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Diazepam Inhibits Electrically Evoked and Tonic Dopamine Release in the Nucleus Accumbens and Reverses the Effect of Amphetamine

Alexander Gomez-A,[†] Amanda M. Fiorenza,[†] Suelen L. Boschen,[†,‡] Adam H. Sugi,[†] Danielle Beckman,[‡] Sergio T. Ferreira,[‡] Kendall Lee,[§] Charles D. Blaha,[§] and Claudio Da Cunha*[,†]

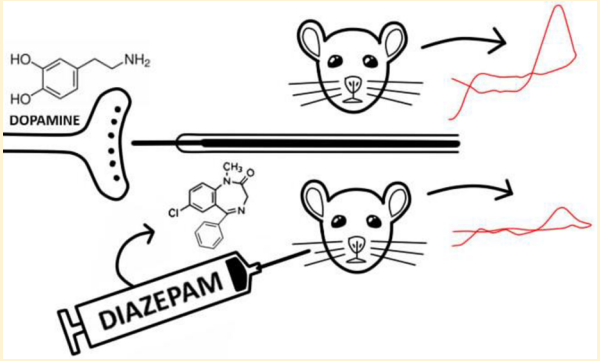
[†]Departamento de Farmacologia, Universidade Federal do Paraná, Curitiba 81.530-980, PR, Brazil

[‡]Institute of Biophysics Carlos Chagas Filho and Institute of Medical Biochemistry Leopoldo de Meis, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

[§]Department of Neurologic Surgery, Mayo Clinic, Rochester, Minnesota 55905, United States

Supporting Information

ABSTRACT: Diazepam is a benzodiazepine receptor agonist with anxiolytic and addictive properties. Although most drugs of abuse increase the level of release of dopamine in the nucleus accumbens, here we show that diazepam not only causes the opposite effect but also prevents amphetamine from enhancing dopamine release. We used 20 min sampling *in vivo* microdialysis and subsecond fast-scan cyclic voltammetry recordings at carbon-fiber microelectrodes to show that diazepam caused a dose-dependent decrease in the level of tonic and electrically evoked dopamine release in the nucleus accumbens of urethane-anesthetized adult male Swiss mice. In fast-scan cyclic voltammetry assays, dopamine release was evoked by electrical stimulation of the ventral tegmental area. We observed that 2 and 3 mg of diazepam/kg reduced the level of electrically evoked dopamine release, and this effect was reversed by administration of the benzodiazepine receptor antagonist flumazenil in doses of 2.5 and 5 mg/kg, respectively. No significant effects on measures of dopamine re-uptake were observed. Cyclic voltammetry experiments further showed that amphetamine (5 mg/kg, intraperitoneally) caused a significant increase in the level of dopamine release and in the half-life for dopamine re-uptake. Diazepam (2 mg/kg) significantly weakened the effect of amphetamine on dopamine release without affecting dopamine re-uptake. These results suggest that the pharmacological effects of benzodiazepines have a dopaminergic component. In addition, our findings challenge the classic view that all drugs of abuse cause dopamine release in the nucleus accumbens and suggest that benzodiazepines could be useful in the treatment of addiction to other drugs that increase the level of dopamine release, such as cocaine, amphetamines, and nicotine.



KEYWORDS: *Dopaminergic neurons, Electrochemistry, Ventral tegmental area, Nucleus accumbens core, GABA, Anxiolytic, Anticonvulsant*

1. INTRODUCTION

Benzodiazepines (BZs) are widely used as anxiolytics, sedative-hypnotics, anticonvulsants, anesthetics, and muscle-relaxants.[1] These effects of the BZs are achieved by binding to a specific site in type A γ-aminobutyric acid ($GABA_A$) receptors.[2] BZs are also used for recreational purposes and can lead to addiction in vulnerable people.[3] It has been proposed that most addictive drugs share the common property of increasing extracellular dopamine concentrations in the nucleus accumbens (NAc).[4,5] There is solid evidence supporting this hypothesis for cocaine,[6,7] amphetamines,[8,9] and nicotine.[10,11] However, although influential studies and review papers propose that this is also true for BZs,[12,13] direct evidence that BZs cause dopamine release is lacking. A microdialysis study by Bentue-Ferrer et al.[14] reported that small doses of the BZs alprazolam and lorazepam caused a modest increase in tonic dopamine levels in the striatum of rats. However, several other microdialysis studies reported that systemic and intrastriatal administration of the BZs diazepam, midazolam, flunitrazepam, and imidazenil caused a decrease in tonic levels of dopamine in the rat frontal cortex and NAc; this effect was also prevented by the BZ receptor antagonist flumazenil.[15−21] Flumazenil also increased the level of tonic dopamine release in the NAc of rats chronically treated with diazepam or imidazenil.[19,20] Those studies that showed that BZs increase levels of dopamine release were conducted using *in vivo* microdialysis, which inherently can detect only relatively slow changes in tonic (basal) extracellular concentrations of dopamine. As such, these studies do not reflect the direct effect of the BZs on phasic

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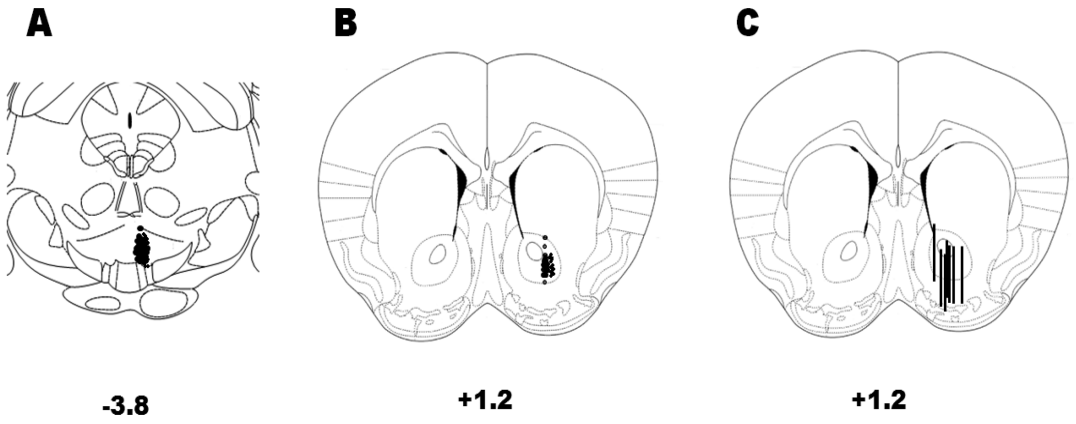

Figure 1. Schematic drawing of coronal sections showing the locations of the electrical stimulating electrodes, carbon-fiber recording microelectrodes, and microdialysis probes according to the mouse brain atlas of Paxinos and Franklin.[47] The approximate AP distances (millimeters) from the bregma are indicated. (A) Site of the stimulating electrodes in the mouse ventral tegmental area. (B) Site of the recording electrodes in the mouse nucleus accumbens. (C) Site of the microdialysis probes in the nucleus accumbens.

