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## Stimulation of glutamate receptors in the ventral tegmental area is necessary for serotonin-2 receptor-induced increases in mesocortical dopamine release

E.A. Pehek<sup>1,2,3</sup> and A.E. Hernan<sup>1,4,5</sup>

<sup>1</sup>Louis Stokes Cleveland Department of Veterans Affairs, Cleveland, OH 44106y

<sup>2</sup>Case Western Reserve University, Department of Psychiatry, Cleveland, OH 44106

<sup>3</sup>Case Western Reserve University, Department of Neurosciences, Cleveland, OH 44106

<sup>4</sup>Case Western Reserve University, Department of Biology, Cleveland, OH 44106

<sup>5</sup>University of Vermont College of Medicine, Burlington, VT 05405

### Abstract

Modulation of dopamine (DA) released by serotonin-2 (5-HT<sub>2</sub>) receptors has been implicated in the mechanism of action of antipsychotic drugs. The mesocortical DA system has been implicated particularly in the cognitive deficits observed in schizophrenia. Agonism at 5-HT<sub>2A</sub> receptors in the prefrontal cortex is associated with increases in cortical DA release. Evidence indicates that 5-HT<sub>2A</sub> receptors in the cortex regulate mesocortical DA release through stimulation of a “long-loop” feedback system from the PFC to the VTA and back. However, a causal role for VTA glutamate in the 5-HT<sub>2</sub>-induced increases in PFC DA has not been established. The present study does so by measuring 5-HT<sub>2</sub> agonist-induced DA release in the cortex after infusions of glutamate antagonists into the VTA. Infusions of a combination of a NMDA (AP-5: 2-amino-5-phosphopentanoic acid) and an AMPA/kainate (CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione) receptor antagonist into the VTA blocked the increases in cortical DA produced by administration of the 5-HT<sub>2</sub> agonist DOI [(±)-2,5-Dimethoxy-4-iodoamphetamine] (2.5 mg/kg s.c.). These results demonstrate that stimulation of glutamate receptors in the VTA is necessary for 5-HT<sub>2</sub> agonist-induced increases in cortical DA.

### Keywords

antipsychotic; prefrontal cortex; microdialysis; schizophrenia; AMPA; NMDA

### Introduction

Multiple studies have demonstrated that dopamine (DA) systems are regulated by serotonin (5-HT) receptors (Alex and Pehek, 2007). The serotonin 5-HT<sub>2A</sub> receptor is of particular interest due to its role in hallucinogenic drug action and its putative involvement in atypical

antipsychotic drug mechanisms. A substantial body of literature has shown that 5-HT<sub>2A</sub> receptors in the prefrontal cortex (PFC) regulate mesocortical DA release (Bortolozzi et al., 2005; Gobert and Millan, 1999; Pehek et al., 2001; 2006). The mesocortical DA system has been implicated particularly in the cognitive deficits observed in disorders such as schizophrenia (Weinberger, 1987). 5-HT<sub>2A</sub> receptors are localized, in part, to the apical dendrites of pyramidal cells in the rat PFC (Cornea-Hebert et al., 1999; Hamada et al., 1998; Jakab and Goldman-Rakic, 1998; Willins et al., 1997; Weber and Andrade, 2010). More recent data has found that a large number of pyramidal cells projecting to the midbrain ventral tegmental area (VTA), the DA cell body site of origin of the mesocortical tract, contain 5-HT<sub>2A</sub> receptors (Vasquez-Borsetti et al., 2009). Furthermore, pyramidal neurons projecting from the PFC innervate DA cells in the VTA (Sesack and Pickel, 1992).

These data suggest that cortical 5-HT<sub>2A</sub> receptor regulation of mesocortical DA is mediated by actions on a “long-loop” neuronal circuit involving glutamatergic corticostriatal projections to DA cells in the VTA (Vasquez-Borsetti et al., 2009). We have previously published data to support such a circuit (Pehek et al., 2006). In addition to increasing DA in the PFC, injections of the 5-HT<sub>2</sub> agonist DOI also increased glutamate efflux in the VTA, which was blocked by intracortical infusions of the selective 5-HT<sub>2A</sub> antagonist M100907. These findings indicate that the effects of DOI are mediated by 5-HT<sub>2A</sub> receptors localized in the PFC. Furthermore, they demonstrate that 5-HT<sub>2A</sub>-induced increases in PFC DA are correlated with increases in VTA glutamate. However, they do not establish causality. A more direct test of the hypothesis is necessary and is the objective of the present work. This experiment determined if increases in glutamatergic tone in the VTA are necessary for 5-HT<sub>2A</sub> agonist-induced cortical DA release.

