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Effect of Vagus Nerve Stimulation on Serotonergic and Noradrenergic Transmission

by

Adrienne E. Dorr and Guy Debonnel

From the Department of Psychiatry,

McGill University, Montréal, Québec, Canada (A.E.D. and G.D.)

Effect of VNS on 5-HT and NE Transmission

Corresponding author: Guy Debonnel

McGill University, Department of Psychiatry, 1033 Pine Avenue West, Montréal, Québec, Canada H3A 1A1

Telephone: (514)-398-4650

Fax: (514) 398-4866

email: guy.debonnel@mcgill.ca

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Non-standard abbreviation list:

VNS, vagus nerve stimulations; 5-HT, 5-hydroxytryptamine; NE, norephinephrine; DRN, dorsal raphe nucleus; LC, locus coeruleus; ECT, electroconvulsive therapy; MAOI, monoamine oxidase inhibitor; SSRI, selective serotonin reuptake inhibitor; NST, nucleus of the solitary tract; i.p., intraperitoneal; LSD, lysergic acid diethylamide; S.E.M., standard error of the mean; $ED_{50,}$ effective dose 50%; HRSD, Hamilton rating scale for depression; NRI, norepinephrine reuptake inhibitor; NK, neurokinin/ substance P, WAY-100635, [2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride .

Abstract

Vagus nerve stimulation (VNS) is an anti-epileptic treatment, which has recently shown promise as an antidepressant. Yet, its antidepressant mechanisms of action are unknown. Serotonergic (5-HT) and noradrenergic (NE) systems are involved in the pathophysiology of depression and in the mechanisms of action of antidepressants. The present study analyzes 5-HT and NE neuronal firing rates in their brainstem nuclei: the dorsal raphe nucleus (DRN) and locus coeruleus (LC) respectively. The basal firing rates in the DRN and LC were significantly increased following long-term treatments with VNS. Following short-term VNS treatments, firing rates were significantly higher for LC (at one hour and 3 days). As changes in their firing rate may have been due to altered autoreceptor sensitivities, the responses of autoreceptors to the acute administration of their respective agonists were assessed. However, no significant difference was seen in the DRN. No significant differences in dose response curves for 5-HT_{1A} somatodendritic and α_2 adrenergic autoreceptors were noticed between long-term VNS and controls. VNS appears to have a novel mechanism of antidepressant action, enabling it's effectiveness in treatment resistant depression. LC firing rates significantly increase earlier than DRN basal firing. As the LC has an excitatory influence on DRN; it is possible that the increased DRN firing rate is secondary to an initial increased LC firing rate from VNS.

Introduction

The vagus nerve (cranial nerve 10) is generally thought of as a group of efferent parasympathetic fibers regulating autonomic functions. However, this nerve consists of 80 % afferent fibers (Foley and Dubois, 1937) from the head, neck, and body, leading up toward the cerebrum. These afferent fibers were targeted for therapeutic use by stimulation in medically and surgically intractable epilepsy, with several patients reporting a large decrease in seizure frequency (reviewed in Groves and Brown 2005).

In addition to decreasing seizure frequency, an improvement in mood was witnessed in patients with vagus nerve stimulation (VNS), even in those with little or no change in seizure frequency (Harden et al., 2000). Clinical studies with VNS in treatment resistant depression have generated positive results, with three month treatments inducing a 31% response rate, and a 15% remission rate, based on Hamilton Rating Scales for Depression scores. Response and remission rates increased further with longer VNS treatments (Nahas et al., 2005). Recently, Krahl and colleagues showed that VNS significantly reduced immobility in rats with the forced-swim test, an animal model used to predict antidepressant treatments efficacy. VNS treated animals' immobility levels were similar to those treated with desipramine and electroconvulsive therapy (ECT), two antidepressant treatments (Krahl et al., 2004). VNS therapy is now approved for treatment resistant depression in the European Union, USA, and Canada but its mechanisms of action in depression are unknown.

Serotonin and norepinephrine are involved in the pathophysiology of depression and mechanisms of action of antidepressant treatments (reviewed in Millan 2004). The major brain stem cell body nucleus for 5-HT is the dorsal raphe nucleus (DRN). Acute MAOI and SSRI treatments, both of which increase extracellular 5-HT levels, show an initial decrease in DRN 5-

HT firing rate in rats, while long-term treatments create a return to basal firing rates. This initial decrease in firing rate was due to negative feedback from inhibitory 5-HT_{1A} somatodendritic autoreceptors, and these receptors become desensitized during long-term exposure to endogenous or exogenous agonists (reviewed in Blier 2003).

NE reuptake inhibitors show beneficial effects with depression treatment (Massana et al., 1999; Montgomery et al., 2003), as do dual 5-HT and NE reuptake inhibitors (Stahl et al., 2002) and α_2 receptor antagonists (Nutt and Pinder, 1996). The locus coeruleus (LC) is the major NE brainstem nucleus that sends projections to many brain areas, including limbic structures. It receives innervation from the nucleus of the solitary tract (NST) (Van Bockstaele et al., 1999), a brainstem nucleus for vagus nerve afferents (Barraco, 1994). In addition, the DRN innervates the LC (Cedarbaum and Aghajanian, 1978; Leger and Descarries 1978) and conversely, the LC gives NE inputs to the DRN (Barbaran and Aghajanian 1980), creating ample opportunity for cross modulation. Antidepressant treatments affecting one system, either NE or 5-HT, can indirectly affect the other based on this cross modulation. For instance, NE acts on 5-HT DRN neurons to induce tonic activation mediated by excitatory α_1 adrenoreceptors (Baraban and Aghajanian, 1980), whereas SSRI treatments produce a net inhibition of LC firing rate after two weeks (Grant and Weiss, 2001; Szabo et al., 2000). NE neurons have inhibitory α_2 adrenergic autoreceptors on the soma and terminals that decrease NE firing rate or NE terminal release, respectively, in the presence of excess endogenous NE or α_2 agonists such as clonidine. Following a long-term treatment with the dual SSRI and 5-HT_{2A} antagonist YM992, NE neurons showed an increase in firing rate induced by a decrease in sensitivity of the α_2 adrenergic autoreceptor (Szabo and Blier, 2002). Interestingly, lesioning the LC in animal studies blocks the anti-epileptic action of VNS, suggesting the LC is involved in its effective circuitry (Krahl et al.,

1998). Recent electrophysiological evidence has come to light showing an increased discharge rate of LC NE neurons in response to acute VNS within a very short time frame of seconds to minutes post-stimulation (Groves et al., 2005). It is interesting to note, however, that no significant change in cerebrospinal fluid metabolites of NE and 5-HT was seen in VNS patients treated for three months compared to pre-treatment levels (Carpenter et al., 2004).

