Reversal of prolonged dopamine inhibition of dopaminergic neurons of the ventral tegmental area

Sudarat Nimitvilai and Mark S. Brodie

Department of Physiology and Biophysics
University of Illinois at Chicago
Department of Physiology and Biophysics
University of Illinois at Chicago
835 S. Wolcott, Room E-202, M/C 901
Chicago, IL 60612-7342
(S.N. and M.S.B.)
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Address for correspondence:
Mark S. Brodie, Ph.D.
Department of Physiology and Biophysics
University of Illinois at Chicago
835 S. Wolcott, Room E-202, M/C 901
Chicago, IL 60612-7342
Telephone: 312-996-2373
Fax: 312-996-1414
E-mail: mbrodie@uic.edu

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Non-Standard abbreviations:; pDAergic: putative dopaminergic; DAergic: dopaminergic; VTA: ventral tegmental area; LTP: long term potentiation; NMDA: N-methyl-D-aspartate; AMPA: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; DA: dopamine

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Abstract:

Drug abuse-induced plasticity of putative dopaminergic (pDAergic) ventral tegmental area (VTA) neurons may play an important role in changes in the mesocorticolimbic system that lead to the development of addiction. In the present study, extracellular recordings were used to examine time-dependent effects of dopamine (DA) on pDAergic VTA neurons in rat brain slices. Administration of DA (2.5-10 μM) for forty minutes resulted in inhibition followed by partial or full reversal of that inhibition. The reduced sensitivity to DA inhibition lasted 30-90 min after washout of the long-term dopamine administration. The inhibition reversal was not observed with 40 min administration of D2 agonist quinpirole (25-200 nM), so this phenomenon was not the result of desensitization induced solely by stimulation of D2 DA receptors. Inhibition reversal could be observed with the co-application of quinpirole and the D1/D5 agonist SKF38393, suggesting a D1/D5 mechanism for the reversal. Furthermore, D1/D5 antagonists given in the presence of prolonged DA exposure prevented the inhibition reversal. Application of 3 μM quinpirole caused desensitization to low quinpirole concentrations which was blocked by a D1/D5 antagonist. These data suggest that co-activation of D1/D5 receptors and D2 receptors in the VTA result in desensitization of autoinhibitory D2 receptors. Prolonged increases in pDAergic tone in the VTA, such as may occur in vivo with drugs of abuse, could reduce the regulation of firing by D2 dopamine receptor activation, producing long-term alteration in information processing related to reward and reinforcement.
Introduction:

Putative dopaminergic (pDAergic) neurons of the ventral tegmental area (VTA) are important for the rewarding and reinforcing properties of numerous drugs of abuse (Wise, 1996). Drugs of abuse increase DAergic neurotransmission (Di Chiara and Imperato, 1988; Imperato and Di Chiara, 1986; Imperato et al., 1986). Most studies have examined dopamine (DA) release in the terminal target regions of the DA VTA neurons, the nucleus accumbens and the prefrontal cortex, and have found that drugs of abuse increase the DA concentrations in these regions (Di Chiara et al., 2004; Di Chiara and Imperato, 1988). As there is dendritic DA release in response to increased activity of mesencephalic DA neurons (Cragg et al., 1997), it is likely that the DA concentrations in the VTA are also increase in response to most drugs of abuse. The effect of these elevated dopamine concentrations in the VTA is not known, but it is known that elevated dopamine can produce long-term changes in neurotransmission; for example, elevated dopamine can increase glutamatergic receptor expression in prefrontal cortex (Gao and Wolf, 2008; Sun et al., 2008).