Dual probe in vivo microdialysis in conscious rats was employed followed by measurements of dialysate DA with HPLC and electrochemical detection. We tested if concurrent blockade of both AMPA and NMDA receptors in the VTA would attenuate the increase in dialysate DA produced by administration of the 5-HT<sub>2</sub> agonist DOI. A mixture of the NMDA antagonist AP-5 and the AMPA/kainate receptor antagonist CNQX was infused by reverse dialysis into the VTA. It was hypothesized that such infusions would block DOI-induced increases in cortical DA.

## Experimental Procedures

### Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing between 200–350 gms at the time of surgery, were used for all experiments. Rats were housed two per cage in a temperature controlled room with a 12hr/12hr light/dark cycle. Food and water were available *ad libitum*. All animal procedures were in strict accordance with the *NIH Guide for the Care and Use of Laboratory Animals* and were approved by the local animal care committee.

## Surgery

Rats were anesthetized with a mixture of xylazine and ketamine (6 and 70 mg/kg, respectively; administered i.m.) and mounted in a stereotaxic frame. Microdialysis probes were implanted into the PFC (+3.2 AP, ML 0.8, DV -5.5) and the VTA (-5.60 AP, ML 0.6, DV -8.4) (Paxinos and Watson, 1998; see Fig. 1). Placements were ipsilateral and approximately half of the placements were on the right and half on the left. The probes were then secured in place with three set screws covered with cranioplastic cement. Probe locations were verified histologically at the completion of the experiments. If improperly placed, animals were excluded from the experiments.

## Microdialysis

Microdialysis probes were of a concentric flow design (Yamamoto and Pehek, 1990). Average recovery for DA was 10–15%. PFC probes were constructed with a 5.0 mm active dialyzing surface membrane (Spectra/Por Hollow, MW cutoff = 13,000, diameter = 240  $\mu$ m) to effectively dialyze from the dorsal anterior cingulate to the most ventral region of the infralimbic PFC. VTA probes were constructed with a 1.0 mm active dialyzing surface at the most ventral extension of the probe to effectively dialyze the mediolateral parabrachial and paranigral VTA (see Fig 1). The tips of the probes (approximately 0.3 mm) were plugged with glue and thus did not recover analyte. The afternoon prior to microdialysis experiments animals were placed in clear Plexiglas microdialysis chambers (Harvard Apparatus, Hollister, MA, USA) with food and water available *ad libitum*. Animals were tethered to counterbalance arms (Instech, Plymouth Meeting, PA, USA) that permitted free movement about the chamber. 18–24 hrs after probe insertion, a micro-infusion pump (PHD 2000<sup>TM</sup>, Harvard Apparatus) and liquid swivel (Instech) were used to perfuse a modified Dulbecco's artificial cerebrospinal fluid (aCSF) buffer solution (137 mM NaCl, 3 mM KCl, 1.2 mM MgSO<sub>4</sub>, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, with 1.2 mM CaCl<sub>2</sub> and 10 mM glucose; pH 7.4) through the probes. Samples were collected every 30 min after baseline levels of DA were stable (typically 2–3 hrs.). After baseline collections, drugs dissolved in the aCSF were administered by reverse dialysis. Tubing connections were switched manually while maintaining a constant flow rate and collection volume. Samples were immediately analyzed for DA content by HPLC.