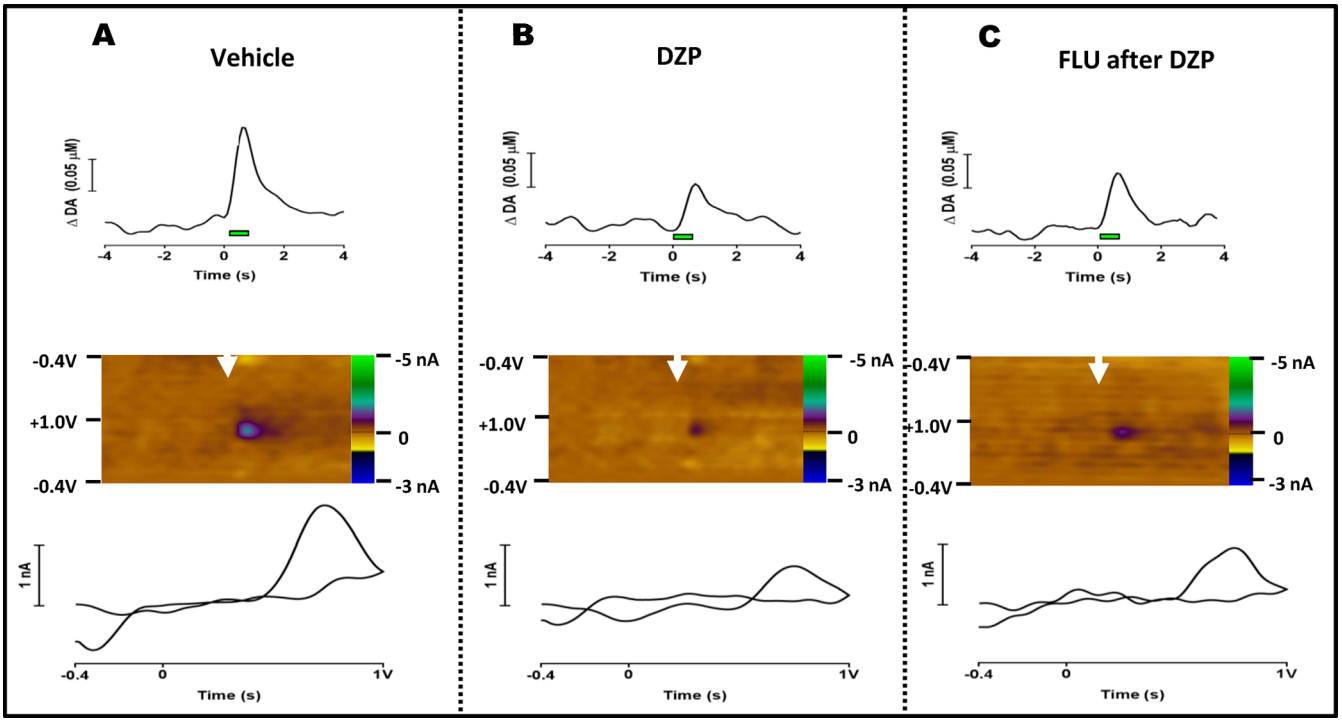

Figure 2. Individual examples illustrating the effect of the intraperitoneal administration of 2 mg of diazepam/kg and 2.5 mg of flumazenil/kg on electrically evoked dopamine release in the nucleus accumbens evoked by electrical stimulation of the ventral tegmental area in urethane-anesthetized mice. Dopamine was monitored by background-subtracted FSCV at carbon-fiber microelectrodes. From top to bottom are shown concentration vs time traces, pseudocolor plots, and cyclic voltammograms taken at the peak oxidation current, respectively. Δ[DA] represents the variation in dopamine concentration. The arrow indicates when the electrical stimulation started, and the green bar indicates the duration of the electrical stimulation.

dopamine release, which results from burst firing activity that occurs on a subsecond time scale.[12]

Here we used subsecond sampling fast-scan cyclic voltammetry (FSCV) recorded with carbon-fiber microelectrodes to show that the BZ diazepam causes a dose-dependent decrease in the level of dopamine release in the NAc evoked by electrical stimulation of the ventral tegmental area (VTA). The VTA and NAc are brain nuclei rich in dopaminergic neurons and dopamine terminals, respectively.[22] We also confirmed this finding using microdialysis and further demonstrated that BZs interact with other drugs of abuse, such as amphetamine, to reverse their facilitator effects on dopamine release.

2. RESULTS AND DISCUSSION

2.1. Histology and FSCV Validation. All stimulating electrodes were located in the VTA (Figure 1A), and all recording electrodes were located in the NAc core (Figure 1B). All microdialysis probes were also located in the NAc (Figure 1C). Thionin-stained midbrain slices showed no signs of electrolytic lesion near the stimulation electrode (Supplementary Figure 1).

The voltammograms obtained *in vivo* (Figure 2) have oxidation and reduction current peaks in the same range as those observed in the flow cell calibration of the electrodes.

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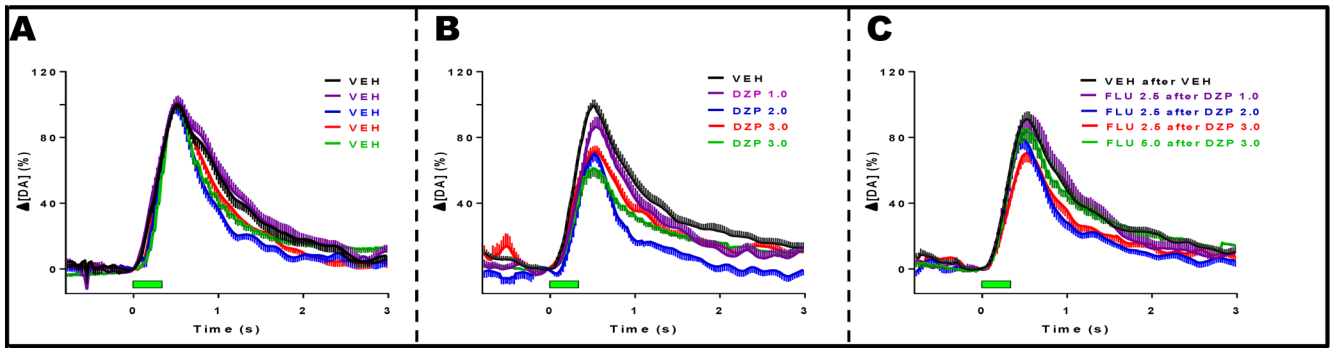

Figure 3. Effect of diazepam and flumazenil on dopamine release and re-uptake. Mice were anesthetized with urethane, and electrically evoked release of dopamine in the nucleus accumbens evoked by electrical stimulation of the ventral tegmental area was monitored by background-subtracted FSCV at carbon-fiber microelectrodes. The green bar indicates the duration of the electrical stimulation. Changes in dopamine concentration (Δ[DA]) were measured: under baseline conditions (not shown), (A) after the administration of vehicle (VEH), (B) after the administration of diazepam (DZP) at doses of 1, 2, and 3 mg/kg, and (C) after the administration of flumazenil (FLU) at doses of 2.5 and 5 mg/kg. Data are expressed as the mean ± SEM (standard error of the mean) percent of the baseline.