There are 5 classes of DA receptors: two “D1-like” receptors (D1 and D5) and three “D2-like” receptors (D2, D3, and D4). D1-like (D5) receptor immunoreactivity on perikarya of mesencephalic DA neurons has been demonstrated (Khan et al., 2000; Ciliax et al., 2000), and mRNA for D5 receptors has been observed in the substantia nigra by some groups (Choi et al., 1995) but not by all groups (Meador-Woodruff et al., 1992). The D2-like mesolimbic DA receptors are predominantly D2 receptors (Sesack et al., 1994). The D1 receptors located in DAergic brain areas appear to be on terminals projecting to the region, not on the DA neurons themselves (Caille et al., 1996). Dopamine D5 receptors are present on the cell bodies of dopaminergic VTA neurons (Ciliax et al., 2000).

pDAergic VTA neurons fire action potentials spontaneously in vivo (Bunney et al., 1973) and in vitro (Pinnock et al., 1979; Brodie and Dunwiddie, 1987). This spontaneous firing is inhibited by
the action of DA at D2 autoreceptors on the cell bodies and dendrites of these neurons (Lacey et al., 1987). Stimulation of D2 autoreceptors activates G protein linked potassium channels, which appear to be activated directly by G-proteins without the involvement of cAMP or adenylate cyclase (Kim et al., 1995).

Drugs of abuse produce increases in dopaminergic neurotransmission, either by increasing firing rate of DA VTA neurons, or by blocking reuptake or reversing DA transporter activity in terminal regions (Mueller et al., 2004), and it is likely that the DA concentration in the VTA, released in the somatodendritic area (Cragg et al., 1997; Rice et al., 1997) can remain elevated during drug abuse episodes. For this reason, we tested the effects of sustained administration of exogenous DA on DA inhibition of DA VTA neurons. In vitro electrophysiological experiments using whole cell patch clamping cannot reliably maintain healthy neuronal recordings for periods longer than one hour. We used extracellular recording from DA VTA neurons in brain slices, a technique which avoids disrupting the intracellular milieu, and which makes it possible to monitor spontaneous firing of these neurons for long continuous time periods (1-4 hours).
Methods:

**Animals:** Fischer 344 (F344; 90 - 150 gm) used in these studies were obtained from Harlan Sprague-Dawley (Indianapolis, IN). All rats were treated in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and all experimental methods were approved by the Animal Care Committee of the University of Illinois at Chicago.

**Preparation of brain slices:** Brain slices containing the ventral tegmental area (VTA) were prepared from the subject animals as previously described (Brodie et al., 1999a). Briefly, following rapid removal of the brain, the tissue was blocked coronally to contain the VTA and substantia nigra; the cerebral cortices and a portion of the dorsal mesencephalon were removed. The tissue block was mounted in the vibratome and submerged in chilled cutting solution. Coronal sections (400 μm thick) were cut and the slice was placed onto a mesh platform in the recording chamber. The slice was totally submerged in aCSF maintained at a flow rate of 2 ml/min; the temperature in the recording chamber was kept at 35º C. The composition of the aCSF in these experiments was (in mM): NaCl 126, KCl 2.5, NaH₂PO₄ 1.24, CaCl₂ 2.4, MgSO₄ 1.3, NaHCO₃ 26, glucose 11. In some experiments, a HEPES-aCSF hybrid solution was used; composition of this solution was (in mM): NaCl 106, KCl 2.5, NaH₂PO₄ 1.24, CaCl₂ 2.4, MgSO₄ 1.3, HEPES 20, NaHCO₃ 26, glucose 11. The composition of the cutting solution was (in mM): KCl 2.5, CaCl₂ 2.4, MgSO₄ 1.3, NaHCO₃ 26, glucose 11, and sucrose 220. Both solutions were saturated with 95% O₂/ 5% CO₂ (pH=7.4). Equilibration time of at least one hour was allowed after placement of tissue in the recording chamber before electrodes were placed in the tissue.

**Cell identification:** The VTA was clearly visible in the fresh tissue as a grey area medial to the darker substantia nigra, and separated from the nigra by white matter. Recording electrodes were placed in the VTA under visual control. pDAergic neurons have been shown to have distinctive electrophysiological characteristics (Grace and Bunney, 1984; Lacey et al., 1989). Only those
neurons which were anatomically located within the VTA and which conformed to the criteria for pDAergic neurons established in the literature and in this laboratory (Lacey et al., 1989; Mueller and Brodie, 1989) were studied. These criteria include broad action potentials (2.5 msec or greater, measured as the width of the bi- or tri-phasic waveform at the baseline), slow spontaneous firing rate (0.5 - 5 Hz), and a regular interspike interval. Cells were not tested with opiate agonists as has been done by other groups to further characterize and categorize VTA neurons (Margolis et al., 2006). It should be noted that some neurons with the characteristics we used to identify DA VTA neurons may not, in fact, be DA-containing (Margolis et al., 2006).

