the writing of the manuscript: C.A.Oosterhof, M. El Mansari, P. Blier.

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11. Footnotes.

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12. Legends to figures.

Figure 1: effect of systemic brexpiprazole and aripiprazole administration on VTA DA neurons. A, B: illustrative trace of DA neurons and response to intravenous administration of brexpiprazole (black arrows, figure A) and aripiprazole (grey arrows, figure B). C, D: brexpiprazole (n=11) did not change firing activity of VTA DA neurons, whereas aripiprazole (n=6) significantly reduced both firing and bursting activity of these neurons. Data were analyzed using linear regression and are presented as mean \pm S.E.M. *Significant effect of brexpiprazole in comparison to saline administration, **slope: $p < 0.01$, ##intercept: $p < 0.01$.

Figure 2: effect of systemic brexpiprazole administration on VTA DA neurons inhibited by the dopamine agonist apomorphine. A: illustrative trace of a DA neurons and response to intravenous administration of apomorphine and brexpiprazole. B: brexpiprazole reversed the inhibitory effect of apomorphine (n=9). The ED₅₀ value was obtained with a sigmoidal curve fit; data are presented as mean \pm S.E.M.

Figure 3: effect of systemic brexpiprazole and aripiprazole administration on 5-HT neurons in the DRN. A: illustrative trace of a 5-HT neuron and response to intravenous administration brexpiprazole. B: brexpiprazole (n=11) is a more potent 5-HT_{1A} agonist than aripiprazole (n=15). Data were analyzed using linear regression and are presented as mean \pm S.E.M.

Figure 4: effect of iontophoretically applied brexpiprazole, 5-HT, and concomitant ejection of 5-HT and brexpiprazole on pyramidal neurons in the hippocampus CA3 region. A: illustrative trace of discharge activity of a pyramidal neuron in the CA3 region of hippocampus, and effects of iontophoretically applied agents before and after systemic WAY 100.635 (WAY) administration. B, C: the inhibitory response to iontophoretic application of brexpiprazole and 5-HT is significantly reduced after WAY administration. D: There was no different inhibitory effect of co-ejection of brexpiprazole and 5-HT

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compared to 5-HT ejection alone. The number of neurons (n) and animals (a) is presented in histograms; data were analyzed with a paired t-test and are presented as mean \pm S.E.M. *Significant effect of WAY 100.635 administration on the inhibitory effect of brexpiprazole and 5-HT; *p<0.05, **P<0.01.

Figure 5: effect of systemic brexpiprazole on LC NE neurons inhibited by the preferential 5-HT_{2A} agonist DOI. A: illustrative trace of an NE neuron inhibited by intravenous administration of DOI and reversal of inhibition by brexpiprazole. Between 720-740 seconds the electrical signal of the neuron decreased below the level of the differential amplitude discriminator. Following this decrease, the electrode was quickly lowered by ~20 micrometer and the signal was restored to its prior amplitude. Wavemark analysis confirmed that the same neuron was recorded before and after this period. B: graphic presentation of the reversing effect of brexpiprazole on neurons inhibited by DOI (n=10). The ED₅₀ value was obtained with simple non-linear regression; data are presented as mean \pm S.E.M.

Figure 6: effect of brexpiprazole on the excitatory effect of iontophoretically applied NE on LGN neurons. A: illustrative trace of the electrical activity of an LGN neuron, its excitation by iontophoretically applied NE, ineffectiveness of the selective α_{1A} -adrenergic receptor antagonist SNAP 5089 to alter this excitation, blockade of this excitation by systemic brexpiprazole administration, and brisk excitatory response to light flashes, confirming proper recording electrode positioning. B: effect of brexpiprazole on the excitatory response of LGN neurons to iontophoretic application of NE. The number of neurons tested is presented in histograms; data were analyzed with repeated measures ANOVA and Tukey *post-hoc*, and are presented as mean + S.E.M.

*Significant effect of brexpiprazole administration; *p<0.05, ***P<0.001.

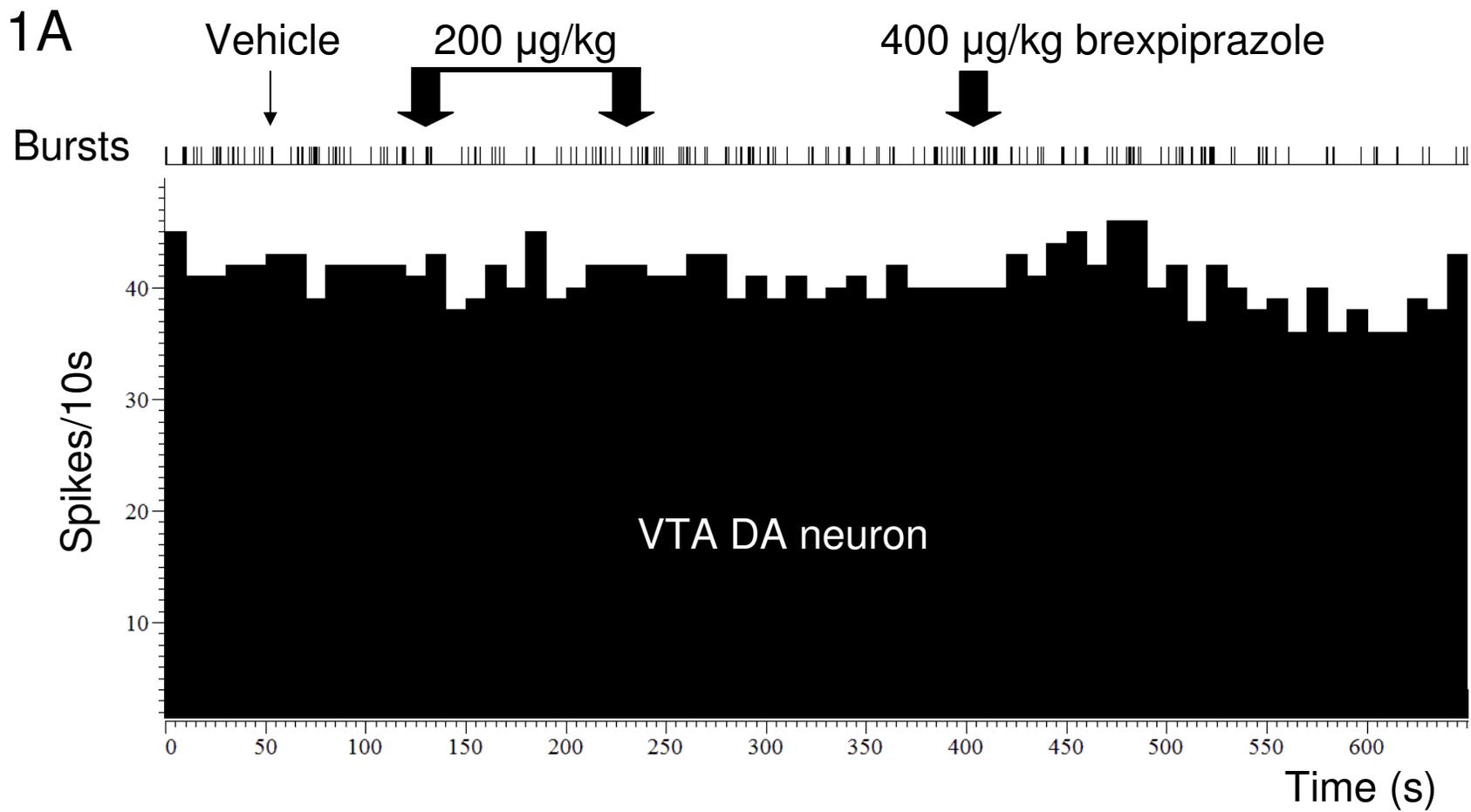
N.B. Data from the neuron recorded in (A) were not included in (B) due to the injection of SNAP 5089 prior to brexpiprazole administration.