## Drugs

The 5-HT<sub>2</sub> agonist ( $\pm$ )-DOI hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO, USA). DOI was injected systemically (s.c.) and was dissolved in water (2.5 mg/kg/ml, dose refers to the salt). The AMPA/kainate antagonist CNQX disodium salt was obtained from Sigma-Aldrich. The NMDA antagonist (+/-)AP-5 was obtained from Tocris Bioscience (Ellisville, MO, USA). Since both AMPA and NMDA receptors have been implicated in the regulation of DA release, a combination of CNQX (50  $\mu$ M) and AP-5 (200  $\mu$ M) was dissolved in the aCSF and infused into the VTA by reverse dialysis. This drug combination, infused into the VTA at these concentrations, has been used previously by others (Karreman and Moghaddam, 1996; Taber et al., 1995). Empirical evidence indicates that the concentrations of drugs crossing the dialysate membrane during reverse dialysis are extremely small. For example, the amounts of various drugs (DA uptake blockers) that

crossed the dialysate membrane *in vitro* ranged from 2.0 – 8.6% (Nomikos et al., 1990). However, *in vitro* studies of recovery fail to account for impediments to drug diffusion that normally occur *in vivo* in the brain tissue. One study that calculated the true *in vivo* diffusion of an antiviral nucleoside demonstrated that the recovery was one-third of that observed *in vitro* (Wang et al., 1993). Thus, what appear to be relatively high concentrations of drugs must be used in most microdialysis studies.

The antagonists were initially dissolved in water containing 1.5 – 5  $\mu$ l of glacial acetic acid to make a 10 mM stock solution. They were then further diluted to the appropriate  $\mu$ M concentrations with aCSF. The pH of all aCSF solutions was adjusted to 7.4. Following the achievement of stable baseline dialysate DA, CNQX + AP-5 were infused for one hr. before the injection of DOI.

### Chromatography

DA concentrations in dialysate samples were measured by reverse phase HPLC coupled with electrochemical detection. Twenty  $\mu$ l samples were injected immediately after collection onto a 2  $\times$  100 mm Phenomenex Ultracarb™ (Torrance, CA, USA) column (3  $\mu$ m particle size, ODS 20). The column was maintained at a temperature of 37°C. The mobile phase consisted of 32 mM anhydrous citric acid, 54 mM sodium acetate trihydrate, 0.074 mM EDTA, 0.215 mM octylsulfonic acid, and 3% methanol (vol/vol), pH 4.2. To maintain separation of DA from its metabolites and 5-hydroxyindoleacetic acid, the pH of the mobile phase and the concentration of the octylsulfonic acid were adjusted as needed. A BAS LC-4C electrochemical detector with a glassy carbon electrode, maintained at a potential of +0.60 V relative to an Ag/AgCl reference electrode, was employed.

### Histology

After microdialysis experiments were completed, probe placements were verified histologically. Only animals whose probe placements were verified to be in the PFC and the VTA were used in this study.

### Data analysis

Microdialysis data were expressed as a percentage of the average of the last three baseline samples prior to drug treatment. The SPSS statistical program was employed to analyze the data by a 2-way repeated measures ANOVA with time as the within factor and drug treatment as the independent factor. This was followed by Simple Effects tests to probe a significant interaction between treatments (Winer, 1971).

### Results

A 2-way ANOVA with time and drug treatment as the factors yielded a significant interaction [ $F(10,102) = 3.1, p = 0.003$ ] as well as a significant main effect for time [ $F(6,102) = 7.07, p < 0.001$ ]. (see Fig. 1). Simple Effects Tests revealed that only the vehicle + DOI group changed significantly over time [ $F(6,59) = 39.32, p < 0.0000$ ]. The only timepoint that showed a difference between groups was at 120 min [ $F(3,69) = 7.27, p = 0.0003$ ]. These results show that DOI administration significantly increased DA at the 120

min time point on the graph [60 min after DOI injection) The combination of antagonists (CNQX/AP-5) did not alter basal DA (i.e. did not change over time) but did block the effects of DOI (CNQX/AP-5 + DOI group did not change over time).

Basal levels of DA (pg/20  $\mu$ l) were: vehicle + vehicle:  $0.28 \pm 0.08$ , n = 5; CNQX/AP-5 + DOI:  $0.22 \pm 0.1$ , n = 5; DOI + vehicle:  $0.27 \pm 0.07$ , n = 5; CNQX/AP-5 + vehicle =  $0.36 \pm 0.08$ , n = 6.