The present study examines the effect of VNS on 5-HT and NE systems. In vivo extracellular unitary recordings were obtained in anaesthetized rats to asses the basal firing rates of 5-HT and NE in the DRN and LC respectively. The sensitivity of the 5-HT_{1A} somatodendritic autoreceptor in the DRN and the α_2 somatodendritic autoreceptor in the LC was assessed by administration of their agonists, LSD and clonidine respectively.

Methods

Animals

All experiments were carried out on Sprague Dawley rats (Charles River, St. Constant, Quebec, Canada). Rats weighed a minimum of 275g for surgery and electrophysiological experiments, and were housed under standard laboratory conditions (12:12 light-dark cycle with access to food and water ad libitum). Body temperature was kept at 37 degrees during the surgery and electrophysiological experiments. The electrophysiological experiments were carried out by standard mounting of anaesthetized rats in a stereotaxic apparatus (David Kopf Instruments) and inserting a catheter into the lateral tail vein to for systemic intravenous administration of drugs. Rats were kept hydrated during the experiments by intravenous injection of 0.1 ml saline every half hour.

Surgery

Using sterile techniques, male Sprague Dawley rats were operated on under equithesine anesthetic, 1 ml i.p. per 300 gram rat (Chloral hydrate, 4.26%; sodium pentobarbitol, 0.96%). Supplemental doses of equithesine were given i.p., 0.1 ml at a time, when a nociceptive response to hind paw pinch was noticed, to ensure the rat was fully anaesthetized.

A horizontal incision was made, slightly above the clavicle. The left omohyoid and sternomastoid muscles were carefully separated, to allow a clear view of the left vagus nerve. In the rat, the vagus nerve lies lateral to the carotid artery. Bipolar leads were wrapped around the left carotid artery and vagus nerve, allowing close contact between the vagus nerve and the electrodes. This method of wrapping the electrodes around the carotid and the nerve has been performed before, with success (Handforth and Krahl, 2001). The electrodes were sutured to the underlying muscle, in order to keep the leads in place. The leads were tunneled subcutaneously, within and around the neck toward a horizontal dorsal incision made in the back. For stimulator placement, a pocket was made under the skin of the back and wiped with iodine. The tunneled leads were then connected to the stimulator, the stimulator is placed in the dorsal pocket, and antibiotics and fluid replacements are given to ease recovery. A sham control group went under the same surgical procedure with leads and a dummy 102 pulse stimulator in place. The lead impedance was checked to assure a tight connection between the nerve and coils, using the device diagnostic setting on the NCP handheld computer and programming wand. A recovery period of two days was allowed before the stimulator was turned on in treated rats. The stimulator was programmed with output similar to those used in humans (30 second on, 5 minute off; continuous cycle; 20 Hz, pulse width of 500 microseconds, 0.25 milliamps) (Sackeim et al., 2001). One hour, 24 hour, and three day stimulator groups were used for short-term VNS

treatment. Two week, three week, and three month treatment groups were used for long-term treatment groups. Sham treated groups ranged from 3 days post surgery to three months. No significant difference was noticed between different groups of shams, allowing all sham treatments to be grouped together into one sham group (data not shown).

Electrophysiological experiments: Dorsal raphe nucleus

Experiments were carried out with the VNS device in place; however, the device was inactivated for the duration of the experiment due to electrical interference. Rats were anaesthetized with Chloral Hydrate i.p at 400 mg/kg. Extracellular unitary *in-vivo* recordings were conducted with a pulled single barrel glass micropipette. The electrode was filled with 2% pontamine sky blue solution, 0.5 M, with an impedance range of 2-4 megaohms. For the DRN, a burr hole of 4 mm in diameter was drilled 1 mm anterior to lambda centered on the midline (Paxinos and Watson, 1986). The electrode was lowered to a depth within the range encompassing the DRN, after passing the Sylvius aqueduct until 1mm below it. 5-HT neurons in the DRN were identified by their special characteristics outlined by Aghajanian (1978). DRN 5-HT neurons were found starting at 5.0 mm below the dura surface. For averaging the firing rate for each group, at least 5 descents were made per rat with the electrode at 100 μm from previous descents. Each neuron was recorded for at least 60 seconds. Neuronal firing rates were calculated by adding each discharge per 10 second histogram (as obtained by the Spike2 program during recording) and dividing by length of time recoded in seconds. All neuronal firing rates for all rats in one group were added together and divided by number of neurons recorded per group. Lysergic acid diethylamide (LSD), an agonist for 5-HT_{1A} receptor was administered intravenously with the last 5-HT neuron, after at least 60 seconds of recording for baseline firing rate determination, to test the sensitivity of the 5-HT_{1A} somatodendritic autoreceptor. LSD was

used instead of 8-OH-DPAT as the latter 5-HT_{1A} receptor agonist can also inhibit 5-HT firing through a long feedback loop from the frontal cortex (Romero et al., 1994). The 5-HT_{1A} receptor antagonist WAY 100635 was administered intravenously, after at least 60 seconds of LSD inhibition, to bring the firing rate back to basal levels, confirming the inhibition was indeed due to the agonistic action at the somatodendritic 5-HT_{1A} receptor. Percent inhibition for each neuron was calculated by first determining the basal firing rate, then the firing rate after agonist administration, expressing the firing rate due to the agonist as a percentage of the basal firing rate. The percent inhibition was 100 minus this percentage.

Electrophysiological experiments: Locus Coeruleus

Similarly to the DRN experiments, LC experiments were done with the inactivated stimulator in place. Rats were anaesthetized with Chloral Hydrate i.p at 400 mg/kg. Extracellular unitary *in-vivo* recordings were conducted with a pulled single barrel glass micropipette. The electrode was filled with 2% pontamine sky blue solution, 0.5 M, with an impedance range of 2-4 megaohms. A burr hole was drilled 1.1 mm posterior to lambda and 1.1 mm lateral to the midline. The electrode was lowered at 0.7 mm interaural, and 1.1 to 1.4 mm lateral (Paxinos and Watson 1986).

Spontaneously active NE cells were identified by their regular 1-5 Hz firing rate, a positive action potential of long duration (0.8-1.2 ms), and their burst discharge due to nociceptive pinch of the contralateral hind paw (Aghajanian, 1978). For averaging the firing rate for each group, at least 5 descents were made per rat with the electrode at 100 μm from previous descents. Each neuron was recorded for at least 60 seconds. Neuronal firing rates were calculated by adding each discharge per 10 second histogram (as obtained by the Spike2 program during recording) and dividing by length of time recoded in seconds. All neuronal firing rates for all rats in one

group were added together and divided by number of neurons recorded per group. Clonidine, an agonist for α_2 adrenergic receptors, was administered intravenously with the last NE neuron, after at least 60 seconds of recording for baseline firing rate determination, to test the sensitivity of LC α_2 autoreceptors. The α_2 adrenergic receptor antagonist idazoxan was administered intravenously, after clonidine inhibition of at least 60 seconds, to bring LC firing rates back to basal levels confirming the inhibition was indeed due to the agonistic action at the α_2 adrenergic autoreceptor. Percent inhibition for each neuron was calculated by first determining the basal firing rate, then the firing rate after agonist administration, expressing the firing rate due to the agonist as a percentage of the basal firing rate. The percent inhibition was 100 minus this percentage.