**Drug Administration:** Drugs were added to the aCSF by means of a calibrated infusion pump from stock solutions 100 to 1000 times the desired final concentrations. The addition of drug solutions to the aCSF was performed in such a way as to permit the drug solution to mix completely with aCSF before this mixture reached the recording chamber. Final concentrations were calculated from aCSF flow rate, pump infusion rate and concentration of drug stock solution. The small volume chamber (about 300μl) used in these studies permitted the rapid application and washout of drug solutions. Typically drugs reach equilibrium in the tissue after 2 to 3 minutes of application.

DA hydrochloride, quinpirole, sulpiride and most of the salts used to prepare the extracellular media were purchased from Sigma (St. Louis, MO). SKF38393 ((±)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8- diol hydrobromide), SCH39166 ((6aS-trans)-11-Chloro-6,6a,7,8,9,13b-hexahydro-7-methyl -5H-benzo[d]naphth[2,1-b]azepin-12-ol hydrobromide), and SCH23390 ((R)-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-te trahydro-1H-3-benzazepine hydrochloride) were purchased from Tocris (Ellisville, MO).

**Extracellular recording:** Extracellular recording was chosen for these studies as this method permits the recordings to be of long duration (routinely > 4 hours) and allows us to assess the effects of extended exposure (>30 minutes) to drugs. The limitation of only measuring
spontaneous action potential frequency (rather than membrane potential or other electrophysiological parameters) is counterbalanced by the advantage of being able to determine the time course of drug actions and interactions. Extracellular recording electrodes were made from 1.5 mm diameter glass tubing with filament and were filled with 0.9% NaCl. Tip resistance of the microelectrodes ranged from 2 – 4 MΩ. A Fintronics amplifier was used in conjunction with an IBM-PC-based data acquisition system (ADInstruments, Inc.). Offline analysis was used to calculate, display and store the frequency of firing 1 minute intervals. Additional software was used to calculate the firing rate over 5 second intervals. Firing rate was determined before and during drug application. Firing rate was calculated over 1 minute intervals prior to administration of drugs and during the drug effect; peak drug-induced changes in firing rate were expressed as the percentage change from the control firing rate according to the formula ((FRD - FRC) / FRC) X 100, where FRD is the firing rate during the peak drug effect and FRC is the control firing rate. The change in firing rate thus is expressed as a percentage of the initial firing rate, which controls for small changes in firing rate which may occur over time. This formula was used to calculate both excitatory and inhibitory drug effects. Peak excitation was defined as the peak increase in firing rate produced by the drug (e.g., DA) greater than the pre-drug baseline. Inhibition was defined as the lowest firing rate below the pre-drug baseline. Inhibition reversal was observed as a statistically significant reduction in the inhibition.

**Data collection:** For comparison of the time course of effects on firing rate, the data were normalized and averaged. Firing rates over one minute intervals were calculated and normalized to the one-minute interval immediately prior to the DA administration. These normalized data were averaged by synchronizing the data to the DA administration period, and graphs of the averaged data were made.

**Statistical analysis:** Averaged numerical values were expressed as the mean ± the standard error of the mean (S.E.M.). The differences among firing rates during the long drug administration intervals
in these studies was assessed with repeated measures ANOVA. For effects of multiple drug concentrations or more than one drug, an appropriate one- or two-way analysis of variance (ANOVA) was used, followed by Student-Newman-Keuls or Tukey post hoc comparisons when needed (Kenakin, 1987). Statistical analyses were performed with SigmaStat (Systat, San Jose, CA.).
Results:

VTA neuron characteristics

A total of 233 neurons were used in this study. The firing rate of VTA neurons in this study ranged from 0.6118 to 4.3833 Hz, with a mean firing rate of $1.88 \pm 0.09$ Hz. All of the neurons tested had regular firing rates and were inhibited by DA. DA sensitivity was initially assessed by administering dopamine (0.5 – 5 $\mu$M) for five minutes, and then washing out the DA for at least 10 minutes. Note that blockers of the dopamine transporter were not used, as there is normally sufficient dopamine release in brain slices to cause complete cessation of spontaneous firing in the presence of dopamine transporter blockers, such as cocaine (Brodie and Dunwiddie, 1990). In the absence of DA transporter blockers, dopamine has been shown to produce inhibitory effects in the concentration range from 0.5 to 100 $\mu$M, although in dissociated DA VTA neurons, concentrations as low as 50 nM can completely inhibit spontaneous action potential firing (Brodie et al., 1999b). Cells which did not return to the pre-DA firing rate during this washout were not used. One benefit of the extracellular recording method used in these studies is that long duration recordings can be made reliably; the average recording duration was $96 \pm 18$ minutes, with a range of 28 to 280 minutes.

Concentration-response curve: stepwise increases

It is well-known that acute application of dopamine inhibits spontaneous firing of putative dopaminergic (pDAergic) neurons of the VTA. Figure 1A illustrates the effect of acute application of a single high concentration of dopamine to the spontaneous firing frequency of a typical pDAergic VTA neuron. In this case, a four minute application of 100 $\mu$M dopamine produced complete cessation of firing which subsided upon washout of the dopamine. In the first set of experiments that formed the basis for the present study, concentration-response curves for dopamine
were generated by adding DA to the superfusate in a stepwise manner; that is, the lowest concentration of DA (0.5 μM) was given for 5 minutes, and then the concentration was increased immediately to the next highest concentration (1 μM) and so on. An illustration of the firing rate of a typical pDAergic neuron of the VTA is shown in Figure 1B. As shown in this rate meter graph, the firing rate was inhibited by the lower concentrations of DA tested, but as the concentration of dopamine was increased, the inhibition of the firing rate by dopamine was slowly reversed, until at the highest concentrations, the firing rate was higher than prior to dopamine administration. The effect of 100 μM dopamine administered in this protocol as the last of several dopamine concentrations can easily be contrasted with the effect of the acute application of dopamine shown in Figure 1A. Due to the number of concentrations of DA tested in this protocol, some of the slices were treated with some concentration of DA for about 40 minutes. Not all cells received all DA concentrations; only cells tested with at least two concentrations were included (n=63). The pooled results of this experiment are shown in Figure 1C. Low concentrations of DA produced characteristic concentration-dependent inhibition of firing, but as the concentration was increased above 10 μM, the inhibitory effect of DA decreased, until at the highest concentrations in this protocol, the firing rate in the presence of DA was higher than before exogenous DA was added. There was a significant difference between the firing rate in the presence of 50 and 100 μM DA compared to the firing rate at 1-10 μM DA, and, in addition, the DA effects at 2.5-10 μM (inhibition) and 100 μM (excitation) were significantly different from the baseline pre-DA firing rate (one-way repeated measures ANOVA P<0.001, Student-Newman-Keuls post-hoc test p< 0.05 for significant differences, n=63). This suggests a second mechanism emerging in the presence of DA that counteracted the DA-induced inhibition.