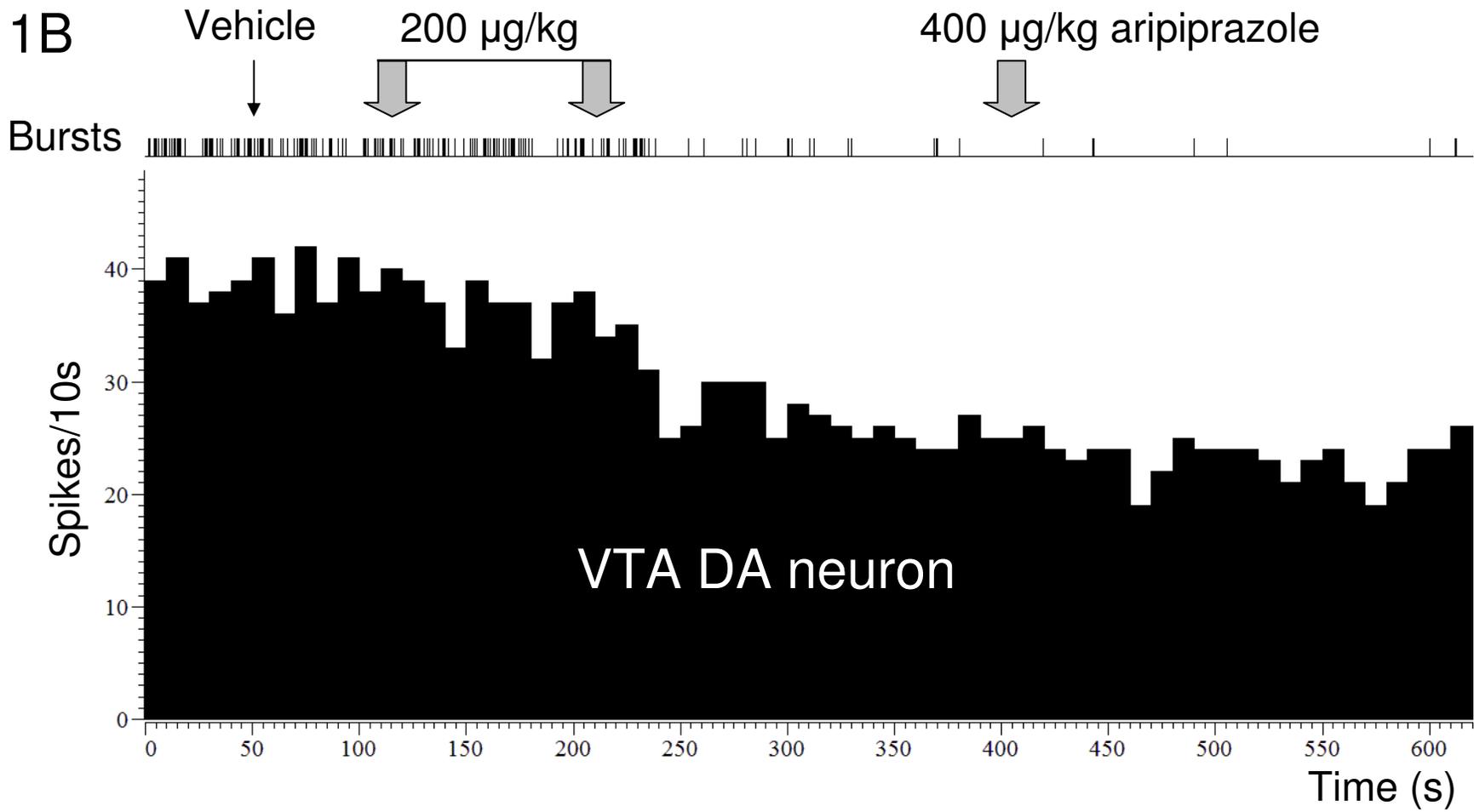
Figure 7: effect of brexpiprazole on α_2 -heteroceptors located on 5-HT terminals. A: schematic presentation of the effect of three cumulative doses of brexpiprazole (500