## Discussion

Results showed that injections of the 5-HT<sub>2</sub> agonist DOI increased DA release in the PFC and this was blocked by infusions of a combination of an AMPA and a NMDA antagonist into the VTA. These findings demonstrate that enhanced glutamatergic tone in the VTA is necessary for 5-HT<sub>2</sub> modulation of mesocortical DA release. It agrees with our previous work that DOI administration increases glutamate release in the VTA; an effect that is blocked by infusions, directly into the PFC, of the selective 5-HT<sub>2A</sub> antagonist M 100,907 (Pehek et al., 2006). Thus the 5-HT receptor subtype regulating glutamate release in the VTA is the 5-HT<sub>2A</sub> subtype and it is localized cortically. These results agree with other evidence that suggests that cortical 5-HT<sub>2A</sub> receptors modulate cortical DA release by actions on a “long-loop” feedback loop involving glutamatergic projections to the midbrain VTA (Vasquez-Borsett et al., 2009). However, the present work is the first to show that 5-HT<sub>2</sub> modulation of cortical DA is dependent on the stimulation of glutamate receptors in the VTA.

The agonist DOI has affinity for all three of the 5-HT<sub>2</sub> receptor subtypes: A, B, and C. However, the present results are unlikely to result from binding to 5-HT<sub>2B/C</sub> receptors because studies have shown that 5-HT<sub>2B/C</sub> receptor agonism decreases cortical DA release (Gobert et al., 2000; Millan et al., 1998; Pozzi et al., 2002). Furthermore, our previous study showed that DOI-induced glutamate release in the VTA was blocked by the selective 5-HT<sub>2A</sub> antagonist M100907 (Pehek et al., 2006).

### Putative circuitry underlying 5-HT<sub>2</sub>/DA interactions

One neuronal circuit is the aforementioned “long-loop” monosynaptic feedback circuit from the PFC to the VTA and back. 5-HT<sub>2A</sub> receptors are dense in the PFC and are localized there, in part, to the apical dendrites of pyramidal neurons (Cornea-Hebert et al., 1999; Hamada et al., 1998; Jakab and Goldman-Rakic, 1998; Weber and Andrade, 2010; Willins et al., 1997). 5-HT<sub>2A</sub> receptor stimulation can produce excitation in pyramidal neurons (Araneda and Andrade, 1991; Aghajanian and Marek, 1997; Puig et al., 2003). A large proportion of pyramidal neurons that project to the VTA contain 5-HT<sub>2A</sub> receptors (Vasquez-Borsett et al., 2009). Furthermore, there are monosynaptic connections between PFC pyramidal neurons and DA cells in the VTA (Sesack and Pickel, 1992). These corticotegmental projections synapse on mesocortical DA, but not mesolimbic DA, cell bodies (Carr and Sesack, 2000). Microinjections of glutamate agonists into the VTA increase in vivo extracellular concentrations of dopamine in the rat PFC (Kalivas et al., 1989). Thus, 5-HT<sub>2A</sub> receptor stimulation could cause the release of glutamate from corticotegmental projections that in turn excite VTA DA cell firing and subsequent release of DA in the PFC.

Systemic administration of the 5-HT<sub>2</sub> agonist DOI increases mesocortical DA release that is blocked by *intracortical* infusions of the selective 5-HT<sub>2A</sub> antagonist M100907 (Pehek et al., 2006). This latter finding indicates that the effect of DOI is due specifically to stimulation of 5-HT<sub>2A</sub> receptors within the PFC. Administration of DOI also increased *glutamate* efflux in the VTA, and this was blocked by *intracortical* infusions of M100907 (Pehek et al., 2006), suggesting that the increases in cortical DA release may be due to increases in glutamate release in the VTA. This latter study also indicates that the effects of DOI on VTA glutamate are dependent on a circuit involving the PFC. The current study demonstrates that stimulation of glutamate receptors in the VTA is necessary for 5-HT<sub>2</sub> agonist-induced increases in cortical DA. Previous work by others has shown that application of DOI to the PFC increases burst firing of VTA DA neurons and DA release in the PFC (Martin-Ruiz et al., 2001; Bortolozzi et al., 2005). Taken together, these findings provide strong support for the hypothesis that cortical 5-HT<sub>2A</sub> receptor-induced increases in mesocortical DA are causally related to increases in glutamate efflux in the VTA. We suggest that cortical 5HT<sub>2A</sub> agonism stimulates corticotegmental glutamatergic projections that, in turn, stimulate mesocortical DA neurons.