**Long-term administration of single dopamine concentrations**
As the reversal of DA inhibition could have been the result of either the high and increasing DA concentrations or the long-duration application of DA, we tested whether the inhibitory effect of a single concentration of DA would change over time. Short (5 min) applications of DA were used at the beginning of each recording to assess the magnitude of DA inhibition; concentrations were selected that would produce inhibition of 10%, 25%, 65% 75% or 100% during a 5 minute test application. Selecting DA concentrations according to their inhibitory potency rather than the exact DA concentration controlled for cell-to-cell variability in sensitivity to DA inhibition. The results of these studies are shown in Figure 2A. Concentrations of DA that produced 10% (mean DA concentration = 0.29 ± 0.05 µM; range=0.25 – 0.5 µM; n=7) or 25% (mean DA concentration = 0.61 ± 0.11 µM; range= 0.25-1 µM; n=7) inhibition did not exhibit reversal over the 40 minutes during which they were applied. In contrast, concentrations of DA producing 65% (mean DA concentration = 2.54 ± 0.7 µM; range = 0.5-10 µM ;n=12), 75% (mean DA concentration = 2.82 ± 1.25 µM; range = 0.5-10 µM; n=7) or 100% (mean DA concentration = 8.09 ± 1.02 µM; range = 2.5-10 µM; n=11) inhibition showed clear decreases in inhibitory potency over the 40 minute time course of application, with the inhibition reduced to about 25-30% at the end of the 40 minute time period. When DA inhibition was approximately 50%, the responses were more variable; cells more sensitive to DA (DA concentration < 1.25 µM, mean DA concentration = 0.56 ± 0.08 µM, range = 0.25-1 µM ;n=13) did not exhibit inhibition reversal, whereas cells less sensitive to DA (DA concentration > 1.25 µM, mean DA concentration = 2.25 ± 0.13 µM; range = 1.5-2.5 µM; n=8) demonstrated inhibition reversal (Figure 2B). Inhibition reversal was indicated by a statistically significant difference between the firing rate in the presence of DA at 5 minutes and at 40 minutes (one-way repeated measures ANOVAs, P<0.05; Student-Newman-Keuls post-hoc test, P < 0.05). Again, the inhibition reversal observed with the long application of a single concentration of DA suggests that a
second mechanism emerges with long-term DA administration, and this mechanism requires higher DA concentrations (> 2 μM in this set of experiments).

We also observed that the apparent decrease in DA potency was sustained for a relatively long time after the 40 minute DA application. We observed that there was a long-lasting change in response to lower DA concentrations produced by prolonged application of 10 μM DA (Figure 3). Prior to long duration administration of 10 μM DA, low concentrations of DA (0.42 ± 0.12 μM, range = 0.25-0.5 μM; n=6) produced 28.1 ± 3.8% decrease in firing rate. After administration of 10 μM DA for 40 min, the inhibitory effect of the low concentration of DA was tested at 30 min intervals after washout of the 10 μM DA; at 30 min, the effect was reduced significantly to 10.0 ± 4.5% (one-way ANOVA, F=5.53, p<0.01, Tukey test , p<0.05 n=7); the effects of low dopamine concentrations at 60 min (17.0 ± 1.7%), and 90 min (18.2 ± 1.6%) were not significantly different from control (Figure 3). The inhibitory effect of short applications of the low concentration of DA increased over time, suggesting a recovery from the altered DA response produced by long-term exposure to 10 μM DA.

In a separate set of cells, we carefully examined the percentage of cells which exhibited dopamine inhibition reversal. Putative dopaminergic neurons (n=13) were tested with concentrations of dopamine that produced a mean 70% inhibition in the first 5 minutes (range -43% to -97%), and dopamine was left on for 40 minutes. The effect of dopamine at 5 min (-70.0 ± 4.8%) was significantly different from that at 40 min (-39.7 ± 6.7%) (t-test, p<0.002, n=13). Using the criterion of a reduction in inhibition by dopamine of at least 15%, 12 of the 13 cells exhibited reversal, whereas in the non-reversed neuron, dopamine-induced inhibition went from -82% at 5 min to -73% at the end of 40 min. Results in HEPES-aCSF hybrid buffer were similar. The effect of dopamine at 5 min (-76.0 ± 3.8%) was significantly different from that at 40 min (-44.9 ± 5.6%) (t-test, p<0.005,
Using the criterion of a reduction in inhibition by dopamine of at least 15%, 10 of the 12 cells exhibited reversal, whereas in the non-reversed neuron, one dopamine-induced inhibition went from -84% at 5 min to -70.5% at the end of 40 min, and the other went from -66% at 5 min to -100% at the end of 40 min.