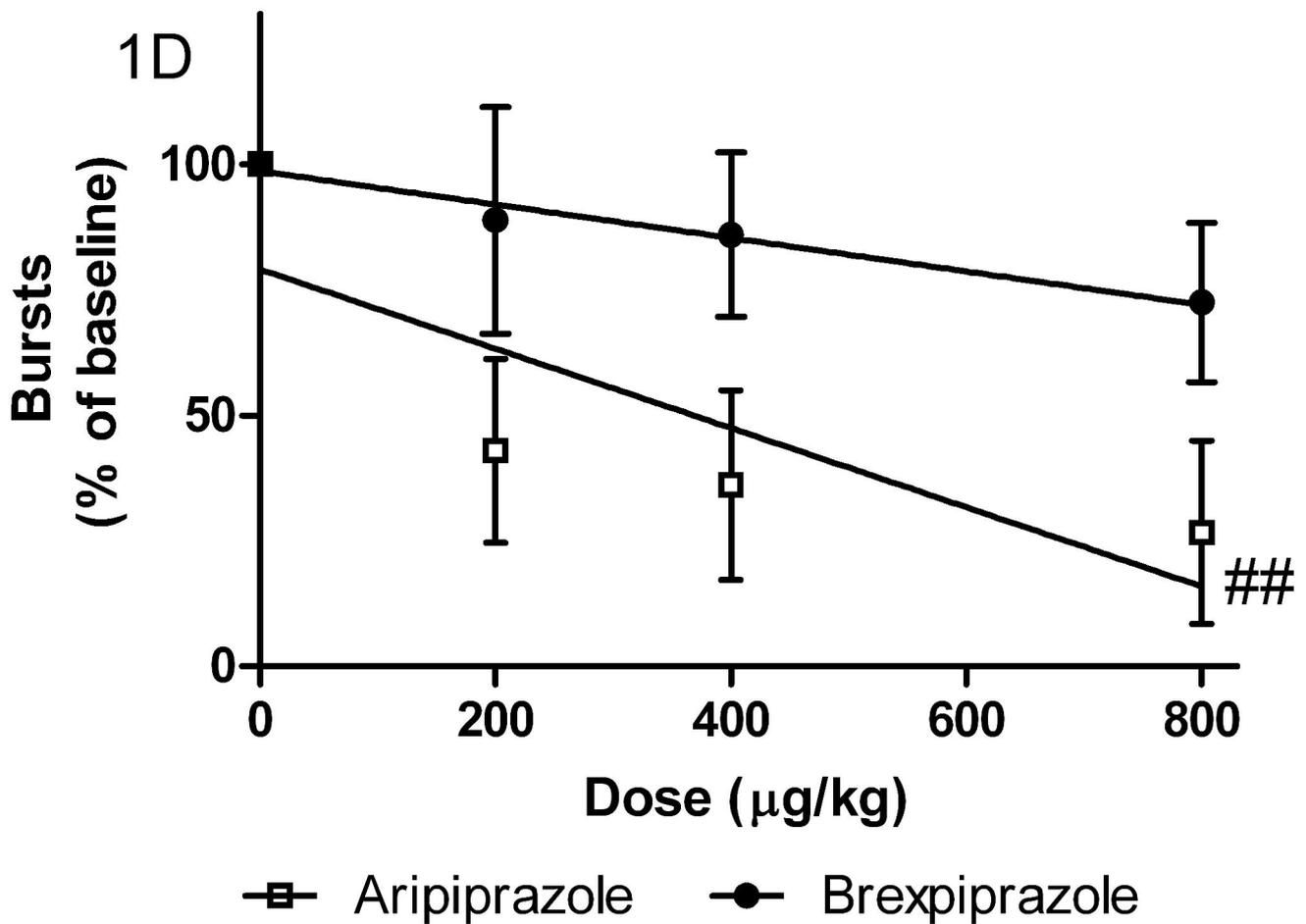
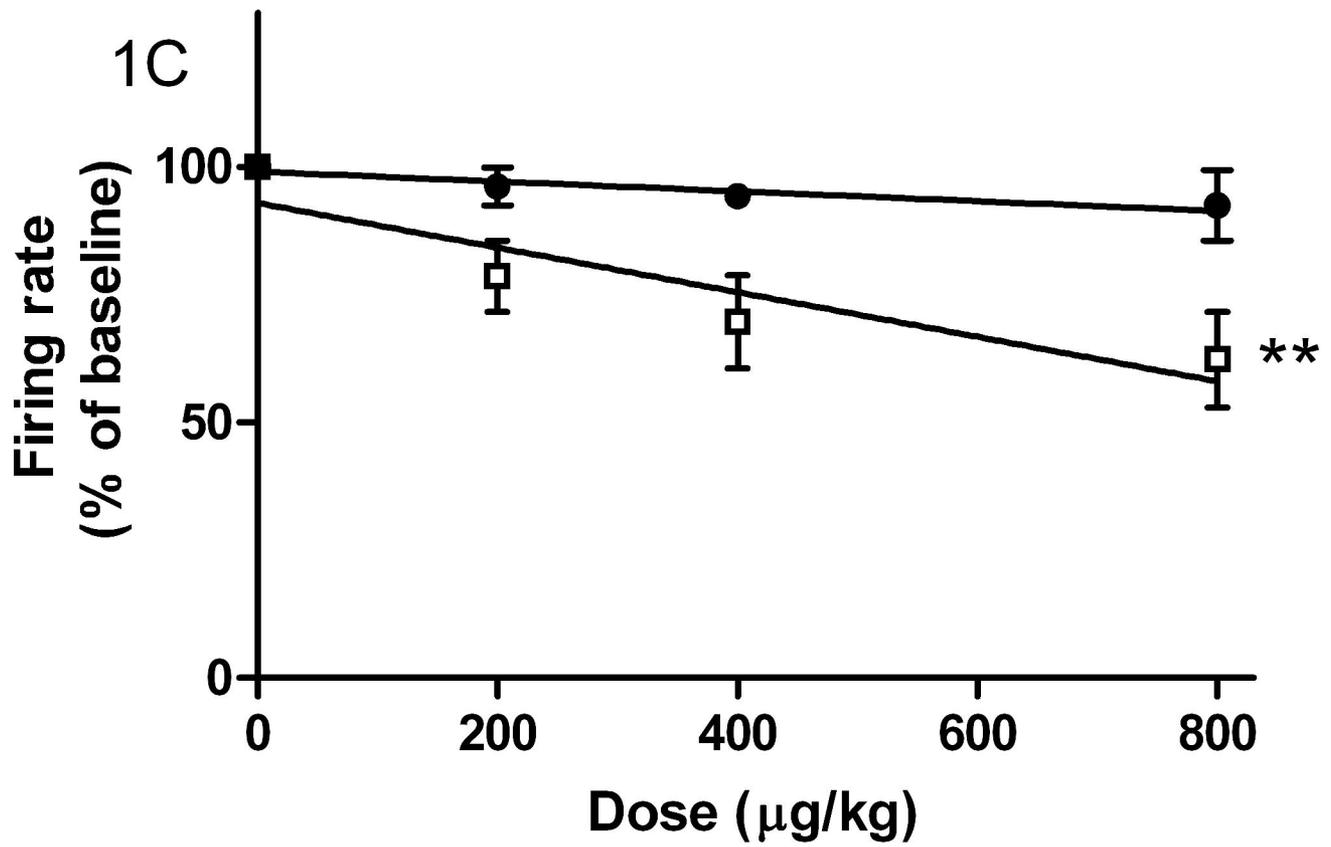
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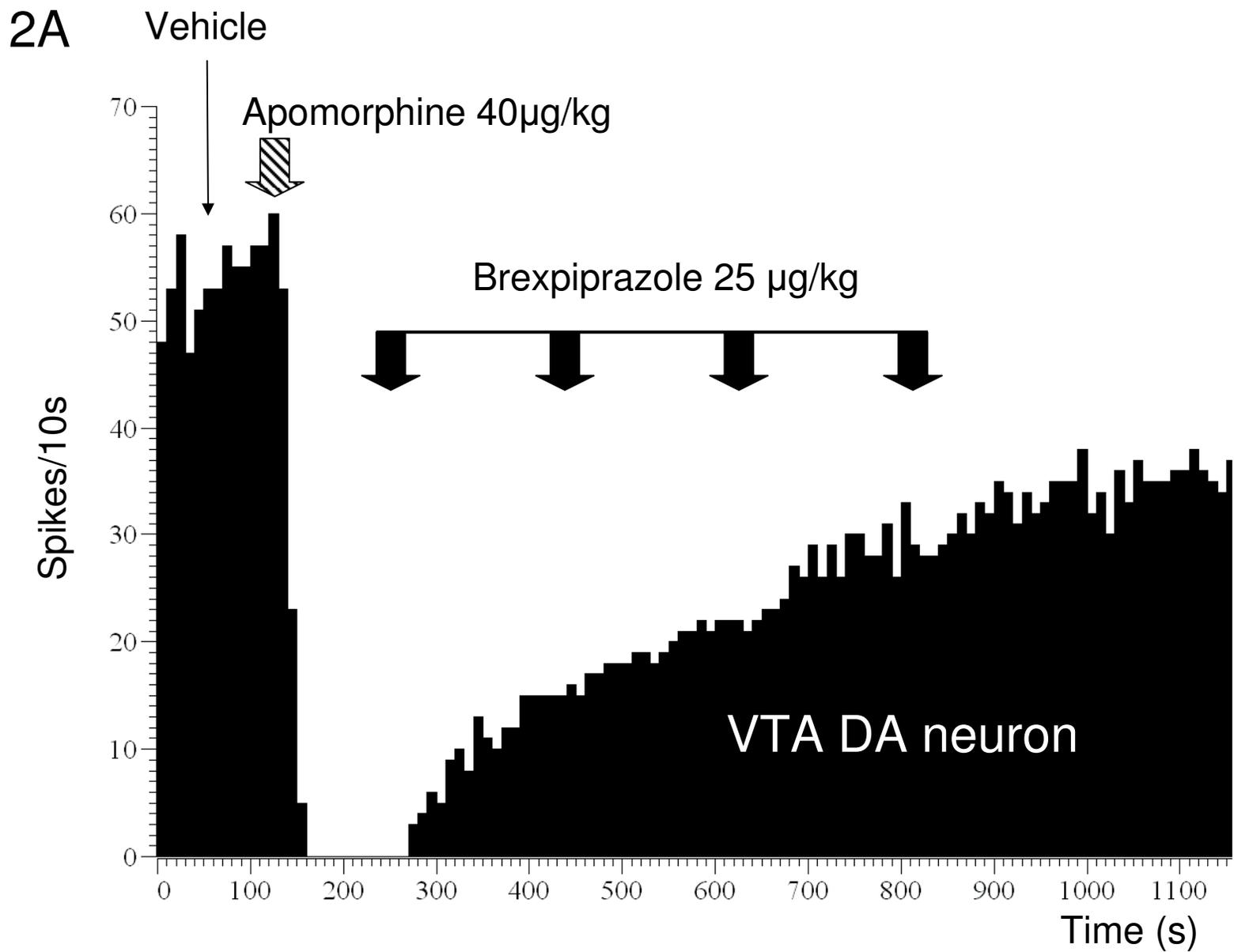
µg/kg, i.v.) after clonidine (10 and 400 µg/kg, i.v.) on DOS (n=6). B: effect of three cumulative doses of brexpiprazole (3 x 500 µg/kg, i.v.) on DOS alone, and absence of effect of clonidine on DOS after brexpiprazole administration (n=5). Brex; brexpiprazole, Clo; clonidine, numbers after abbreviations indicate doses in