Drugs and Materials

The following drugs were used: lysergic acid diethylamide (LSD) (Sandoz, Boucherville, QC); WAY 100635 (Sigma, Oakville, ON); Clonidine (Sigma); Idazoxan (Sigma). All drugs were dissolved in distilled water. Concentration ranges were chosen based on previous successful experiments in our laboratories. Cyberonics® (Houston, Texas, U.S.A.) provided the leads, 102 pulse stimulators and dummy stimulators.

Statistical Comparisons

Basal firing rates are expressed as standard error of the means (±S.E.M.). Non-parametric tests were used as firing rates were not normally distributed. Average firing rates for control and sham were compared using Mann-Whitney rank sum test. Average firing rates for control and treated groups were compared using the Kruskal Wallis one-way analysis of variance on ranks test. Post hoc tests were performed using the Dunn's multiple comparisons test to assess the difference between control and treated groups. The numbers of neurons encountered per track

were analyzed using the Kruskal-Wallis one-way analysis of variance on ranks test. Regression analysis was used to asses any difference between dose response curves for control and treated groups and to find the effective dose for inhibiting neuronal activity by 50% (ED_{50}).

Results

Effect of sham VNS surgery on basal DRN 5-HT and LC NE firing rates

The sham data from all time lengths of VNS treatments were pooled as no significant difference in firing rates were noticed between different sham VNS groups (data not shown). Comparing control and sham basal 5-HT firing rates in anaesthetized rats revealed no significant difference [Mann-Whitney rank sum test, T(174) = 6670.51, P=0.340] (figure 1a). Comparing control and sham basal NE firing rates in anaesthetized rats also showed no significant difference [Mann-Whitney rank sum test, T(139)=4158.50, P=0.111] (figure 1b). Therefore the surgery itself, following a sufficient recuperation time of two days, had no effect on DRN 5-HT or LC NE neuronal basal firing rates.

Effect of short-term and long-term VNS treatment on basal 5-HT firing rates

To determine the basal 5-HT firing rate changes due to short and long-term VNS, *in-vivo* extracellular neuronal activity was sampled from 5 to 6 rats per treatment group, with at least 10 neurons per rat. No significant difference for short-term VNS (one hour, one day, and 3 day treatments) was found when compared with control 5-HT firing rates [Kruskal-Wallis one-way ANOVA on ranks, H(3)=6.86; P=0.076] (figure 2a). However, there was a clear significant group effect for long-term VNS (14 day, 21 day, and 90 day treatments) when compared with control 5-HT firing rates [Kruskal-Wallis one-way ANOVA on ranks, H(3)=41.87, p<0.001].

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Post hoc analysis of long-term VNS groups revealed a statistically significant increase in firing rate for 14 day, 21 day and 90 day treatments versus control [Dunn's method, $p<0.05$] (figure 2b). 90 day treated rats had a mean 5-HT firing rate that was two-fold higher when compared with control (2.22 ± 0.42 Hz. for 90 day VNS, versus 1.11 ± 0.18 Hz for control), indicating a substantial increase in 5-HT firing due to long-term VNS. In addition, mean firing rates showed a trend to increase as length of stimulation increases (slope = 0.18) (figure 2c).

Assessment of $5HT_{1A}$ somatodendritic autoreceptor activity

LSD dose response curves for control and long-term VNS were constructed to compare the sensitivity of the $5\text{-}HT_{1A}$ somatodendritic autoreceptor. The sensitivity of the $5\text{-}HT_{1A}$ receptor was not assessed after short-term VNS treatment, as there was no significant difference in 5-HT firing rate for short-term VNS versus control. After recording a 5-HT neuron for 60 seconds (figure 3a), various doses of LSD (ranging from 1 μg/kg to 60 μg/kg) were administered intravenously. The ED_{50} value for control was 11.22 μg/kg while the ED_{50} for long-term VNS was 10.47 μg/kg (figure 3b). There is no significant difference between the dose response curves for control and long-term VNS ($r2_{control}=0.94$, $r2_{VNS}=0.95$; $F(12,2)=3.85$, $p=0.688$) (Figure 3c).

In all cases, the suppression of the firing activity induced by LSD was reversed by the subsequent administration of WAY 100635 (Figure 3A).

Effect of short-term and long-term VNS treatment on basal NE firing rates

To determine the basal NE firing rate changes due to short and long-term VNS, *in-vivo* extracellular neuronal activity was sampled in the LC from 5 to 6 rats per treatment group, with at least 10 neurons per rat. Following a short-term treatment with VNS, a significant main group effect was observed between VNS and controls [Kruskal-Wallis one-way ANOVA on ranks, $H(3)=9.60$, $P=0.022$] (figure 4a). Dunn's method for post hoc analysis revealed a significant

difference in the direction of higher NE firing rate when comparing acute ($p<0.05$) and 3 day ($p<0.05$) treatments with control; however one day (24 hour) treatment showed no significant difference.

Long-term VNS treatment showed a highly significant difference in firing rates when compared with controls ($H(3)=57.20$, $p<0.001$). Dunn's method for post hoc analysis revealed significant differences for 14 day, 21 day, and 90 day versus control ($p<0.05$) (figure 4b). NE firing rate proved to be over two fold higher for 90 day treatment when compared with control (4.75 ± 0.13 Hz for 90 day VNS, versus 2.23 ± 0.36 Hz for control) indicating a large facilitory effect on NE firing due to VNS. These data show a trend for a time dependant increase in firing rate (slope = 0.34) (figure 4c).

Assessment of α_2 adrenergic autoreceptor activity on NE soma

Clonidine dose response curves were compared between control and long-term VNS treatments to assess the sensitivity of the α_2 adrenergic autoreceptors on the LC NE cell bodies. After recording a LC neuron for 60 seconds (figure 5a), various doses of clonidine (ranging from 1 μg/kg to 60 μg/kg) were administered intravenously. The ED_{50s} were 7.50 μg/kg and 8.13 μg/kg for control and long-term VNS, respectively (figure 5b). Regression analysis revealed no significant difference between control and VNS dose response curves ($r2_{control}=0.47$, $r2_{VNS}=0.60$; $F(10,2)=0.27$ $p=0.769$) (figure 5c) suggesting there is no desensitization of the α_2 adrenergic autoreceptors.