Cyclic voltammograms and corresponding pseudocolor plots show clear dopamine oxidation peaks occurring between 0.65 and 0.79 V and a reduction peak between −0.20 and −0.36 V (vs the $Ag/AgCl^{-}$ reference electrode) with relatively low currents at other potentials. On average, the oxidation of 0.5 μM dopamine at an electrode with a 100 μm exposed tip caused a current of 4.8 ± 0.4 nA. The average length of the carbon-fiber electrodes used was 91 ± 12 μm. Background noise, defined as the variance of oxidation current measured between −65 and 5 s before the electrical stimulation, was 0.04 ± 0.02 nA and did not vary significantly among groups [$F(4,23) = 0.73$; $p = 0.58$]. Data from 35 of 564 electrically evoked dopamine signals were discarded because they overlapped with obvious stimulation artifacts. The remaining data were averaged by animal, and the composite data of all animals were used for statistical tests.

As shown in Supplementary Figure 2, intraperitoneal (ip) administration of the dopamine transport inhibitor (DAT) blocker nomifensine (20 mg/kg) did not change the potentials at which dopamine oxidizes or reduces (Supplementary Figure 2A,C). In addition, nomifensine administration caused a significant time-dependent increase in extracellular dopamine concentration [calculated from the height of the oxidation peak (Supplementary Figure 2D)], an increase in the decay half-life ($T_{1/2}$) (Supplementary Figure 2E), and a decrease in the decay rate constant (K) (Supplementary Figure 2F). These findings support the use of the FSCV oxidation current as a measure of variation of extracellular dopamine release and re-uptake in this study.[22]

2.2. Dose-Dependent Inhibition of Electrically Evoked Dopamine Release by Diazepam. Individual examples of FSCV measurements of the effects of diazepam and flumazenil on dopamine release in the NAc evoked by electrical stimulation of the VTA are demonstrated in Figure 2. The selected examples are representative of the recordings obtained from all animals and show that 2 mg of diazepam/kg reduced the level of VTA stimulation-evoked dopamine release. Figure 3 shows the temporal variation of the dopamine concentration, and Figure 4 shows the average height of the peaks in response to VTA stimulation. Average $T_{1/2}$ and K (decay constant) values are shown in Table S1.

As shown in Figure 4A, the height of the dopamine peaks (Δ[DA]) in the control group did not significantly decrease during the 1 h duration of the experiment. This control is important to show that any observed decrease in the peak heights in the groups treated with diazepam was not caused by electrode desensitization. Panels A and B of Figure 4 show that diazepam caused a dose-dependent and significant reduction in the size of the peaks. A repeated-measures two-way analysis of variance (ANOVA) showed a nonsignificant drug treatment factor [$F(4,24) = 2.25$; $p = 0.09$], a significant time effect [$F(3,72) = 17.34$; $p < 0.001$], and a significant interaction between these factors [$F(12,72) = 1.89$; $p < 0.05$]. Post hoc tests showed that diazepam at 2 and 3 mg/kg, but not at 1 mg/kg, caused a significant reduction in the dopamine peak height (Figure 4B). Post hoc tests also showed that 2.5 mg of flumazenil/kg reversed the effect of diazepam at 2 mg/kg, but not at 3 mg/kg (Figure 4A,C). However, flumazenil at a higher dose (5 mg/kg) reversed the effect of 3 mg of diazepam/kg (Figure 4A,C). Supplementary Figures 3 and 4 show that 2.5 mg of flumazenil/kg also prevented the effects of 2 mg of diazepam/kg when the administration occurred before that of diazepam.

The shape of the dopamine peaks, notably the descending trace corresponding to re-uptake of the neurotransmitter, suggests that diazepam did not affect dopamine re-uptake (Figure 3). This was confirmed by analysis of $T_{1/2}$ and the corresponding decrease in K. While nomifensine caused a significant and time-dependent increase in $T_{1/2}$ and corresponding decrease in K (Supplementary Figure 2), these factors were not significantly affected by any dose of diazepam (Table S1).

2.3. Diazepam Inhibits Tonic Release of Dopamine. Figure 5 shows that the tonic concentrations of dopamine measured in the microdialysis samples also were significantly reduced by the ip administration of 2 mg of diazepam/kg. Figure 5 also shows that this effect was reversed by the ip administration of 2.5 mg of flumazenil/kg. A repeated-measures two-way ANOVA showed a nonsignificant drug treatment factor [$F(1,5) = 1.40$; $p = 0.29$], a significant time effect [$F(10,50) = 2.62$; $p < 0.05$], and a significant interaction between these factors [$F(1,50) = 4.50$; $p < 0.001$]. Bonferroni's post hoc tests showed that diazepam caused a significant reduction in the tonic dopamine concentration. These post hoc tests also showed that 2.5 mg of flumazenil/kg significantly reversed this reduction.

2.4. Diazepam Reverses Increases in Electrically Evoked Dopamine Release Induced by Amphetamine.

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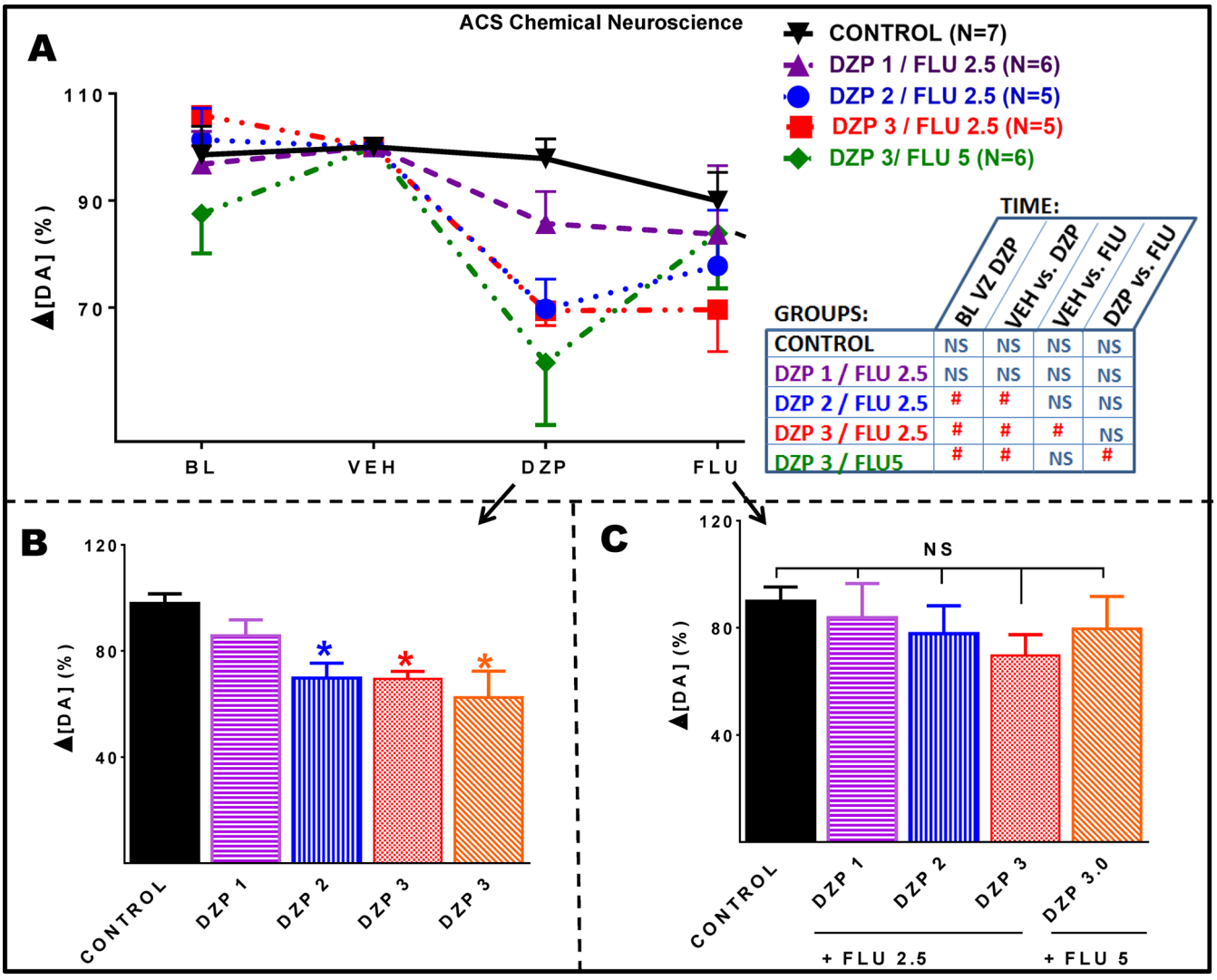