µg/kg, numbers in brackets indicate first, second or third administration of brexpiprazole. Data were analyzed with repeated measures ANOVA followed by a Tukey *post-hoc* test, and are presented as mean + S.E.M.

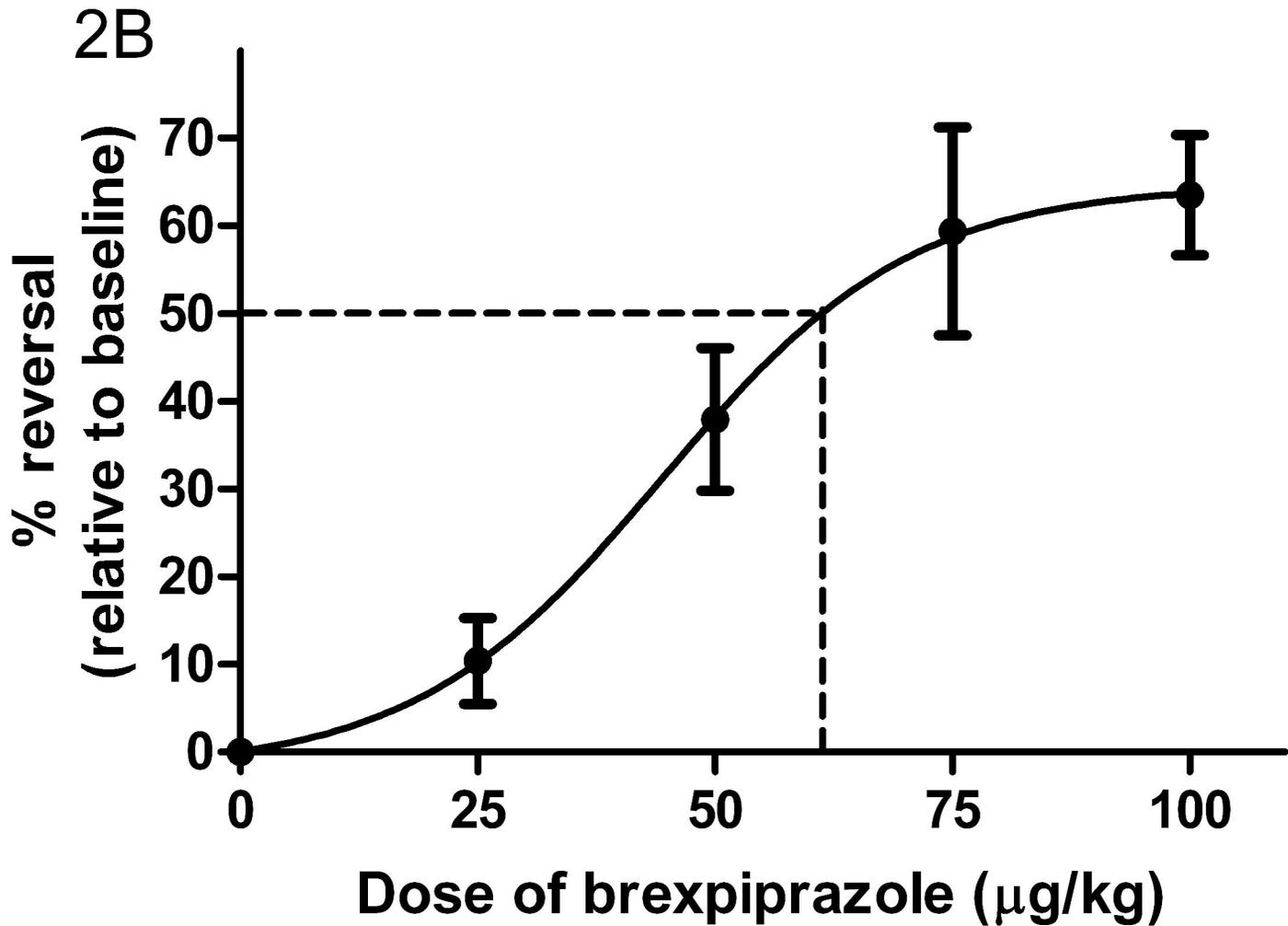
*Significant effect of brexpiprazole administration on DOS relative to clonidine 400 µg/kg (A) and baseline values (B), respectively. #significant effect of clonidine administration on DOS compared to baseline. *p<0.05, **P<0.01, ***P<0.01; ##P<0.01).