The present work did not determine the endogenous source of glutamate in the VTA. Our previous work showed that DOI injections increased glutamate in the VTA and this was blocked by infusions of M100907 into the cortex, implicating projections specifically from the PFC. It is likely that the same projections were active in the present study. However, there are 5-HT<sub>2A</sub> containing pyramidal neurons that project to areas other than the VTA. There is evidence that some of these are callosal/commissural neurons that innervate the contralateral cortex (Avesar and Gullledge, 2012). Thus, in the present study, we cannot rule out a non-PFC source of 5-HT<sub>2</sub> regulation of VTA glutamate. There are non-PFC glutamate afferents to the VTA (Yetnikoff et al., 2014) as well as glutamate cell bodies within the VTA (Morales and Root, 2014). There is also evidence for the co-release of glutamate from VTA DA neurons (Broussard, 2012). Recent work has also demonstrated a glutamatergic projection from the raphe to the VTA that is involved in reward (Qi et al., 2014). In addition, while other studies have implicated corticotegmental glutamate projections in the regulation of PFC DA, other non-direct pathways may have been involved. For example, VTA DA neurons may be regulated indirectly through PFC projections to the nucleus accumbens, which projects to the VTA via the ventral pallidum (Floresco et al., 2003; Vasquez-Borsett et al., 2009). Thus, the involvement of projections from the PFC to other brain areas that in turn innervate the VTA cannot be ruled out. 5-HT<sub>2A</sub> receptors are also localized on DA and GABA cells in the VTA (Nocjar et al., 2002). Thus it is possible that the present effects of DOI are due to direct actions on the VTA. These receptors could directly modulate VTA glutamate by activating glutamate-containing neurons in the VTA. However, we think this is unlikely since our previous research found that DOI-induced increases in glutamate were dependent on a circuit involving the PFC, i.e. blocked by infusions of M100907 into the cortex (Pehek et al., 2006).

In short, 5-HT<sub>2A</sub> receptor modulation of mesocortical DA release requires the stimulation of glutamate receptors in the VTA. This may be mediated by a “long-loop” feedback system involving 5-HT<sub>2A</sub>-containing pyramidal cells that project to the VTA and stimulate mesocortical DA neurons. This circuitry may be relevant to the mechanism of atypical

antipsychotic and other drugs with 5-HT<sub>2A</sub> antagonist properties. Knowledge of the underlying neurocircuitry is essential for our understanding of the mechanisms of action of these drugs as well as agents such as hallucinogens that are agonists at the 5-HT<sub>2A</sub> receptor. Furthermore, DA/5-HT interactions may be generally important in PFC cognitive functions such as working memory and selective attention that are altered in syndromes such as schizophrenia.

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## Abbreviations

<b>aCSF</b>	artificial cerebrospinal fluid
<b>AMPA</b>	$\alpha$ -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
<b>AP-5</b>	2-amino-5-phosphopentanoic acid
<b>CNQX</b>	6-cyano-7-nitroquinoxaline-2,3-dione
<b>DA</b>	dopamine
<b>DOI</b>	( $\pm$ )-2,5-Dimethoxy-4-iodoamphetamine
<b>HPLC</b>	high performance liquid chromatography
<b>5-HT</b>	serotonin
<b>NMDA</b>	<i>N</i> -Methyl-D-aspartic acid
<b>PFC</b>	prefrontal cortex
<b>5-HT<sub>2</sub></b>	serotonin-2
<b>VTA</b>	ventral tegmental area