**Long-term administration of single quinpirole concentrations**

One possibility is that there is DA D2 receptor desensitization as a result of the long application of higher DA agonist concentrations. To test this possibility, we assessed the effect of a long administration of the D2 receptor agonist quinpirole (concentration causing a greater than 50% inhibition at 5 min, mean concentration of quinpirole = 43.5 ± 3.42 nM; mean change in firing rate at 10 min = -73.6%) (Figure 4). Unlike the effect of DA, prolonged application of quinpirole produced inhibition that was not reversed with time; inhibition became significantly greater between 5 and 15 minutes, and then no significant change in inhibition between 15 and 40 minutes (one-way repeated measures ANOVA P<0.001, Student-Newman-Keuls post-hoc, significant differences P<0.05). This observation suggests that the reversal of DA inhibition was not induced solely by desensitization of D2 receptors.

As there is evidence for D1/D5 receptors in the VTA and activation of these receptors results in depolarization of DA VTA neurons(Schilstrom et al., 2006), we tested whether concomitant stimulation of D1/D5 receptors are involved in the reversal of DA inhibition. A D1/D5 agonist, SKF38393 (10 μM), was added to the superfusate with quinpirole (102 ± 20 nM, n=10). We do not know the reason for the need for higher quinpirole concentrations in the presence of SKF38393 (102 nM) compared to the quinpirole concentration needed in the absence of SKF38393 (43.5 nM); it is possible that this is due to partial blockade of D2 receptors by SKF38393, as the affinity of SKF38393 for the D1 receptor is only 150 times greater than its affinity for the D2 receptor (Seeman
and Van Tol, 1994). This concomitant stimulation of D2 receptors (by quinpirole) and D1/D5 receptors (by SKF38393) should mimic the stimulation of these receptors by dopamine. With the treatment with SKF38393, some reversal of the quinpirole inhibition was observed; there was a significant difference between the firing rate at 10, 15 and 20 minutes compared to the firing rate at 40 minutes (Friedman repeated measures ANOVA on ranks P<0.001, Tukey post-hoc, significant differences P<0.05). Treatment with SKF38393 alone produced a small but significant increase in the firing rate at 40 minutes compared to that at 5, 10 and 15 minutes (one-way repeated measures ANOVA P<0.001, Student-Newman-Keuls post-hoc, significant differences P<0.05) (Figure 4). These results suggest that stimulation of D1/D5 receptors is required for dopamine inhibition reversal.

We also tested whether long-term treatment with dopamine itself can produce excitation in the absence of D2-mediated inhibition. We assessed the effect of a 40 min application of dopamine in the presence of the specific D2 receptor antagonist sulpiride. With co-application of sulpiride (35 ± 5.67 μM) and a single concentration of DA (9.4 ± 5.61 μM), the DA inhibition was blocked, and dopamine over 40 min produced no statistically significant increase in firing rate (one-way repeated measures ANOVA, P > 0.05, n=4) (data not shown).

Effect of quinpirole after induction of dopamine inhibition reversal

It is clear from the results described above that quinpirole alone does not induced inhibition reversal. We also tested whether there was alteration in sensitivity to D2 receptor stimulation after induction of dopamine inhibition reversal by dopamine itself. Quinpirole (25 nM) was applied for 10 min to a population of pDA VTA neurons and reduced the spontaneous firing rate (range 46-99%, mean 78.6 ± 7.3%, n=9). Quinpirole was washed out for at least 30 minutes, and the firing rate was
restored to the pre-quinpirole baseline. Then, dopamine (10 µM) was applied for 40 minutes, and dopamine inhibition reversal was induced (Figure 5A). Dopamine was washed out for 30 min and then quinpirole was again tested to assess any change in the effect of this D2 agonist following the dopamine treatment. After dopamine inhibition reversal was induced by dopamine, the effect of acutely administered quinpirole was significantly reduced compared to the effect of quinpirole before dopamine treatment (Figure 5B). The mean inhibition produced by quinpirole before dopamine was 78.6 ± 7.3%; however, 30 min after washout of dopamine, the quinpirole-induced inhibition was 49.1 ± 8.6% (t-test, P<0.02, n=9). This indicates that there is desensitization of D2 receptors after dopamine inhibition reversal. In order to compare our studies with those of Bartlett, et al (2005), we tested the effect of low concentrations of quinpirole before and after a 25 min exposure to 3 µM quinpirole. Following exposure to this high quinpirole concentration, there was significant desensitization to the inhibitory effect of low quinpirole. We repeated the experiment in the presence of D1/D5 antagonist SCH39166, and found that the D1/D5 antagonist blocked the desensitization induced by high quinpirole exposure (Figure 5C). Interestingly, the lower concentration of quinpirole needed to produce about 45% inhibition was almost 8 times higher in the presence of SCH 39166 (285 ± 77 nM) than in its absence (36 ± 13 nM).