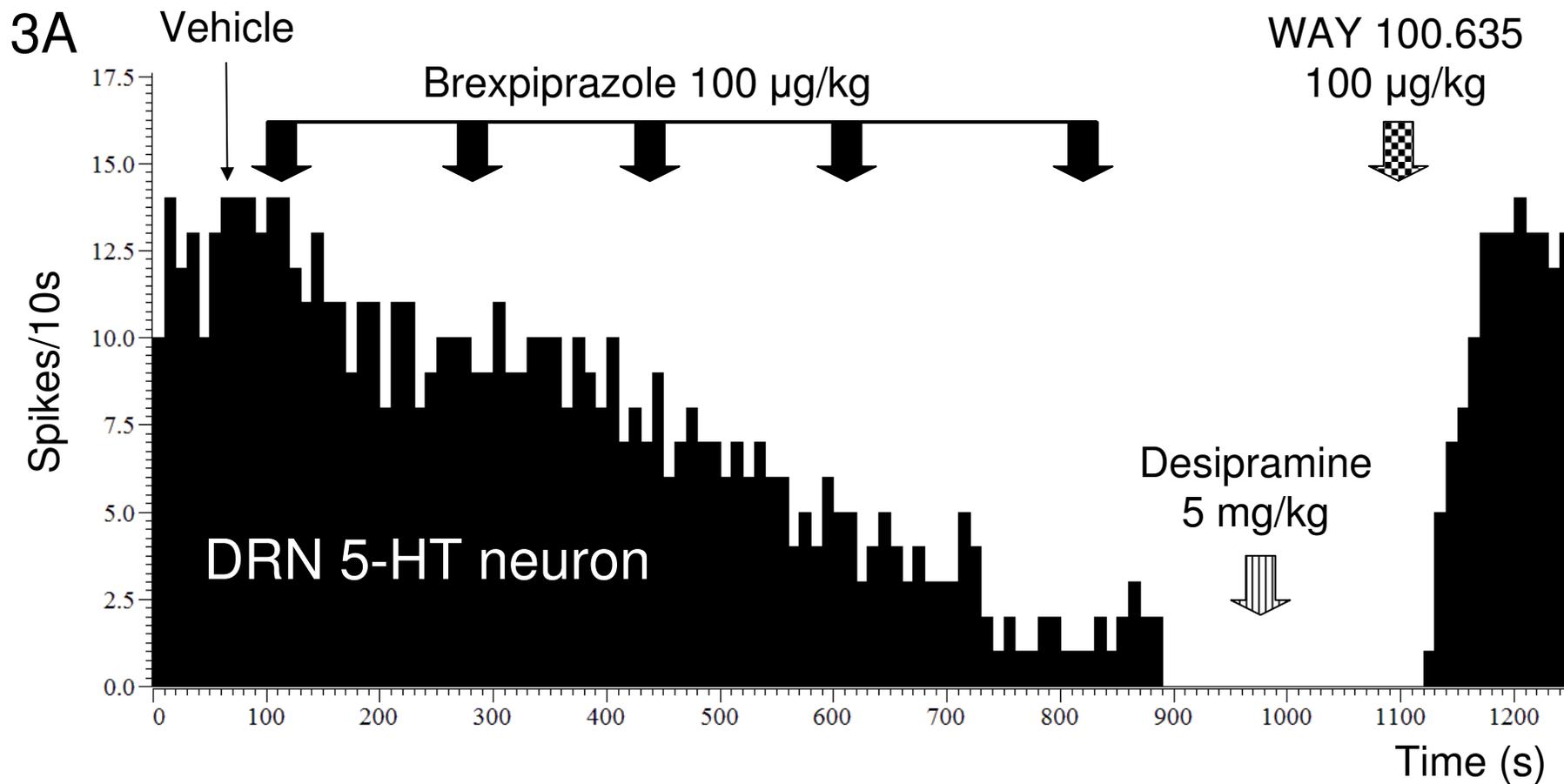


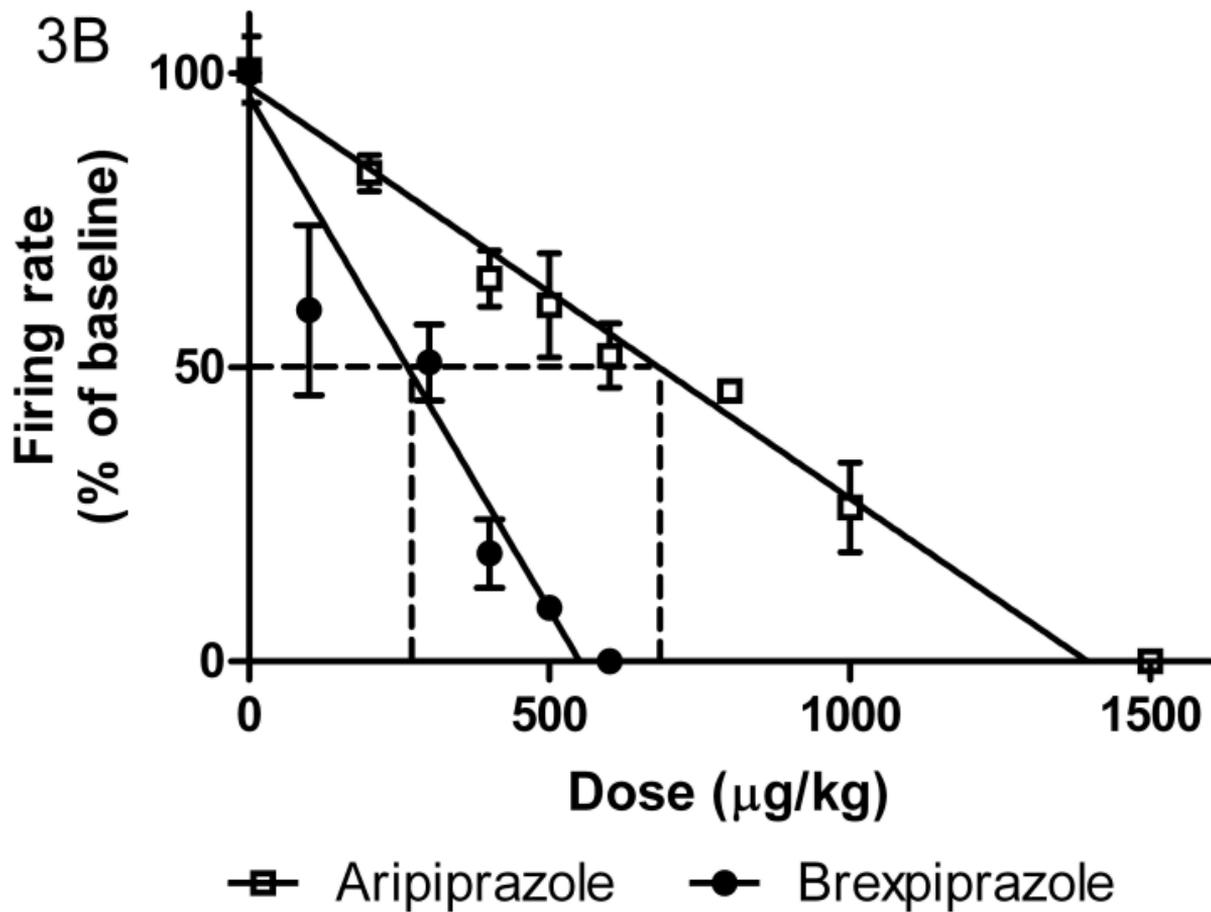




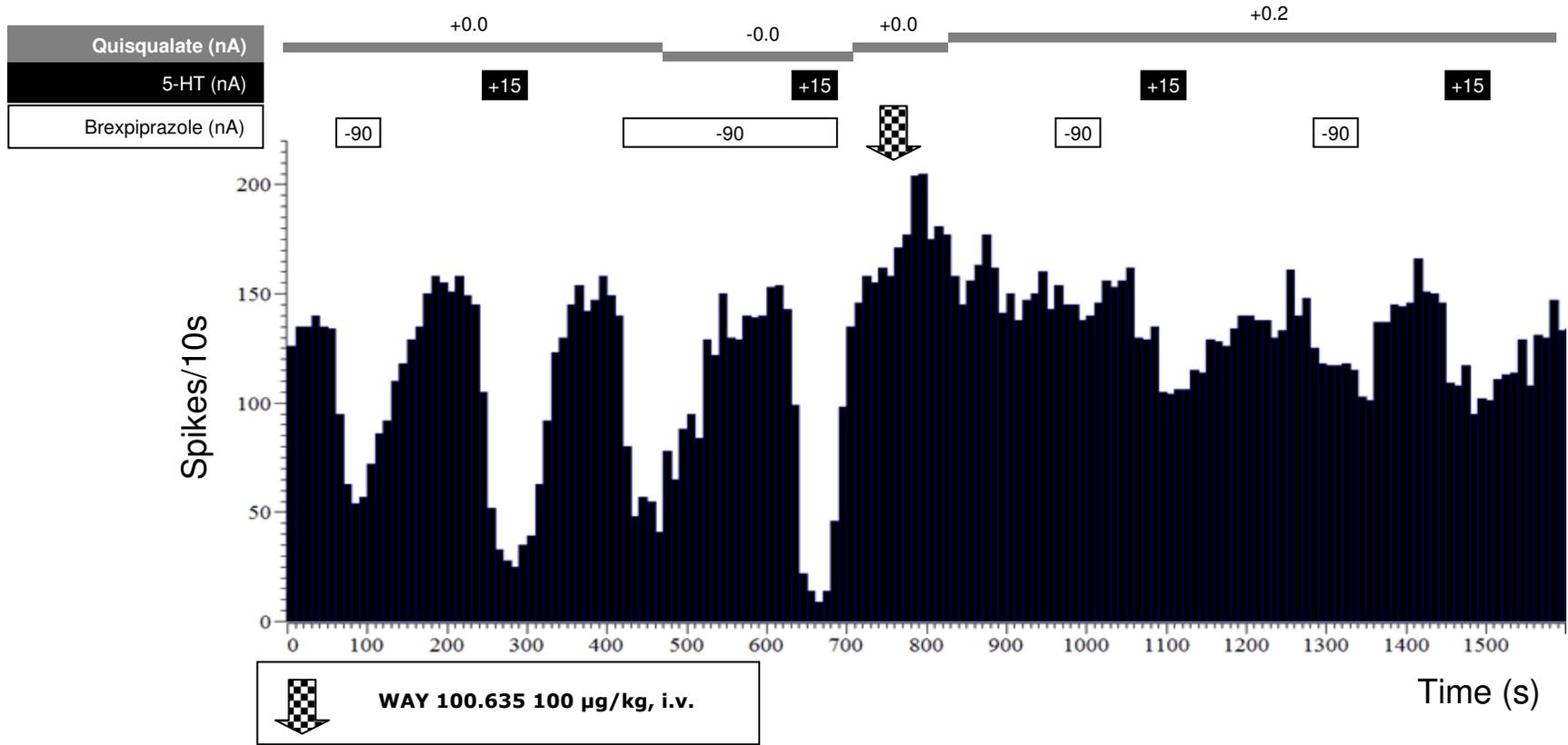