Figure 4. Effect of diazepam and flumazenil on dopamine release. Mice were anesthetized with urethane, and electrically evoked release of dopamine in the nucleus accumbens evoked by the electrical stimulation of the ventral tegmental area was monitored by background-subtracted FSCV at carbon-fiber microelectrodes. Dopamine release (Δ[DA]) was estimated by the heights of the peaks shown in Figure 3. Changes in dopamine concentration (Δ[DA]) were measured: under baseline conditions (BL), after the administration of vehicle (VEH), after the administration of diazepam (DZP) at doses of 1, 2, and 3 mg/kg, and after the administration of 2.5 mg of flumazenil/kg (FLU 2.5) or 5 mg of flumazenil/kg (FLU 5). The mice of the control group received vehicle when the mice of the other groups received diazepam and flumazenil. Temporal variations are shown in panel A, and comparisons between groups are shown in panels B and C. Data are expressed as the mean ± SEM percent of the vehicle. NS, not significant; *$p < 0.05$ compared to the control group at the same time; #$p < 0.05$ compared to other time (Bonferroni test after two-way analysis of variance).

Representative examples of FSCV measurements showing that diazepam reverses the effect of amphetamine on electrically evoked dopamine release in the NAc evoked by electrical stimulation of the VTA are shown in Figure 6. Figure 7 shows the temporal variation of the dopamine concentration, and Figure 8 shows the average height of peaks in response to VTA stimulation. A repeated measures ANOVA of the data presented in Figure 8 revealed significant drug treatment [$F(3,22) = 32.61$; $p < 0.001$] and time [$F(2,44) = 33.60$; $p < 0.001$] effects and a significant interaction between these factors [$F(6,44) = 25.51$; $p < 0.001$]. Post hoc analysis showed that administration of 5 mg of amphetamine/kg caused a significant increase in the height of the peaks (Figure 8A,B), and the subsequent administration of 2 mg of diazepam/kg reversed this effect (Figure 8A,C).

2.5. Diazepam Does Not Alter the Effects of Amphetamine on Dopamine Re-Uptake. Figure 9 shows that diazepam did not alter the inhibitory effects of amphetamine on dopamine re-uptake. A repeated measures ANOVA of the $T_{1/2}$ data (Figure 9A) showed significant drug treatment [$F(3,22) = 5.43$; $p < 0.01$] and time [$F(2,44) = 17.50$; $p < 0.001$] effects and a significant interaction between these factors [$F(6,44) = 2.52$; $p < 0.05$]. The same analysis applied to the decrease in the decay constant (K) (Figure 7B) showed significant drug treatment [$F(3,22) = 8.46$; $p < 0.01$] and time [$F(2,44) = 45.44$; $p < 0.001$] effects and a significant interaction between these factors [$F(6,44) = 6.53$; $p < 0.001$]. Post hoc analyses showed that administration of 5 mg of amphetamine/kg significantly increased the $T_{1/2}$ for dopamine re-uptake (Figure 9A,C) and decreased the decay constant (Figure 9B,D), but the

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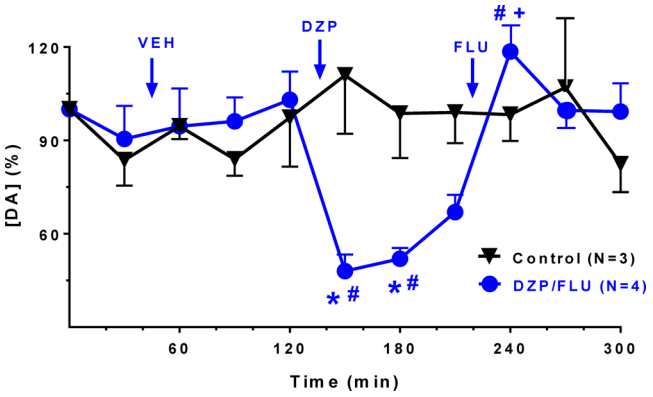

Figure 5. Effect of diazepam on the variation in tonic levels of dopamine in the mouse nucleus accumbens. Mice were anesthetized with urethane; microdialysis samples were collected every 30 min, and dopamine concentrations were measured by HPLC-EC in the following order: under baseline (BL) conditions (before the injections), after the injection of 2 mg of diazepam/kg (DZP), and after the injection of flumazenil (FLU). The mice of the control group received vehicle when the mice of the other groups received diazepam and flumazenil. Data are expressed as the mean ± SEM percent of the baseline. *p < 0.05 compared to the control group at the same time; #p < 0.05 compared to the same group at 120 min; +p < 0.05 compared to the same group at 210 min (Bonferroni test after two-way ANOVA).

administration of diazepam did not alter these effects of amphetamine.

2.6. Summary and Conclusion. BZ drugs increase the affinity of GABA for certain subtypes of GABAA receptors, causing hyperpolarizing inhibition mediated by an increase in the postsynaptic membrane conductance to Cl^- ions.[1] In the study presented here, we present evidence that BZs also affect dopamine neurotransmission in the NAc. More specifically, we showed that the BZ receptor agonist diazepam, used at doses that cause anxiolytic-like effects in rodents,[23] decreased the level of electrically evoked release of dopamine in the NAc. The reversal of this effect by the BZ antagonist flumazenil[24] suggests that the effect of diazepam on electrically evoked dopamine release was mediated by BZ−GABAA complex receptors.