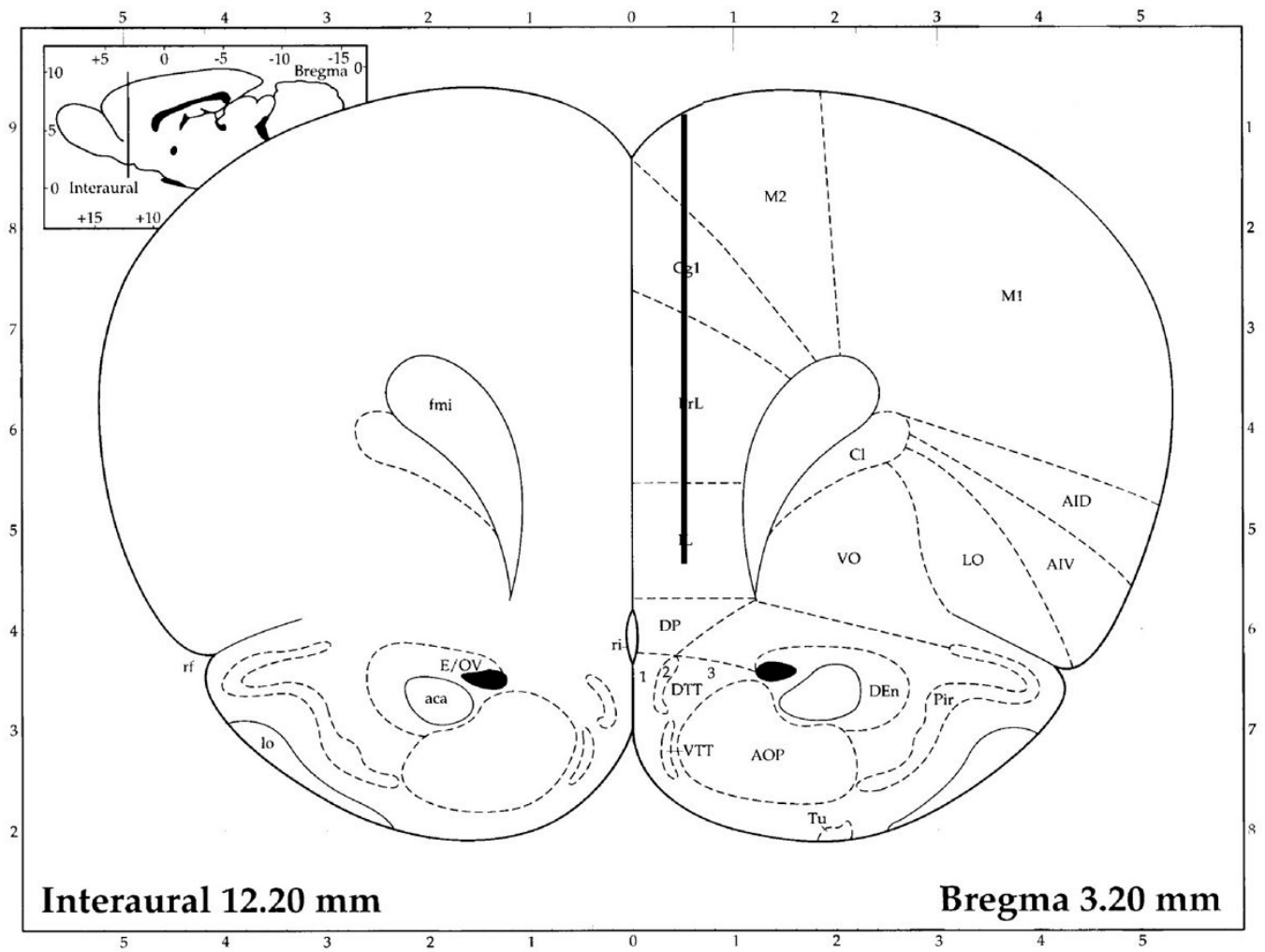
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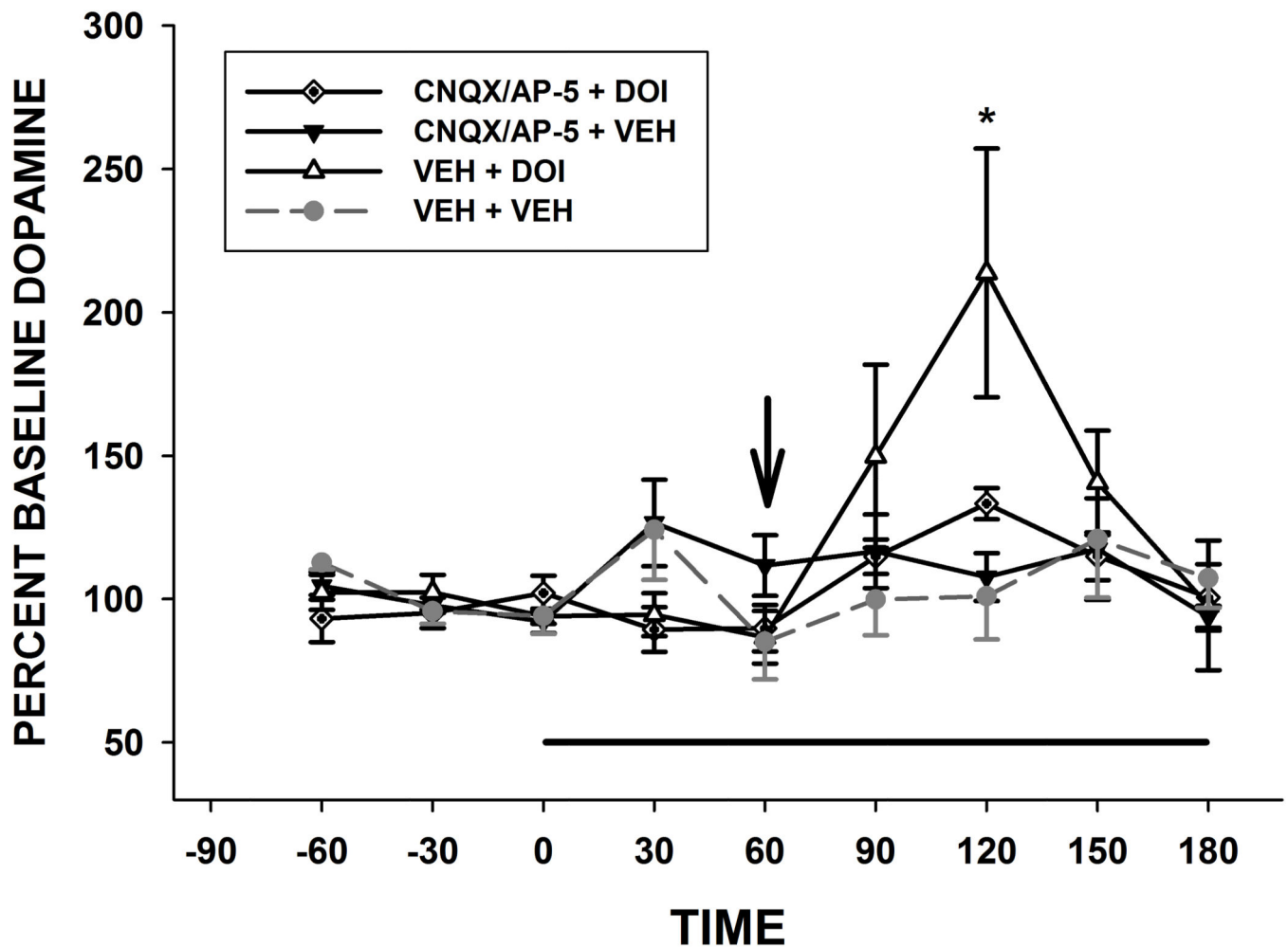
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**Fig 1.** Schematic of the location of the microdialysis probes. The lines in red represent the dialysis membrane. A: prefrontal cortex, B: ventral tegmental area. aca: anterior commissure, anterior; AOP: anterior olfactory nucleus posterior; CA3: field CA3 of the hippocampus; Cg1: cingulate cortex area 1; Cl: claustrum; csc: commissure sup colliculus; cp: cerebral peduncle; DP: dorsal peduncular cortex; fmi: forceps minor corpus callosum; fr: fasciculus retroflexus; IL: infralimbic cortex; M2: secondary motor cortex; ml: medial lemniscus; mp: mammillary peduncle; PrL: prelimbic cortex; SNCD: substantia nigra, compacta, dorsal tier; SNR: substantia nigra reticulata.



**Fig 2.**

Infusion of the glutamate antagonists CNQX (50  $\mu$ M) and AP-5 (200  $\mu$ M) into the VTA blocks 5-HT<sub>2</sub> agonist (DOI)-induced mesocortical DA release. A combination of the glutamate antagonists was infused into the VTA at the time indicated by the bar. DOI was injected (2.5 mg/kg s.c.) at the time indicated by the arrow. \* $p < 0.05$  relative to the VEH + VEH group and the CNQX/AP-5 + DOI group,  $n = 5-6$ /group.