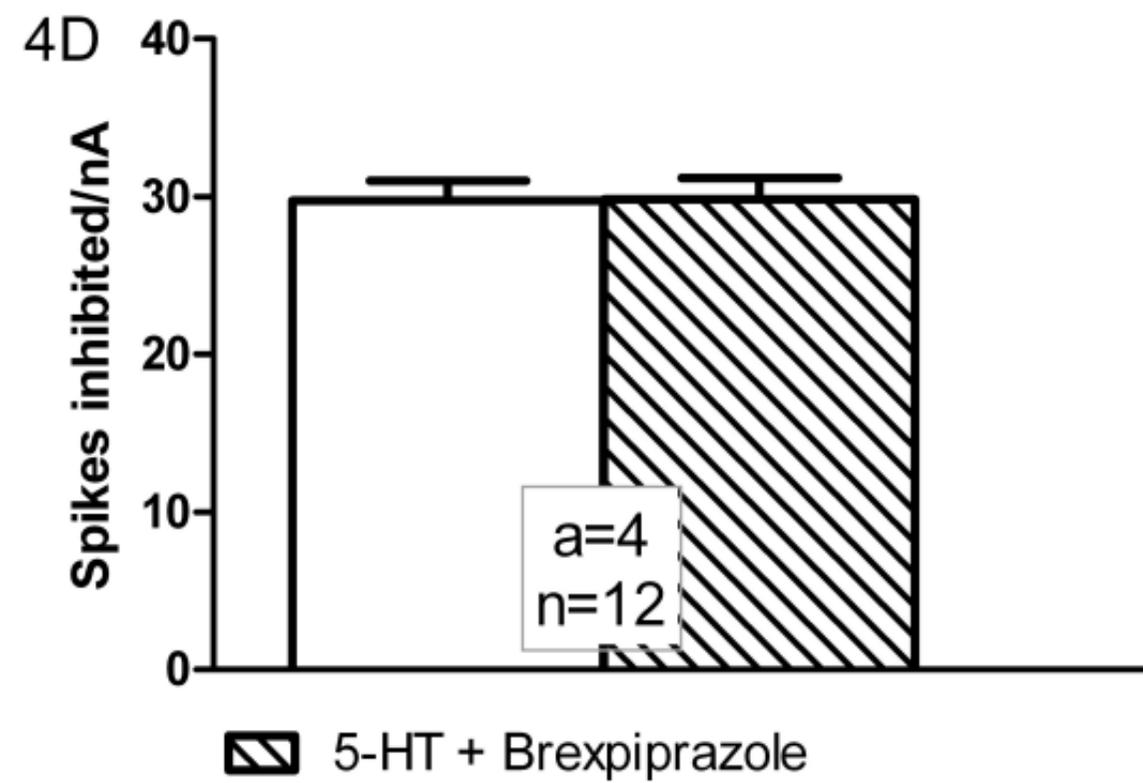
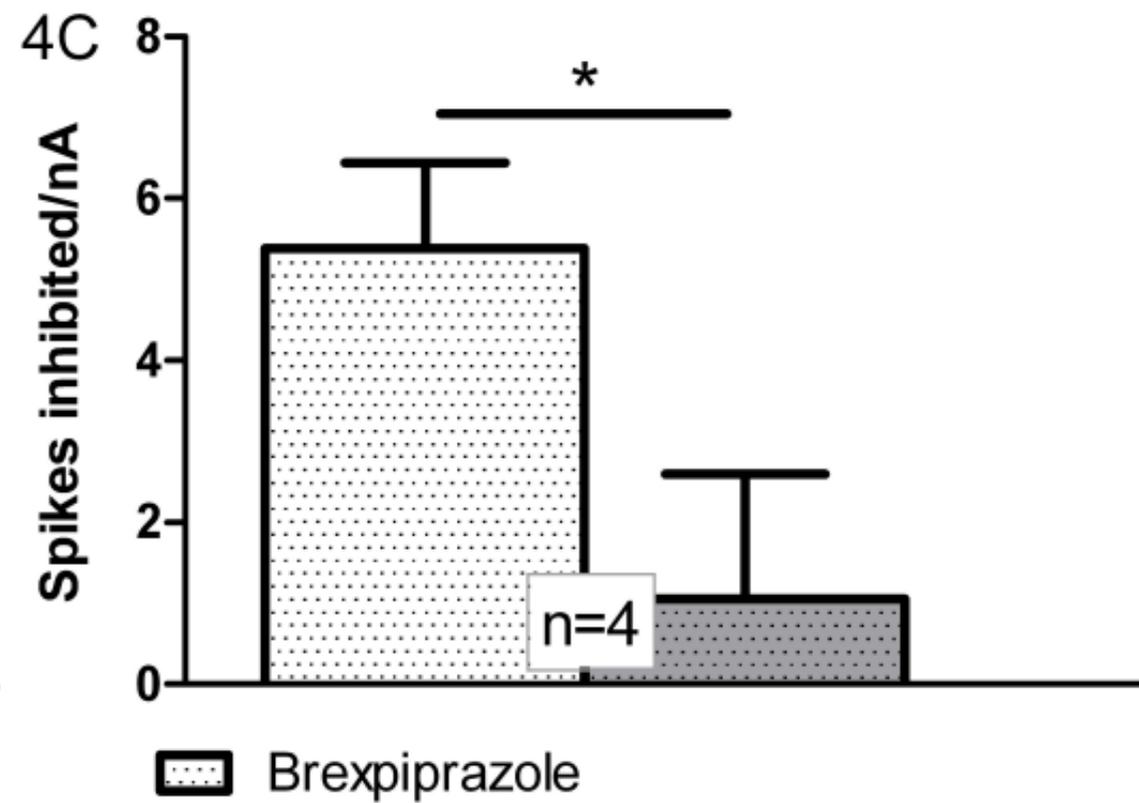
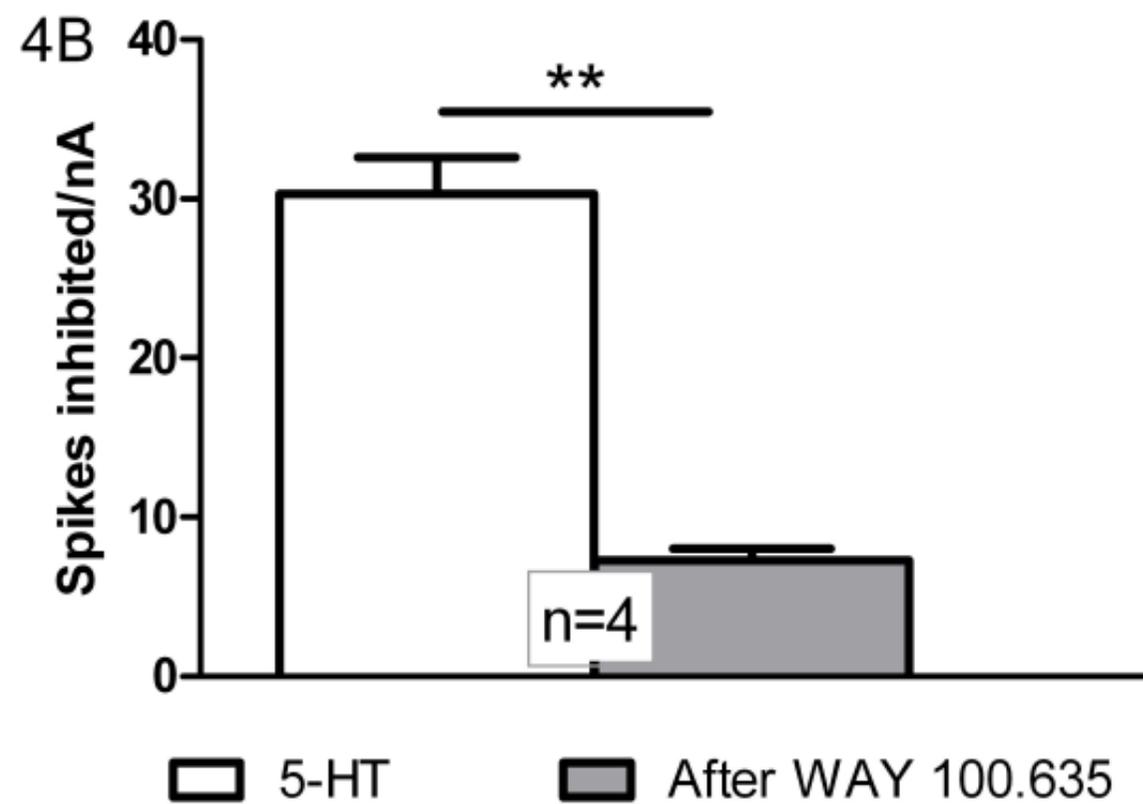