The addictive property of BZs is mediated by the same subunit of the BZ−$GABA_A$ receptor complex involved in their anxiolytic effects, which limits the therapeutic use of BZs.[25] However, as discussed above, while other drugs of abuse such as cocaine, amphetamine, and opioids increase the level of dopamine release in the NAc,[26] BZs cause the opposite effect. Here we further showed that diazepam can also prevent and reverse the increase in the level of dopamine release caused by amphetamine. This suggests BZs as potential candidates for the treatment of the abusive use of opioids and psychostimulants as shown in animal[27−30] and human[31] studies.

Subunit α1 of the $GABA_A$ receptor (necessary for BZ binding) is expressed in GABAergic neurons in the NAc that project to the VTA[32] and in GABAergic interneurons in the VTA.[12] However, $GABA_A$ receptors expressing subunit α1 are not expressed in the dopaminergic neurons of the VTA.[12] This pattern of BZ receptor distribution has been taken as indirect evidence that, rather than decreasing the level of dopamine release in the NAc (as shown in this study), BZs would increase the level of dopamine release in the NAc by disinhibiting GABAergic neurons that make synaptic contacts with dopaminergic neurons in the VTA.[12,13] However, no direct evidence that acute administration of a BZ increases the level of electrically evoked dopamine release in the NAc has been provided. On the contrary, in harmony with the study presented here, all microdialysis studies have shown that diazepam[33] and other BZs[18,34−36] decrease tonic extracellular dopamine levels in the NAc. The mechanism underlying this phenomenon remains unclear.

This is the first time that a suppressive effect of a BZ on dopamine release was shown by using well-controlled FSCV recording. We also used *in vivo* microdialysis to confirm that diazepam decreases the level of dopamine release in the NAc. Here and in previous studies using microdialysis,[18,34−36] samples were collected at intervals of 20−30 min, whereas in FSCV recordings used in our study, changes in extracellular dopamine release were measured every 100 ms. Microdialysis measures tonic variations in extracellular levels of dopamine that can vary on a time scale of minutes to hours because of alterations in the synthesis, metabolism, and tonic firing rate of dopaminergic neurons.[22] However, the relatively low temporal resolution of microdialysis sampling does not permit the measurement of changes in dopamine release due to relatively rapid phasic activation or inhibition of dopamine neurons in response to salient, rewarding, or aversive stimuli.[37,38]

Another advance of this study is that FSCV recording of dopamine release allowed measures of the kinetics of both dopamine release and dopamine re-uptake. Our results clearly show that diazepam decreases the level of electrically evoked dopamine release without affecting dopamine re-uptake even in mice previously treated with amphetamine. The lack of a significant difference among groups was not due to the small sample size or poor quality of the data as, using the same method, we were able to detect significant differences caused by both the DAT inhibitor nomifensine and amphetamine on $T_{1/2}$ and K during the re-uptake phase of the dopamine responses. Amphetamine facilitates monoamine neurotransmission by several mechanisms. These mechanisms include blocking DAT, norepinephrine transporters (NET), and, at a lower level, serotonin transporters (SERT). In addition, amphetamine acts as a substrate for these monoamine transporters, competes with the neurotransmitter substrates, and enters the presynaptic neuron. Inside the nerve terminal, amphetamine displaces monoamines from the cytosolic pool and inhibits monoamine transporter 2 (VMAT2). As a result of these processes, nerve terminals reverse the transport of monoamines, pumping them into the synapse.[39] As observed in the study presented here, amphetamine also augments electrically stimulated efflux of dopamine.[9,40,41]

In the study presented here, diazepam was injected systemically. Thus, it is possible that diazepam affects dopamine release in the NAc by acting on other sites of the brain. However, a microdialysis study by Gruen et al.[42] showed that intrastriatal infusion of BZ receptor antagonist Ro15-1788 or $GABA_A$ receptor antagonist SR 95531 increased the extracellular concentration of dopamine and that this effect was blocked by co-administration of diazepam or GABA. In addition, Takada et al.[21] showed that the BZs midazolam and flunitrazepam decreased the level of extracellular dopamine in the dorsal striatum. Furthermore, an amperometry study reported that the infusion of the $GABA_A$ receptor blocker picrotoxin into the rat amygdala caused an increase in dopamine concentration.[43] These findings suggest that BZs act locally at the BZ−$GABA_A$ receptor complex to decrease the level of tonic dopamine in the striatum. However, two other

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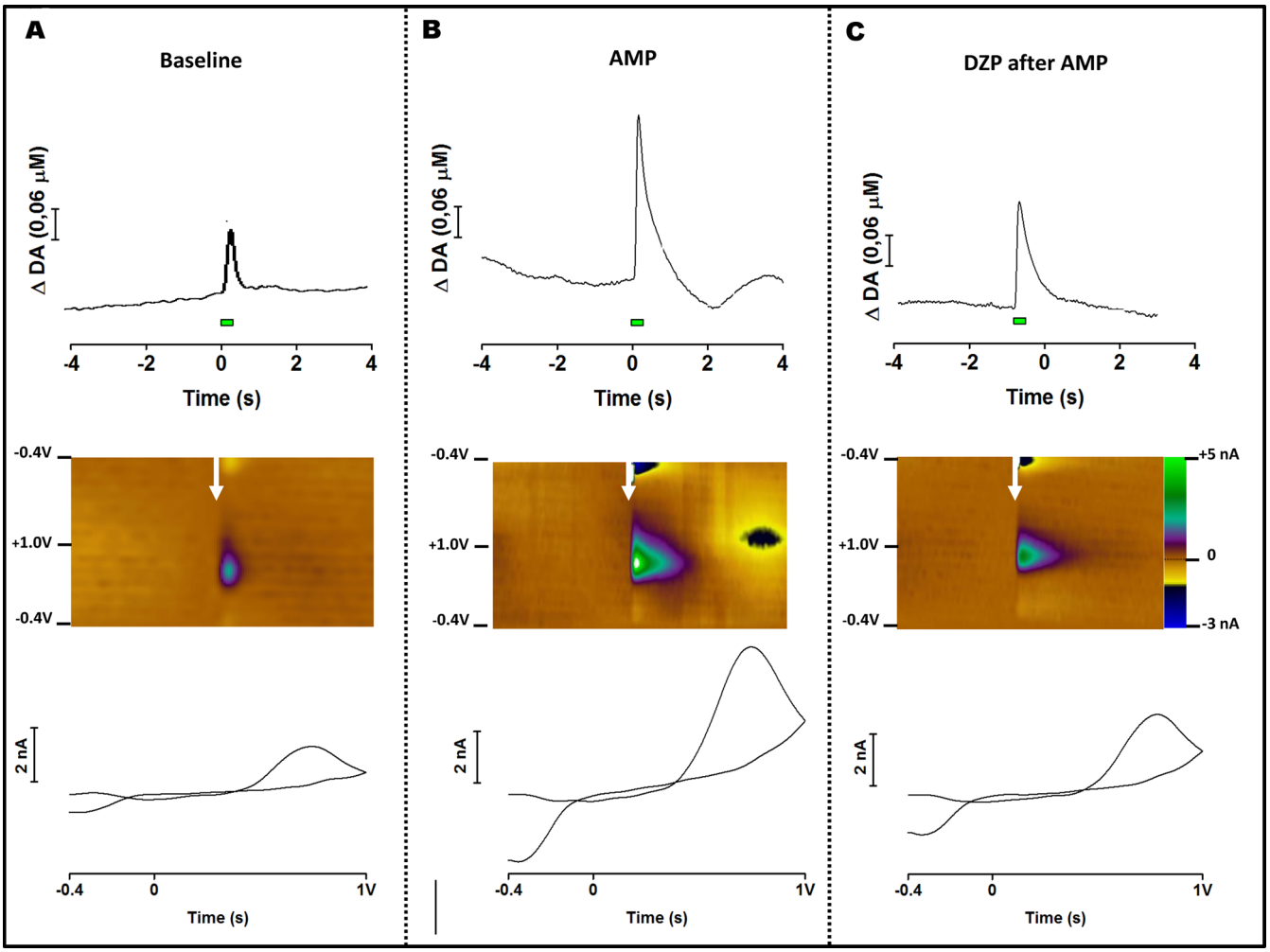