Effect of D1/D5 agents on dopamine inhibition reversal

We also tested the involvement of D1/D5 receptors by applying DA in the presence of D1/D5 receptor antagonists. Both SCH39166 (10 µM n=10) (Figure 6A) and SCH23390 (10 µM, n=8) (Figure 6B) prevented the characteristic reversal of DA inhibition, supporting the idea that pDAergic stimulation of D1/D5 receptors is necessary for inhibition reversal. With SCH39166, there was a statistically significant decrease in the firing rate with time in DA, but no inhibition reversal (Friedman repeated measures ANOVA on ranks, Tukey post hoc P<0.05 for comparison between 5
min time point and 20-35 min time points). With SCH23390, there was no statistical difference in the effect of DA at the 5, 10 and 40 time points, indicating a blockade of the DA inhibition reversal (Friedman repeated measures ANOVA on ranks, Tukey post hoc P>0.05 for comparison between all time points 5-40 min).

We examined the possibility that a D1/D5 antagonist could antagonize dopamine inhibition reversal once it occurs. A concentration of dopamine sufficient to cause inhibition of 60% or greater was applied for 40 min, and then dopamine was continued for an additional 40 min, either alone (n=6, mean dopamine concentration= 5.1 ± 0.9 µM) or in the presence of 10 µM SCH23390 (n=4, dopamine concentration= 4.0 ± 1.4 µM). There was a significant reduction in the effect of dopamine over the 80 minute period in both groups (two-way ANOVA, F=7.37, p < 0.001). In addition, there was a statistically significant difference in the effect of dopamine overall between the cells treated with dopamine alone (n=6) or with dopamine plus SCH23390 (n=4) (two-way ANOVA, p < 0.02), but there was no antagonism of the dopamine inhibition reversal (Figure 6C).
Discussion:

Our results demonstrate that elevation of DA concentration in the VTA initially reduces the firing rate, as shown by numerous laboratories previously, but sustained inhibition of pDAergic neurons by DA exhibits reversal with time. This reversal is apparently due to D2 receptor desensitization induced by concurrent stimulation of D2 and D1/D5 receptors, but not by stimulation of D2 receptors alone. This desensitization is long lasting, affecting the response to dopamine for up to 90 min. Desensitization of D1 receptors has been demonstrated (Ng et al., 1994; Ng et al., 1995). While D2 desensitization has been previously observed (Barton et al., 1991; Bartlett et al., 2005), this is the first report of a necessity of interaction of D1/D5 receptors and D2 receptors that results in a functional decrease in the effect of D2 receptor stimulation in the VTA. We have termed this phenomenon “dopamine inhibition reversal”, as it requires stimulation of both D2 and D1/D5 receptors, rather than classical desensitization that requires stimulation of only the primary receptor, i.e., in this case, D2 receptors.

The results of the present study suggest that reversal of dopamine inhibition depends on a balance between D2 and D1/D5 stimulation. With sufficiently large D2 stimulation (e.g., 3 µM quinpirole (Figure 5C)), the modest stimulation of D1/D5 receptors by ambient concentrations of dopamine may be sufficient to produce desensitization of D2 receptors. The need for D1/D5 stimulation is shown by the blockade of this desensitization by a D1/D5 antagonist (Figure 5C). In cases of more modest D2 receptor stimulation (40 -100 nM quinpirole), stimulation of D1/D5 receptors by ambient dopamine in the brain slice is not sufficient to produce inhibition reversal (Figure 4). Some reversal of the inhibition was observed when these lower concentrations of quinpirole were used in the presence of the D1/D5 agonist (Figure 4). More detailed concentration-response studies using different D2 and D1/D5 agonists are needed to determine the specific ratios.
of D2 and D1/D5 stimulation that result in inhibition reversal and long-lasting desensitization of the D2 receptor.