Effect of VNS on number of neurons per track

The number of neurons encountered per descent into the brain was analyzed for both short-term and long-term VNS in the DRN and LC. Indeed if more neurons are encountered per track, it is possible a previously silent population of neurons have been activated during VNS,

thus affecting the average firing rates and our comparisons with control groups. For the DRN, a Kruskal-Wallis one-way ANOVA on ranks revealed no significant difference versus control for all VNS groups ($H(6)=5.03$; $p=0.541$)(figure 6a). Similarly for the LC, the Kruskal-Wallis one-way ANOVA on ranks revealed no significant difference versus control for all VNS groups ($H(6)=3.03$; $p=0.805$) (figure 6b). It therefore appears VNS does not activate a previously silent population of neurons in either brainstem nuclei of interest in this study.

Discussion

The main results of our studies are that VNS treatments induce a large time-dependant increase in basal neuronal firing in the brainstem nuclei for serotonin and norepinephrine: the dorsal raphe nucleus and locus coeruleus respectively. All classes of antidepressant treatments, including NRIs, ECT, and NK1 antagonists, act at least in part, by increasing 5-HT neurotransmission (reviewed in Haddjeri et al., 1995; Haddjeri and Blier, 2000; Santarelli et al., 2001), however NE probably also plays an important role in antidepressant effects, and NE is thought to be involved in the pathophysiology of depression (Delgado and Moreno, 2000). Long-term SSRIs increase 5-HT neurotransmission, while decreasing spontaneous NE activity (Grant and Weiss 2001; Szabo et al., 2000). Conversely, NRIs are efficient antidepressant treatments and seem to affect 5-HT neurotransmission (Massana et al., 1999; Montgomery et al., 2003) as do dual 5-HT and NE reuptake inhibitors (Stahl et al., 2002). However, to our knowledge VNS represents the first reported antidepressant treatment able to induce an increased firing activity of both serotonergic and noradrenergic neurons.

Indeed, endogenous or local applications of exogenous 5-HT, in the DRN, decrease the 5-HT firing rate through activation of 5-HT_{1A} somatodendritic autoreceptors, as do the 5-HT_{1A}

agonists LSD and 8-OH-DPAT (Aghajanian et al., 1968). Prolonged exposure to increased 5-HT levels or 5-HT agonists progressively desensitizes these receptors leading to a recovery of the 5-HT firing rate. Antidepressant treatments that increase 5-HT concentrations in the vicinity of 5-HT cell bodies, such as SSRIs and MAOIs, initially decrease DRN 5-HT neurons' firing activities, while long-term treatments return the firing rate back to baseline level due to 5-HT_{1A} autoreceptor desensitization while keeping the high synaptic availability of 5-HT (reviewed in Blier 2003). These treatments increase the efficacy of 5-HT neurotransmission via a change in the amount of 5-HT released and altered sensitivity of several subtypes of 5-HT receptors but they never increase the firing activity of 5-HT neurons above their baseline activity.

Our results indicate a large increase in 5-HT firing rate, which is normally associated with increased endogenous 5-HT release, which should activate the somatodendritic autoreceptor. Activation of 5-HT_{1A} somatodendritic autoreceptors would hyperpolarize the cell through $G_{i/o}$ proteins and decrease 5-HT firing rate (Innis and Aghajanian, 1987). We therefore tested the sensitivity of $5HT_{1A}$ somatodendritic autoreceptors after long-term VNS treatments .We chose LSD, as the $5HT_{1A}$ agonist 8-OH-DPAT seems to have a

stronger effect on postsynaptic $5HT_{1A}$ receptors (Romero et al., 1994). The postsynaptic 5-HT_{1A} receptors are involved in a long-loop inhibitory feedback mechanism (Artigas et al., 1996; Hajos et al., 1998), therefore using 8-OH-DPAT may cause DRN 5-HT inhibition which is unrelated to $5HT_{1A}$ somatodendritic autoreceptor inhibition. LSD cannot be considered as highly selective for the 5-HT_{1A} receptor. However, the fact that its suppression of the firing activity of 5-HT neurons was reversed by the selective WAY-100635 (figure 3A), suggests that this effect was indeed mediated by the $5HT_{1A}$ receptor. Despite the significantly increased DRN 5-HT firing rate with long-term VNS (figure 2b), no change in 5-HT_{1A} receptor sensitivity was observed (figure 3b,

3c). In contrast with the SSRIs' results published so far, despite an increase in the firing activity of DRN 5-HT neurons, the $5HT_{1A}$ somatodendritic autoreceptors were fully functional following long-term VNS. The increase in firing rate was therefore not due to a desensitization of these autoreceptors but rather to a distinct mechanism. One possible explanation for the absence of 5-HT_{1A} receptor desensitization could be that VNS increases the release of 5-HT in the terminal regions such as the hippocampus or the medial prefrontal cortex but not in the vicinity of DRN 5-HT cell bodies.

The next step was to examine the basal firing rate of LC NE neurons. We found a progressive and statistically significant increase in NE firing over long-term VNS treatments. This is in keeping with previous data showing that an intact NE system is necessary for effective VNS treatments, at least for epilepsy (Krahl et al., 1998), and that increased NE levels were present in LC terminal areas in VNS treated animals (Hassert et al., 2004). LC firing activity is normally controlled by the tonic activation of somatodendritic α_2 autoreceptors (Mongeau et al., 1997). Similarly to the 5-$HT_{1A,}$ a desensitization of α_2 autoreceptors has been shown to appear progressively, following prolonged exposure to increased NE concentrations. However, if most studies agree on a desensitization of terminal α_2 autoreceptors, there is still some controversy regarding the existence of such desensitization of LC somato-autoreceptors (For a review see Invernizzi and Garattini, 2004).

Thus, the sensitivity of NE α_2 adrenergic receptors on LC NE neurons was also investigated. Our results do not indicate any desensitization of the α_2 adrenergic autoreceptors on the NE cell bodies and thus must result in an increased firing rate through alternative methods. These results are in keeping with those obtained with antidepressants acting selectively on the noradrenergic system (Invernizzi and Garrattini, 2004). However, they differ from those obtained

with compounds acting on both serotonergic and noradrenergic neurotransmissions (Szabo and Blier, 2002). Since VNS is acting by modifying both neurotransmissions, one might expect to find a desensitization of α_2 autoreceptors. Again, similarly to what has been proposed for 5-HT_{1A} autoreceptors, the lack of α_2 desensitization observed following treatment with VNS (up to 90 days), even in the presence of important increases in the firing activity of 5-HT and NE neurons, could suggest that VNS increases NE release in the terminal region but not in the LC. For both neurotransmitters, this hypothesis remains to be verified in microdialysis studies.