Figure 6. Individual examples illustrating the effects of ip administration of 5 mg of amphetamine/kg (AMP) and 2 mg of diazepam/kg on electrically evoked dopamine release in the nucleus accumbens evoked by electrical stimulation of the ventral tegmental area in urethane-anesthetized mice. Dopamine was monitored by background-subtracted FSCV at carbon-fiber microelectrodes. From top to bottom are shown concentration vs time traces, pseudocolor plots, and cyclic voltammograms taken at the peak oxidation current, respectively. Δ[DA] represents the variation in dopamine concentration. The arrow indicates when the electrical stimulation started, and the green bar indicates the duration of the electrical stimulation.

microdialysis studies reported that local infusion of the BZs diazepam (at a dose similar to those used in our study) and flurazepam decrease the level of extracellular dopamine in the NAc, but not in the dorsal striatum.[33,34] On the basis of our current findings, it seems plausible that the proximity of the tip of the microdialysis probes to the NAc in the work of Gruen et al.[42] and Takada et al.[21] might explain the differences between their results[21,42] and those of the other studies.[33,34]

The VTA−NAc pathway plays a critical role in motivation and drug addiction.[37,44] Therefore, the findings reported here suggest that a decrease in extracellular concentrations of dopamine in the NAc impacts the motivational dimension of the anxiolytic effect of BZs. The results of this study also suggest that BZs affect the response to psychostimulant drugs[45] and to novelty, physical, and social stress[46] by decreasing the level of release of dopamine in the NAc. In other words, this study suggests that the anxiolytic properties of the BZs have a dopaminergic component and may be useful for the treatment of drug addiction.

3. METHODS

3.1. Animals. Fifty-six adult Swiss mice (20−40 g) from the colony of the Universidade Federal do Paraná were used for the FSCV recordings. Seven additional mice were used in the microdialysis experiment. They were housed in groups of five in polypropylene cages (41 cm × 34 cm × 16 cm) with sawdust bedding under a 12 h/12 h light/dark cycle (lights on at 7:00 a.m.) and a controlled temperature (22 ± 2 °C). Food and water were available *ad libitum*. After recordings, each mouse was decapitated, and their brains were removed for histology. All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the institutional Ethics Committee for Animal Experimentation of the Universidade Federal do Paraná (Protocol 638) and are consistent with Brazilian law (Bil#11.794/8 October 2008).

3.2. Surgery. Each mouse was anesthetized with urethane (1.5 mg/kg, ip) and mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A scalpel was used to make a 1 cm midline incision exposing the skull bone surface, which was cleaned using the scalpel and sterile cotton swaps. A stainless steel burr (David Kopf Instruments) was used to drill two circular openings 2 mm in diameter above the NAc in the left frontal bone and above the VTA in the parietal bone. The openings were centered in the following

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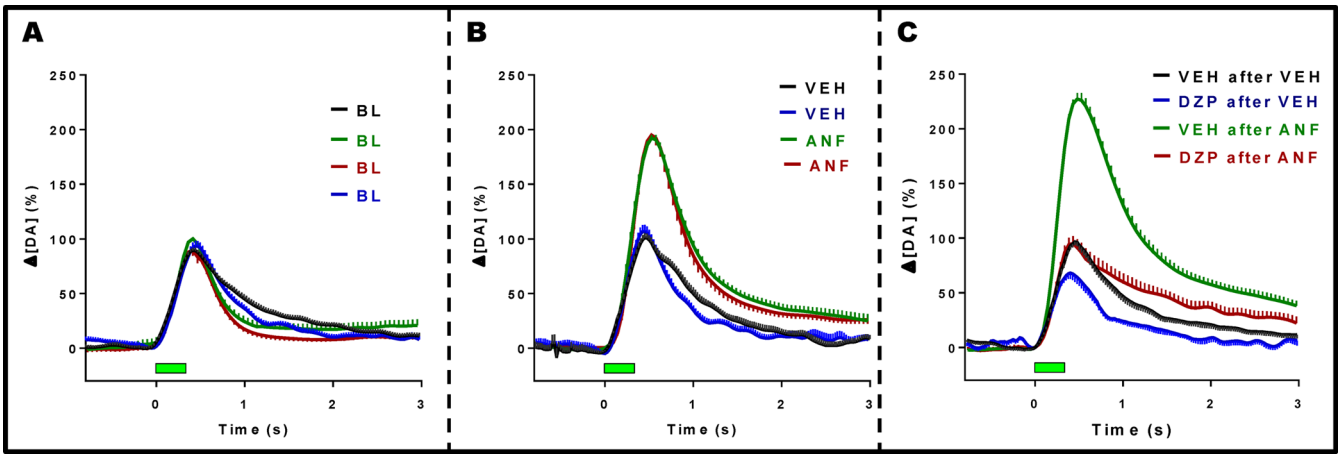

Figure 7. Effects of the administration of diazepam and amphetamine on dopamine release and re-uptake. Mice were anesthetized with urethane, and electrically evoked release of dopamine in the nucleus accumbens evoked by the electrical stimulation of the ventral tegmental area was monitored by background-subtracted FSCV at carbon-fiber microelectrodes. The green bar indicates the duration of the electrical stimulation. Changes in dopamine concentration (Δ[DA]) were measured: (A) under baseline conditions, (B) after the administration of vehicle (VEH) or 5 mg of amphetamine/kg, and (C) after the subsequent administration of vehicle or 2 mg of diazepam/kg (DZP). Data are expressed as the mean ± SEM percent of the baseline.