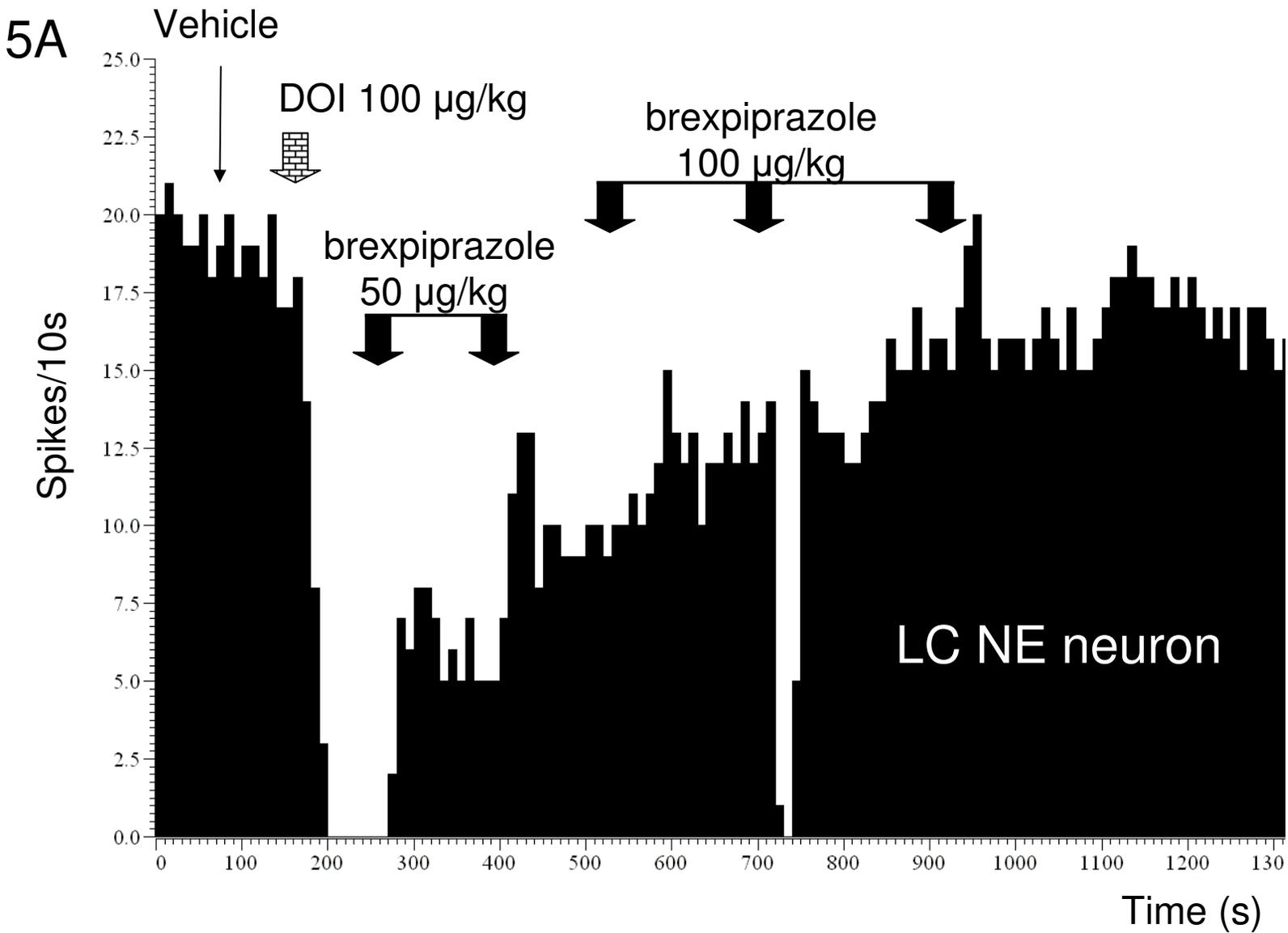




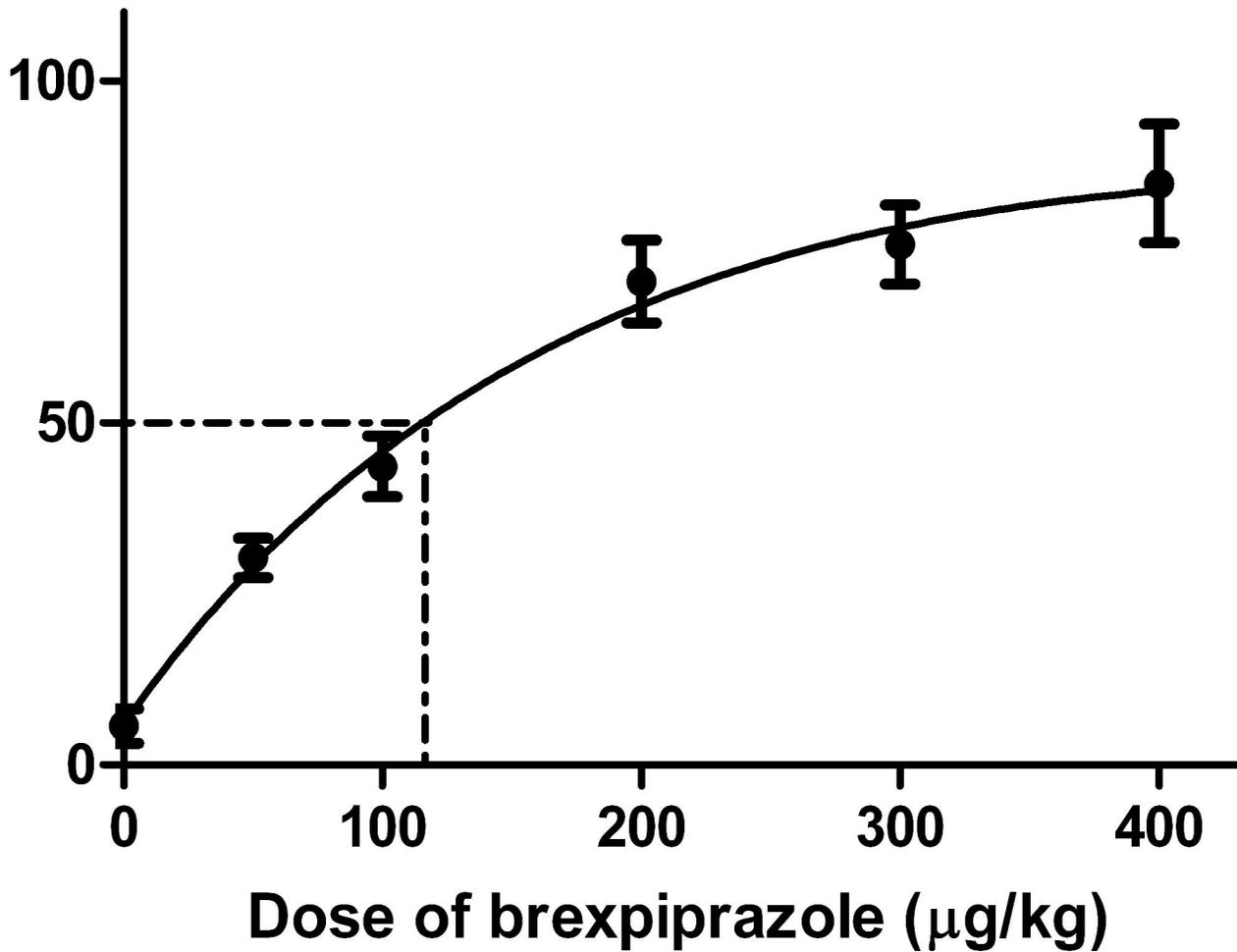
4A



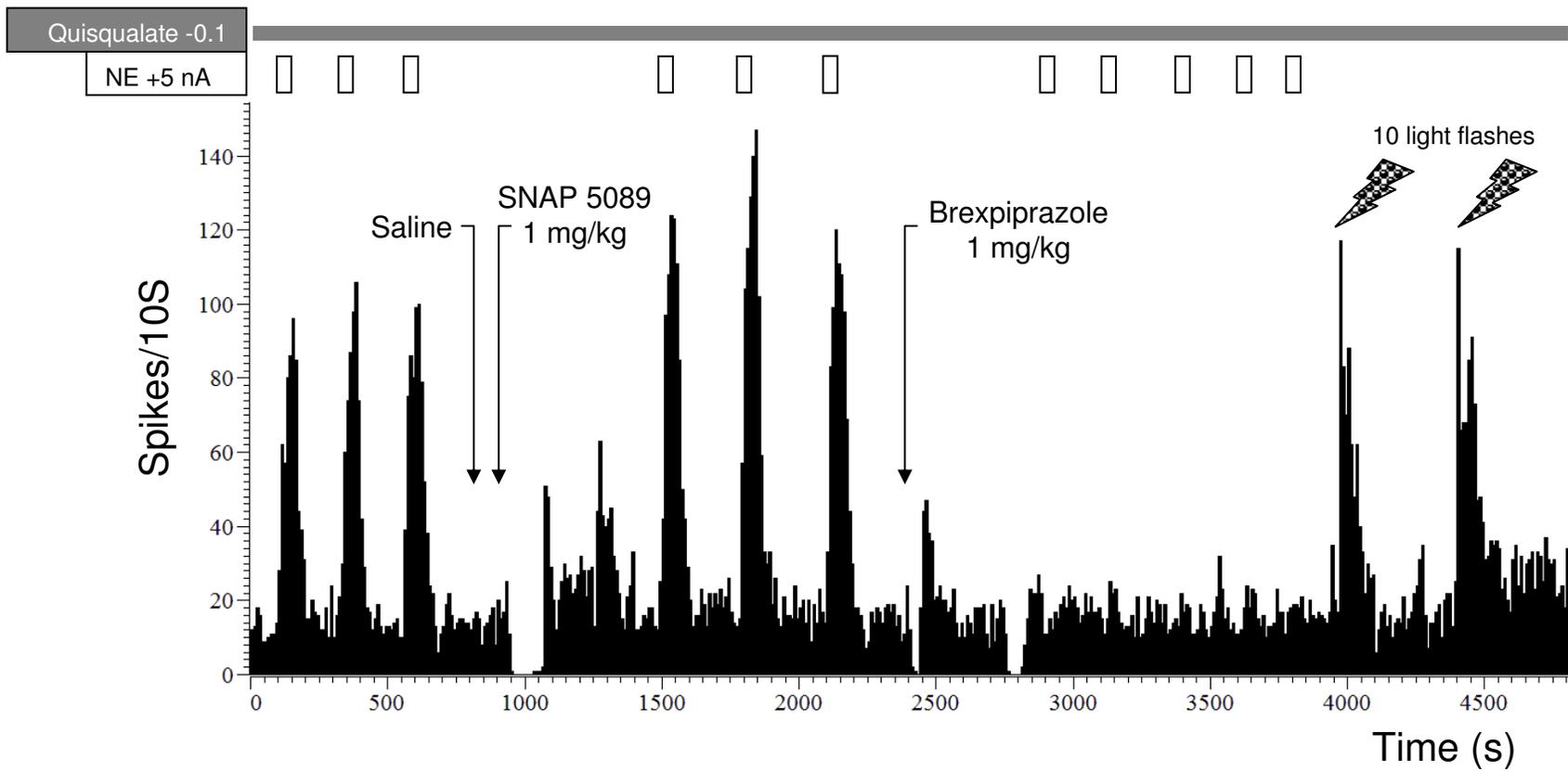


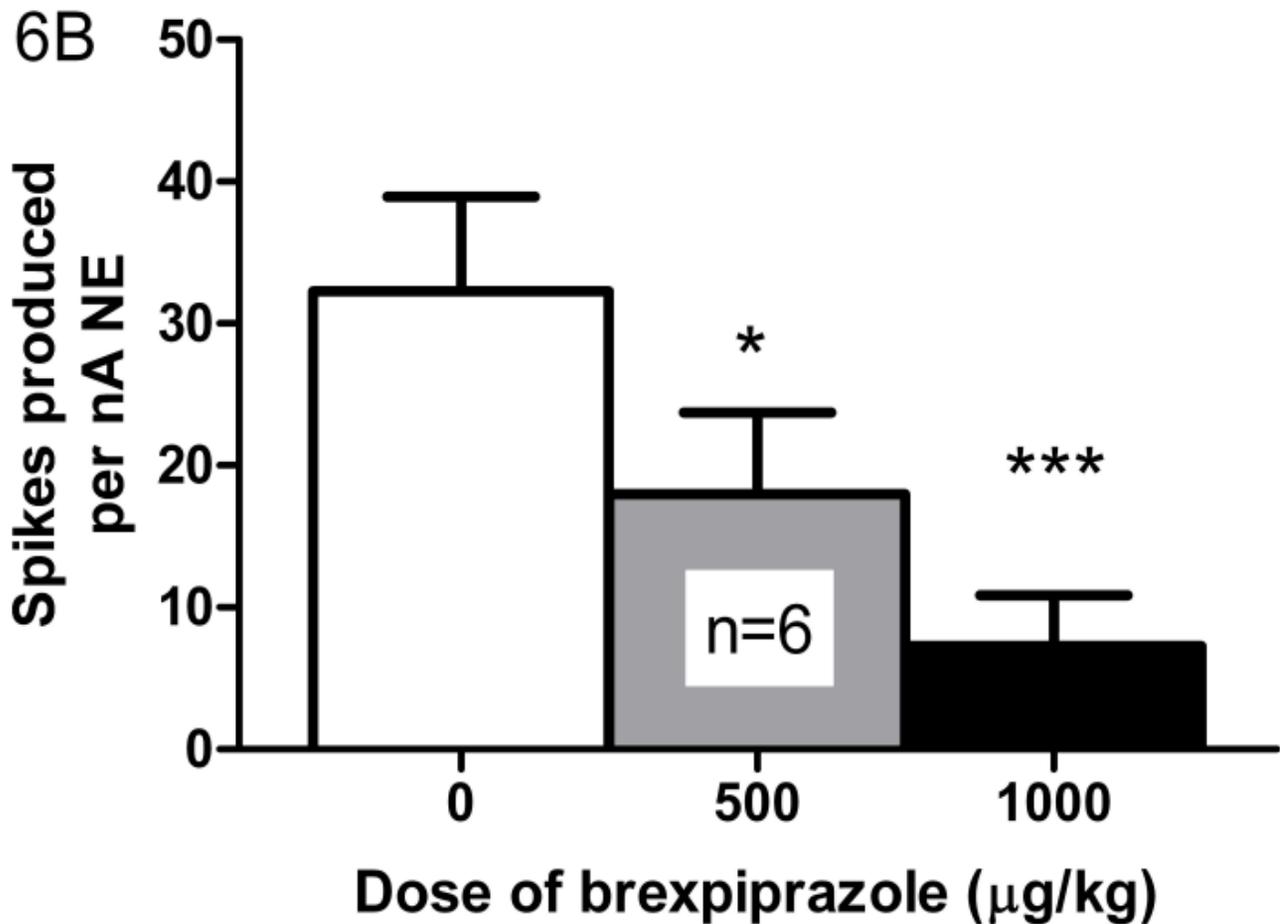


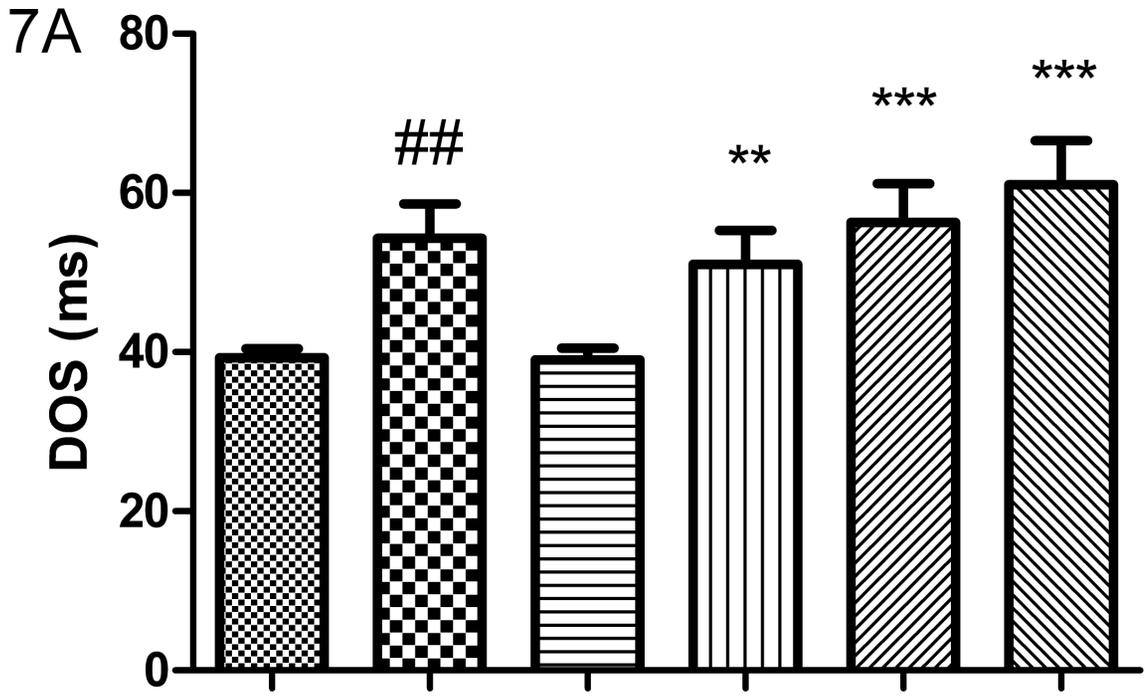
5B
% reversal of DOI
(relative to baseline)