A modification of terminal autoreceptors sensitivity (5-HT_{1B} for 5-HT neurons and α_2 for NE neurons) is unlikely to be involved in the observed firing activity changes as these autoreceptors regulate the amount of neurotransmitter released, however, our present experiments cannot rule-out the possibility of such a desensitization.

One possibility for explaining the increased firing rate of 5-HT and NE neurons following VNS treatments might have been the appearance of fast firing neurons, which would have been silent in control conditions and become activated only after the VNS treatments. However, the number of neurons found per track was identical in all treatment condition, suggesting that we were always recording from the same neuronal population.

The DRN and LC have wide projections within the cortex, acting on areas involved in mood, such as limbic structures (Levitt et al., 1984). In addition, the DRN and LC are highly interconnected, affecting each other's overall activity (Barbaran and Aghajanian, 1980; Cedarbaum and Aghajanian, 1978; Leger and Descarries 1978; Szabo and Blier, 2001), thereby playing a role in each other's downstream target areas. The LC but not the DRN, receives direct inputs from the NTS (Van Bockstaele et al., 1999) which itself receives afferences from the vagus nerve. As we observed a more rapid increase in LC NE firing rate than DRN 5-HT firing

rate (3 days instead of 14 days) it can be postulated that VNS may act initially and/or predominately on the LC, and indirectly with the DRN via afferents from the LC. Indeed, the DRN is tonically activated by the LC by way of excitatory α_1 receptors (Barbaran and Aghajanian, 1980). The activity of the 5-HT neurons may therefore be increased by enhanced noradrenergic tone on α_1 adrenergic receptors. Testing the sensitivity of these receptors on 5-HT DRN neurons in long-term VNS rats would be interesting, as the degree of activation of α_1 receptors may be more in VNS than in control, indicating an important role for the LC in VNS. The NTS directly and indirectly projects to several brain regions, and thus VNS may effect areas that in turn effect the LC or DRN, creating the increased firing rates. Two possibilities include the frontal cortex, which is involved in a long-loop inhibitory feedback on the DRN, and the hippocampus, a terminal area for DRN and LC projections. In the future, it would be interesting to examine the roles of these regions in VNS's effective circuitry.

In conclusion, our results show an overall increase in firing of brainstem cell body nuclei for 5-HT and NE neurons. Both of these neurotransmitters have been implicated in the pathophysiology of mood disorders, and a significant increase in the firing activities of these neurons provides reason for VNS' antidepressant effects. The firing rates of both 5-HT and NE neurons increase as length of treatment increases. This mirrors the trend noticed in clinical VNS studies where mean HRSD scores tend to decrease further over time, indicating clinical improvement (Nahas et al., 2005).

The highly significant dual increase in 5-HT and NE firing rates indicates a substantial increase in neurotransmission for both neurotransmitters, a phenomenon that is not seen with all antidepressant therapies. In addition, the increase in 5-HT neuronal firing does not involve a decreased sensitivity for 5-HT_{1A} receptors, indicating a possible new mechanism of action as an

antidepressant. This may explain why VNS has proved beneficial with treatment resistant depressive patients, where other antidepressant treatments were ineffective (Sackeim et al., 2001; Nahas et al., 2005).

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Portions of the data included in this manuscript were presented at various conferences:

G. Debonnel and A.E. Dorr. Effects of Vagus Nerve Stimulation (VNS) on dorsal raphe serotonergic neurons: An electrophysiological study in the rat. In American College of Neuropsychopharmacology (ACNP). Peurto Rico, 2003.

A.E.Dorr and G. Debonnel. Electrophysiological investigation into the antidepressant effects of vagus nerve stimulation (VNS) Collegium Internationale Neuro-Psychopharmacologicum (CINP). Paris, France, June 2004

A.E. Dorr, G.C. Lucas and G. Debonnel. In-Vivo Effects of Chronic Vagus Nerve Stimulation (VNS) on Serotonin and Noradrenergic Systems. Society for Neuroscience. San Diego, October 2004.

Figure Legends

Fig. 1: Histograms representing the mean (+ or - S.E.M.) spontaneous firing activity. Numbers at bottom of columns represent number of neurons tested. (A) DRN 5-HT neuronal firing rates in control rats and sham surgery rats with the dummy VNS device in place. Five rats were tested per group. Mann-Whitney Rank sum test revealed no statistical significance ($p=0.340$). (B) LC NE neuronal firing rates in control rats and sham surgery rats with the dummy device in place. Five rats were tested per group. Mann-Whitney Rank sum test revealed no statistical significance ($p=0.111$).

Fig. 2: Histograms representing means (+ or - S.E.M.) spontaneous firing activity of DRN 5-HT neurons. The numbers at the bottom of the columns represent the number of neurons tested. (A) Average firing rates for control and short-term VNS groups. Five to six rats were used per group. Kruskal-Wallis one-way ANOVA revealed no significant difference between groups. (B) Average firing rates for control and long-term VNS groups. Five to six rats were used per group. Kruskal-Wallis one-way ANOVA revealed significant differences for all long-term VNS groups versus control ($p<0.05$). (C) Average firing rates showing a trend for a time dependant increase in firing rate.

Fig. 3: (A) Response of DRN 5-HT neurons to the $5HT_{1A}$ agonist LSD and the effect on firing activity. Histograms of one DRN 5-HT neuron in a control (left) and two week VNS treated rat (right). Both show an inhibitory response to a single dose of LSD (10 μg/kg) and antagonist reversal by WAY 100635 (100 μg/kg). (B) Dose response curves for LSD of control

(ED_{50}=11.22 μg/kg) and long-term VNS treatment groups (ED_{50}=10.47 μg/kg). (C) Regression analysis for control and long-term VNS dose response curves. Long-term VNS has a very similar curve as control and is not significantly different (p=0.688)

Fig. 4: Histograms representing means (+ or - S.E.M.) spontaneous firing activity of LC NE neurons. The numbers at the bottom of the columns represent number of neurons tested. (A) Average NE neuronal firing rate in control and short-term VNS. Five to six rats were used per group. Kruskal-Wallis one-way ANOVA revealed a significant difference between one hour and three day treatment when compared to control ($p<0.05$) but not when comparing 24 hour treatment with control. (B) Average NE neuronal firing rate in control and long-term VNS. Five to six rats were used per group. Kruskal-Wallis one-way ANOVA revealed a significant difference for all long-term VNS groups versus control ($p<0.05$). (C) Average firing rates showing a trend for a time dependant increase in firing rate.