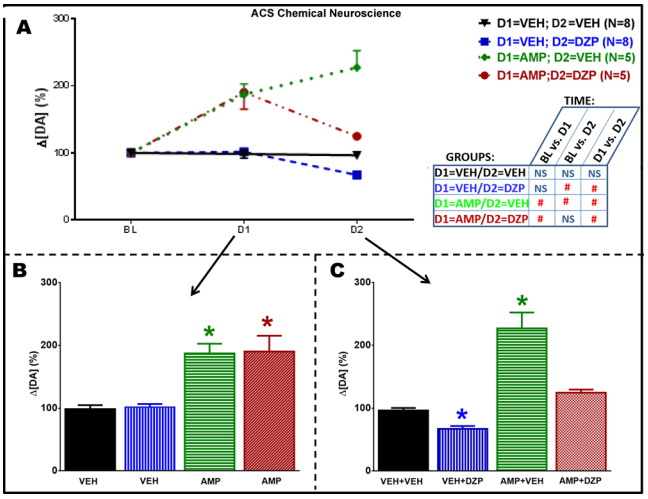

Figure 8. Effects of the administration of diazepam and amphetamine on dopamine release. Mice were anesthetized with urethane and dopamine release was evoked by electrical stimulation of the ventral tegmental area. Variations in extracellular dopamine concentration were monitored by background-subtracted FSCV at carbon-fiber microelectrodes. Dopamine release (Δ[DA]) was estimated by the heights of the peaks measured under baseline conditions (BL), after the administration of vehicle (VEH) or 5 mg of amphetamine/kg (AMP) (D1), and after the administration of vehicle or 2.5 mg of diazepam/kg (DZP) (D1). Temporal variations are shown in panel A, and comparisons between groups are shown in panels B and C. Data are expressed as the mean ± SEM percent of the baseline. NS, not significant; *$p < 0.05$ compared to the control group at the same time; #$p < 0.05$ compared to the other time for the same group (Bonferroni test after two-way ANOVA).

stereotaxic coordinates, according to the atlas of Paxinos and Franklin:[47] NAc, AP +1.2 mm, ML +1.2 mm; VTA, AP −3.8 mm, ML +0.2 mm. A $Ag/AgCl^-$ wire reference electrode was inserted 0.5 mm into a smaller hole drilled in the right parietal bone and fixed to the bone with dental cement.

3.3. Fast-Scan Cyclic Voltammetry Recording. The stimulating electrode was lowered into the VTA in steps of 0.1 mm until the strongest evoked dopamine response was recorded, over the DV range below dura of 3.4−4.1 mm. This procedure was repeated to optimize the location for the recording electrode in the NAc core (3.2−4.0 mm). FSCV measurements were taken with a Wireless Instantaneous Neurotransmitter Concentration Sensor (WINCS, Mayo Clinic) system and processed with WINCSware with MINCS software (version 2.10.4.0, Mayo Clinic). Every 100 ms, a triangular waveform potential of −0.4 V to +1.0 V to −0.4 V was applied at a rate of 300 V/s to the carbon-fiber recording electrode versus the $Ag/AgCl^-$ reference electrode. Oxidative and reductive currents were continuously sampled at 100000 samples/s and 944 samples/scan. The digital output was filtered with a Butterworth low-pass filter (800 Hz, three poles) and smoothed. The triangular waveform potential was applied to the electrode for 10 min before recording commenced to condition the electrode. Next, trains of 20 biphasic pulses (0.5 ms per pulse, 600 μA, 60 Hz) were applied to the stimulating electrode every 180 s via a programmable optical isolator pulse generator (MINCS, Mayo Investigational Neuromodulation Control System, Mayo Clinic).

3.4. Drug Treatments. After the electrochemical recording signal stabilized (did not decay by more than 20%/h), four trains of electrical stimulation 3 min apart were applied in the VTA under the following conditions: baseline (before any drug administration) and 5 min after the administration of vehicle, the BZ receptor agonist diazepam (1, 2, or 3 mg/kg, ip), and then the BZ receptor antagonist flumazenil (2.5 or 5 mg/kg, ip). These drugs were injected ip sequentially in the same animals, but independent groups of mice received different doses of diazepam. Another group of mice was submitted to the same protocol but received drug injections in a different order: baseline, vehicle, 2.5 mg of flumazenil/kg, and 2 mg of diazepam/kg. Another group of mice received vehicle, amphetamine (AMP, 5 mg/kg), and then DPZ (2 mg/kg). A control group received three injections of vehicle at the same time that the other groups received vehicle or the other drugs. At the end of this procedure, the 1 mg of diazepam/kg group also received an ip injection of the dopamine transporter (DAT) inhibitor nomifensine (20 mg/kg, ip), and electrically evoked dopamine release was monitored 5, 8, and 11 min later.

3.5. Fast-Scan Cyclic Voltammetry Data Analysis. Background-subtracted cyclic voltammograms were obtained by subtracting voltammograms collected during stimulation from those collected up to 3 s before the stimulation. Voltammetric responses were viewed as pseudocolor plots with the abscissa as the voltage, the ordinate as the acquisition time, and the current encoded in color. Temporal responses were determined by monitoring the current at the peak oxidation potential for dopamine in successive voltammograms. Current values were converted to concentration based on calibration curves obtained after the experiments with the electrodes immersed in dopamine solutions (0.25, 0.5, and 1.0 μM) in a flow cell. The change

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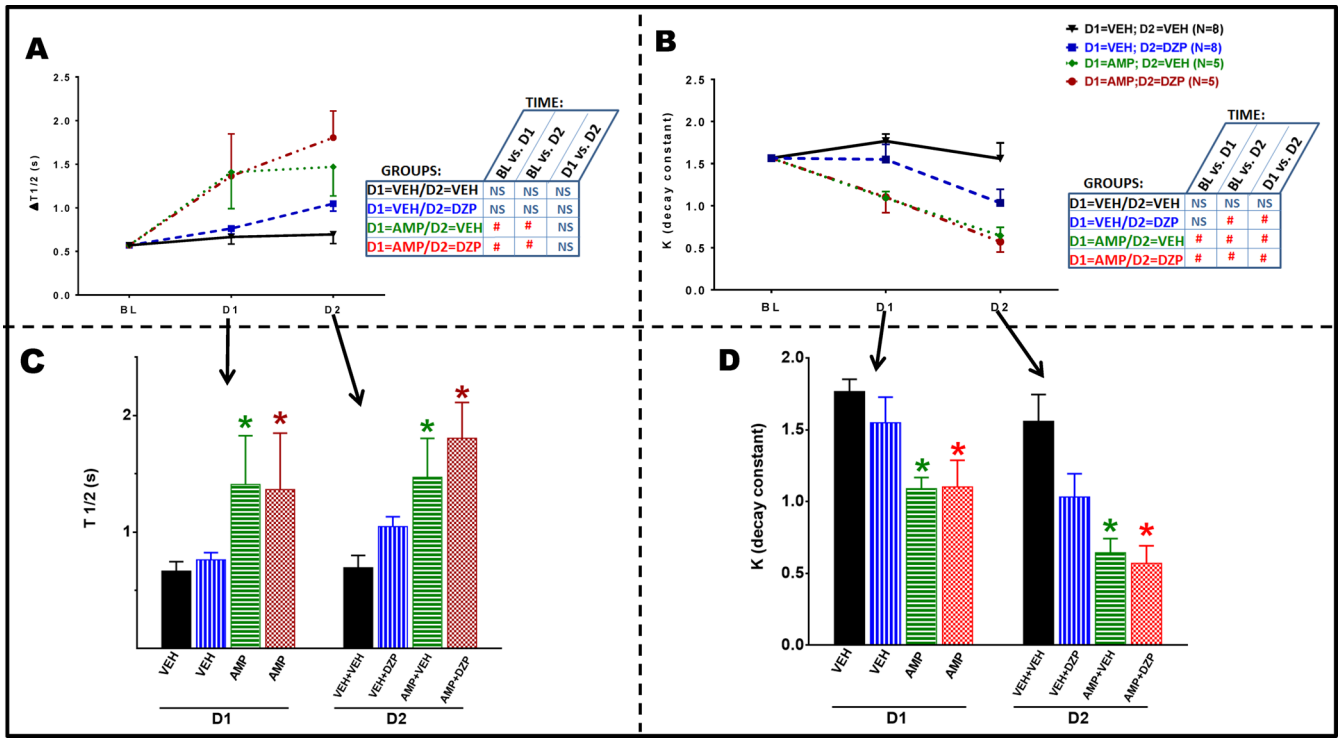