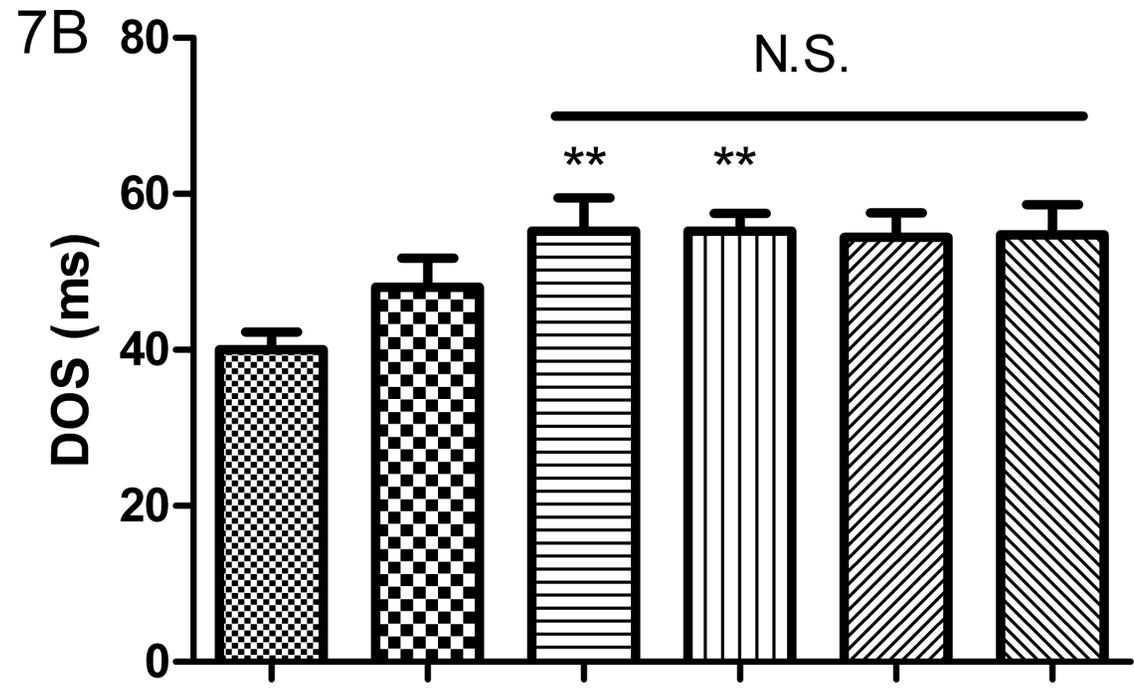
6A







Clo10	-	+	+	+	+	+
Clo400	-	-	+	+	+	+
Brex 500 (1)	-	-	-	+	+	+
Brex 500 (2)	-	-	-	-	+	+
Brex 500 (3)	-	-	-	-	-	+



Brex 500 (1)	-	+	+	+	+	+
Brex 500 (2)	-	-	+	+	+	+
Brex 500 (3)	-	-	-	+	+	+
Clo10	-	-	-	-	+	+
Clo400	-	-	-	-	-	+