Fig. 5: (A) Response of LC NE neurons to the a_2 agonist clonidine and the effect on firing activity. Histograms of one LC NE neuron in a control (left) and two week VNS treated rat (right). Both show an inhibitory response to a single dose of clonidine (20 μg/kg) and antagonist reversal by idazoxan (1mg/kg). (B) Dose response curves for clonidine of control (ED_{50}=7.50 μg/kg) and long-term VNS treatment groups (ED_{50}=8.13 μg/kg). (C) Regression analysis for control and long-term VNS dose response curves . Long-term VNS shows no significant difference from control (p=0.769).

Fig. 6: Histograms representing the mean (+ or - S.E.M.) neurons encountered per track. The numbers at the bottom of the columns represent number of tracks done per group. (A) Average

number of DRN 5-HT neurons per track. ANOVA on ranks reveals no significant difference versus control ($p=0.541$). (B) Average number of LC NE neurons per track. ANOVA on ranks reveals no significant difference.

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Figure 1

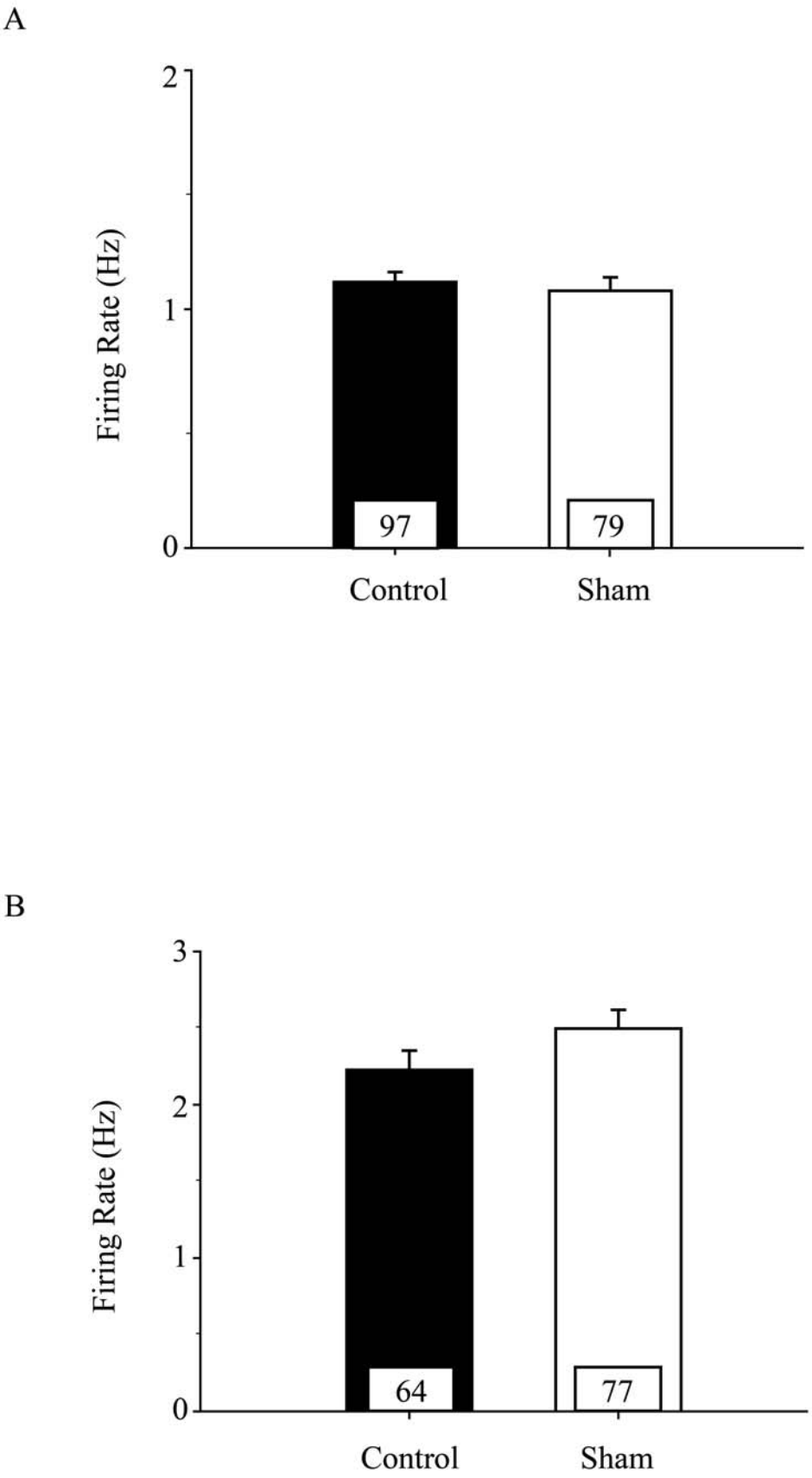

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Figure 2

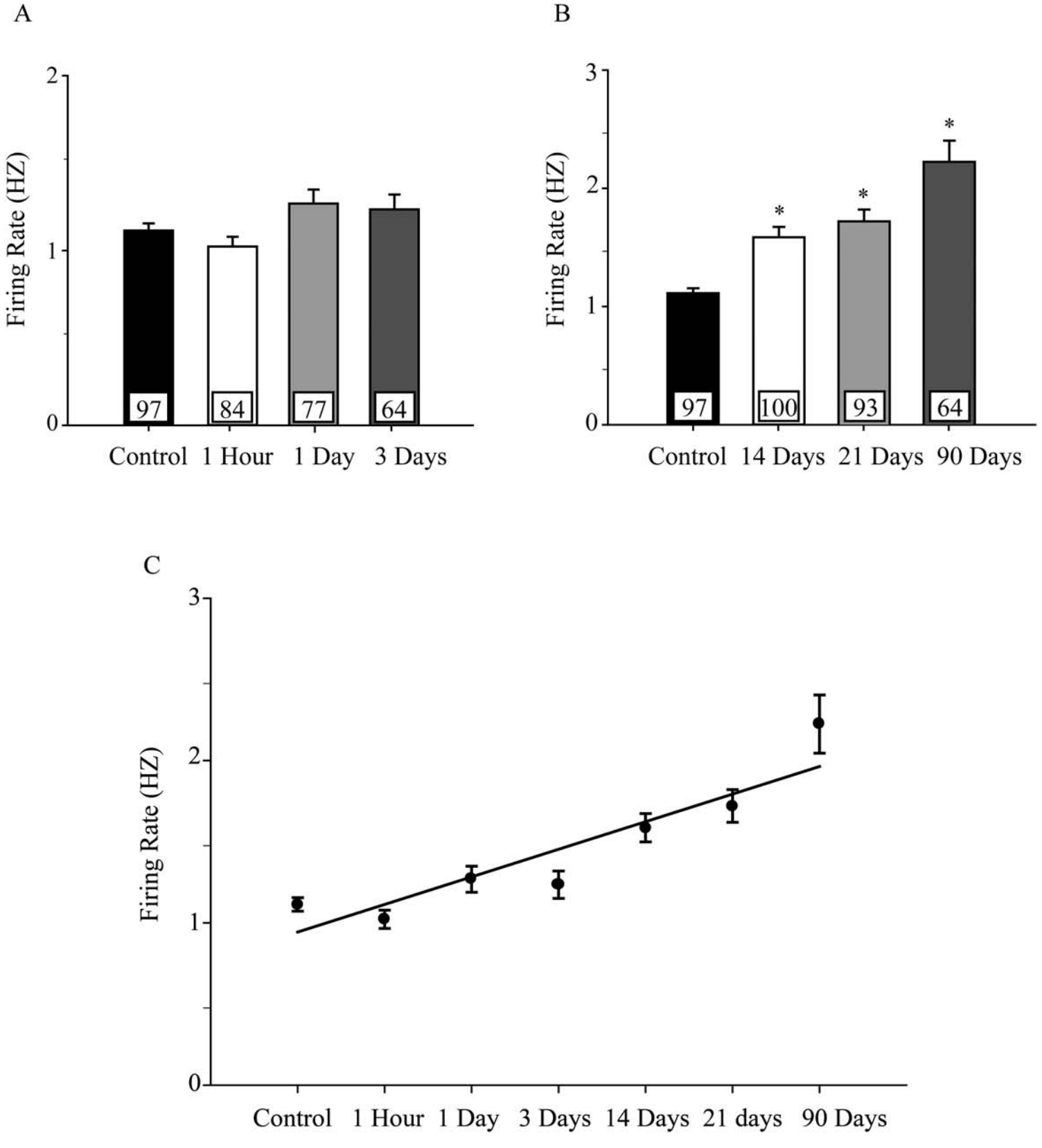

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Figure 3

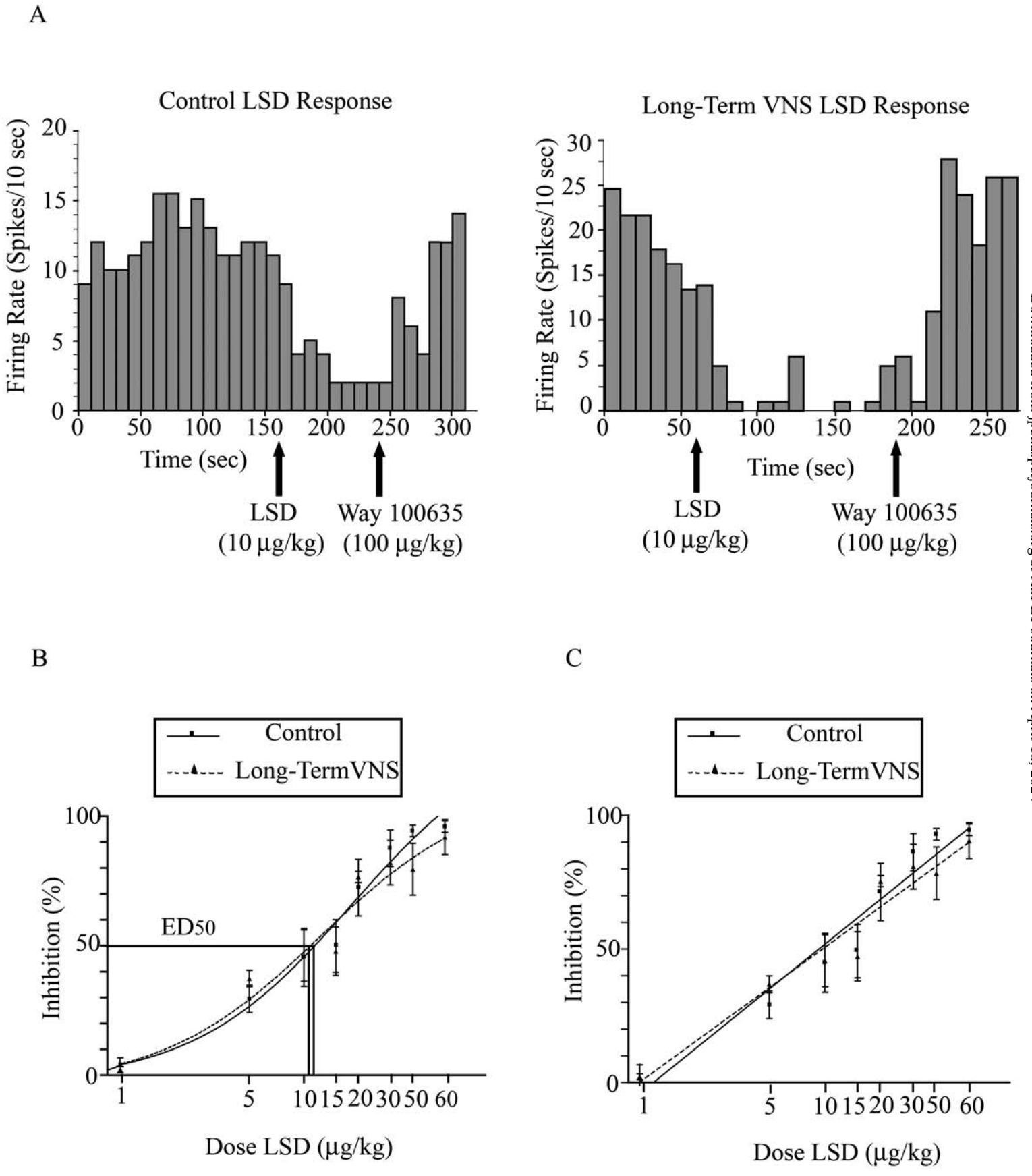

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Figure 4

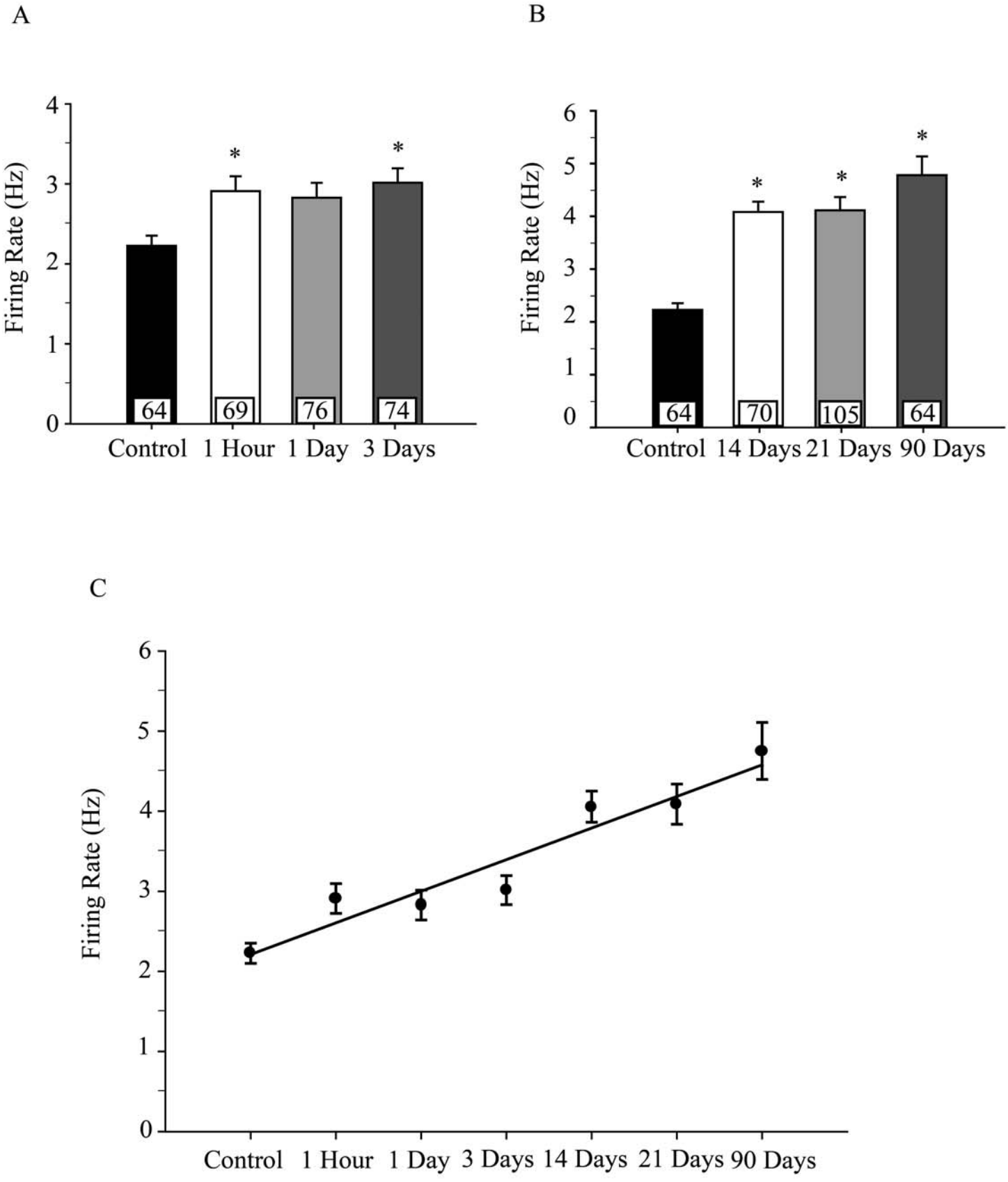

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Figure 5

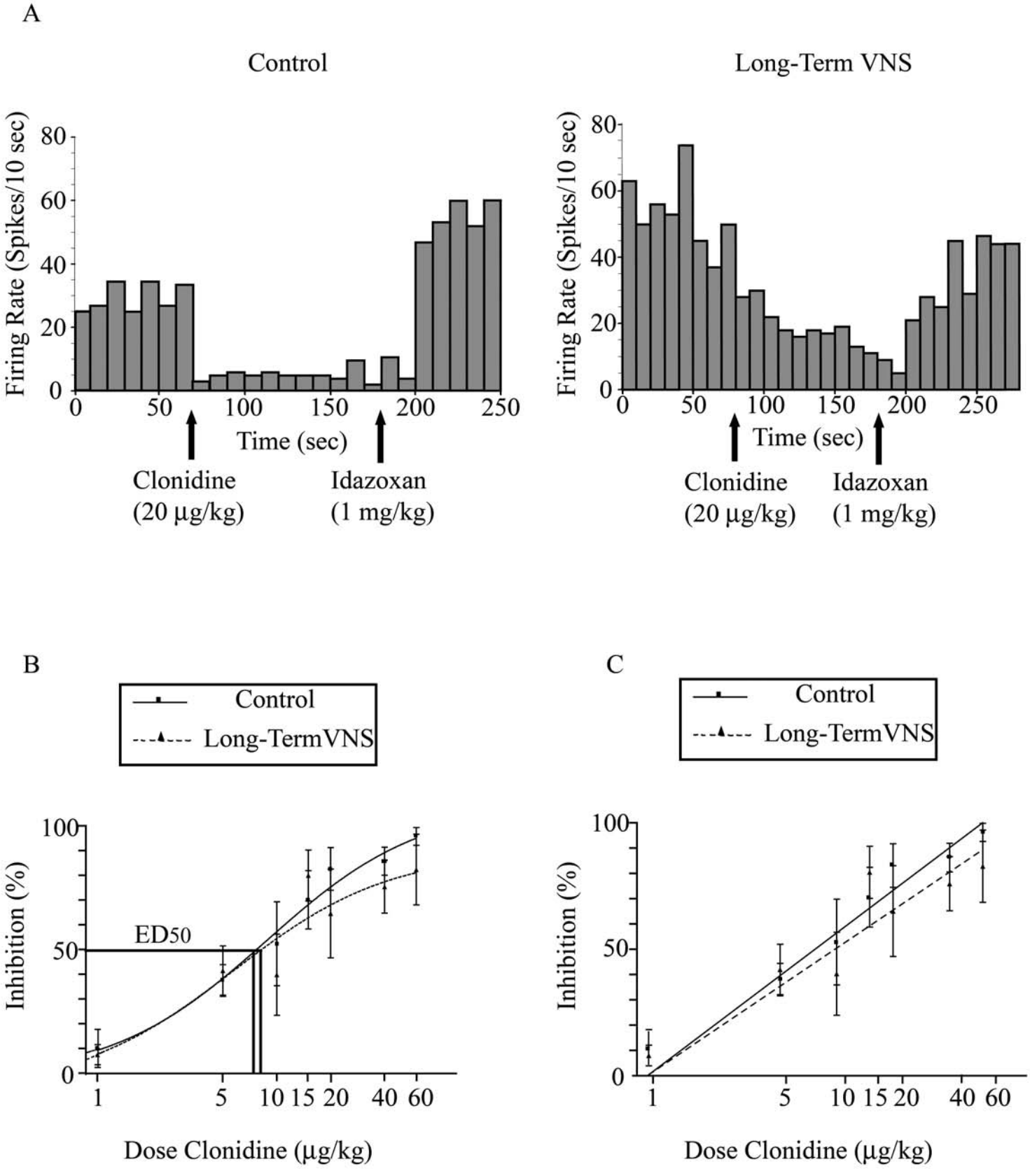

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Figure 6