Figure 9. Effects of the administration of diazepam and amphetamine on dopamine re-uptake. The half-life ($T_{1/2}$) and decay constant for dopamine re-uptake were taken under baseline conditions (BL) and after the administration of vehicle (VEH), 5 mg of amphetamine/kg (AMP), and/or 2 mg of diazepam/kg (DZP). The temporal variations in $T_{1/2}$ and K are compared in panels A and B, respectively. Comparisons between groups are shown in panels C and D. NS, not significant; *$p < 0.05$ compared to the control group at the same time; #$p < 0.05$ compared to the other time for the same group (Bonferroni test after two-way ANOVA).

in dopamine concentration (ΔDA) was measured by subtracting the higher and lower values of current in the optimal voltage for dopamine oxidation (from +0.65 to +0.75 V) recorded between 3 s before and 3 s after the electrical stimulation. Data of four electrically evoked dopamine peaks were collected every 2 min before drug administration (baseline) and after the administration of vehicle, diazepam, flumazenil, and amphetamine for each animal. $T_{1/2}$ (time required for the dopamine signal to decay 50% compared to the initial value) and K (kinetic decay rate constant) were calculated by modeling the descending part of the evoked dopamine peaks to a one-phase exponential decay equation [$Y = (Y_0 - \text{plateau}) \times \exp(-KX) + \text{plateau}$]. The highest point, $T_{1/2}$, and K calculated from the four peaks recorded under the same condition (baseline, vehicle, diazepam, and flumazenil) were averaged.

3.6. *In Vivo* Microdialysis. Mice were anesthetized with urethane (1.5 g/kg, ip) and placed in a stereotaxic frame, where the cranium was exposed and a burr hole was drilled targeting the NAc +1.2 mm from bregma, +0.8 mm from the midline, and −3.8 mm from skull surface, according to the mouse atlas of Paxinos and Franklin.[45] A concentric microdialysis probe (outside diameter of 0.24 mm, permeability of 6 kDa, Cuprophan; AgnTho's, Lidingö, Sweden) with active membrane lengths of 2 mm was inserted unilaterally into the NAc via a polyurethane guide cannula (shaft outside diameter of 0.5 mm, shaft length of 8 mm; AgnTho's) and perfused for 30 min to stabilize the system. Dialysate samples were taken every 20 min. Two dialysate samples were taken under basal conditions; three dialysate samples were obtained after vehicle ip administration, three dialysate samples after ip administration of 2.0 mg of diazepam/kg, and three dialysate samples after ip administration of 2.5 mg of flumazenil/kg. The control group had the same procedure except for vehicle ip administration at the three moments of injection. The full experiment lasted 5 h per mouse. The microdialysis probe was perfused with Ringer's solution [145.0 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, and 1.0 mM $MgCl_2$ (pH 7.4)] at a constant rate of 2 μL/min. All microdialysis samples were collected into polyethylene tubes containing 20 μL of a 0.1 M perchloric acid solution (Merck, Darmstadt, Germany) and 0.06% sodium metabisulfite (Sigma-Aldrich) and stored at −86 °C until high-performance liquid chromatography with electrochemical detection (HPLC−ED) analysis could be performed. Isocratic separation was performed on a reverse phase LC-18 column (4.6 mm × 250 mm; Sigma-Aldrich) using 20 mM Na_2HPO_4, 20 mM citric acid, 10% methanol, and 0.12 mM Na_2EDTA

3.7. Histology. The brains were fixed for 10 days in 4% formaldehyde and transferred to a solution of 30% sucrose with a 4% formaldehyde solution where they were left for an additional 2 days. Coronal slices with a thickness of 50 μm were stained with thionin and compared to the mouse atlas of Paxinos and Franklin[45] to locate damage along the electrode length (DV, and ML coordinates). Electrode tip locations (DV coordinates) were estimated by how far down the electrode was lowered.

3.8. Statistical Analysis. Data were analyzed using a repeated measures two-way ANOVA followed by the Bonferroni test. Differences among groups are considered significant when $p < 0.05$. All statistical data analysis was conducted using Prism for Windows, version 6.01 (GraphPad Software Inc., La Jolla, CA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.6b00358.

A representative illustration of a thionin-stained midbrain slice showing no sign of electrolytic lesion near the stimulation electrode (Supplementary Figure 1), the effect of the administration of the DAT blocker nomifensine on electrically evoked dopamine release recorded by FSCV (Supplementary Figure 2), which was a control experiment to show that the FSCV data is a

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ACS Chem. Neurosci. 2017, 8, 300−309

reliable and selective measure of dopamine, data showing that the administration of flumazenil before diazepam prevents the depressive effects of diazepam on dopamine release (Supplementary Figures 3 and 4), and data that show that diazepam does not affect dopamine re-uptake (Table S1) (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: dacunha.claudio@gmail.com.

ORCID

Claudio Da Cunha: 0000-0001-6920-8561

Author Contributions

A.G.-A and A.M.F. contributed equally to this work. A.G.-A performed the FSCV experiments reported in the first version of the manuscript. A.M.F. performed the FSCV experiments reported in the second and third versions of the manuscript. S.L.B. performed most microdialysis experiments. D.B. and S.T.F. performed the HPLC−ED analysis of the microdialysis samples. A.H.S. wrote Matlab algoritms and ran the FSCV data analysis. K.L. and C.D.B. provided equipment and technical scientific support for the FSCV experiments. C.D.C. proposed the rationale and supervised the experiments. All co-authors made significant contributions to the discussion of the data and writing of the manuscript.

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Notes

The authors declare no competing financial interest.

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