As the excitatory effect produced by the D1/D5 agonist SKF38393 was small in comparison to the difference between maximal DA inhibition and the inhibition after 40 minutes of DA exposure, a synergistic action of DA at D2 and D1/D5 receptors is possible. However, our observation that the inhibitory effect of quinpirole is reduced after 40 min of dopamine exposure (Figure 5A and 5B) indicates that there is a long-term change in D2 receptor sensitivity. This type of interaction has been observed in other preparations. The release of atrial naturetic factor (ANF) is inhibited by DA at lower concentrations, but at higher concentrations, the inhibition of release is reversed. The concentration-dependent reversal of inhibition of ANF release at higher DA concentrations in that study appeared to be due to activation of adenylate cyclase and protein kinase A, possibly as a result of stimulation of D1/D5 receptors (Lee et al., 2000). Future studies will examine possible G-protein interactions that might be involved in the mechanism of the reversal of DA inhibition in the VTA.

Desensitization of D2 receptors has been observed in some expression systems (Cho et al., 2006; Namkung and Sibley, 2004). Desensitization has been well studied in other G-protein coupled receptors (GPCR), like the adrenergic β receptor. The mechanism is generally related to phosphorylation of the GPCR under conditions of agonist occupancy, which results in de-coupling of the receptor and the G-protein, decreasing function of the receptors (Namkung and Sibley, 2004). A similar effect was observed in a study using DA VTA neurons in a brain slice preparation (Bartlett et al., 2005). In that study, 3 µM quinpirole was observed to induce desensitization of DA VTA neurons to subsequent quinpirole administration, an effect dependent upon G protein-coupled receptor-associated sorting protein (GASP). In the present study, quinpirole alone (< 200 nM) did not produce desensitization but required concomitant stimulation of D1/D5 receptors (Figure 4).
higher concentration of quinpirole in the Bartlett, et al, study may partially account for this difference. Endogenous dopamine may provide sufficient D1/D5 stimulation in combination with very large quinpirole concentrations to produce desensitization, as even 3 µM quinpirole in our study failed to produce significant desensitization in the presence of the D1/D5 antagonist SCH39166 (Figure 5c). It is unclear why, in the presence of SCH39166, higher concentrations of quinpirole were needed to produce a similar level of inhibition, as we did not need higher DA in the presence of D1/D5 antagonists (Figure 6). Additional studies will be necessary to determine if GASP, or other cell factors, participates in the interaction between D2 sensitivity and D1/D5 receptor activation.

In the present study, in addition to D2 receptor occupancy, D1/D5 receptor stimulation was needed to reduce the inhibitory action of the D2 agonist. Once produced, the desensitization is persistent for a long time after removal of inhibitory concentrations of dopamine. In addition, once the inhibition reversal is achieved, addition of a D1/D5 antagonist was ineffective in antagonizing the desensitization. These observations suggest a long-duration event, such as phosphorylation, occurs as a result of concurrent D2 and D1/D5 stimulation; stimulation of D1/D5 receptors is apparently necessary for induction but not maintenance of this long-duration event. Candidates for such possible phosphorylation are the receptor itself, proteins of the post-synaptic density (Carlsson and Carlsson, 2008), or dopamine-receptor interacting proteins (DRIPs) (Kabbani and Levenson, 2007). The precise mechanism, and the need for D1/D5 stimulation for induction but not maintenance of this phenomenon, will be the subject of future studies.

The regulation of DA autoinhibition shown above could be functionally important to neurons of the VTA under conditions in which persistent and prolonged release of DA might otherwise produce sustained inhibition of DA VTA firing. Under those conditions, DA could begin to stimulate the D1/D5 receptors, resulting in desensitization not apparent with D2 stimulation alone and could
serve to overcome the persistent inhibition of DA VTA neurons. Although it seems to require concentrations of DA that cause significant inhibition of firing (> 50%), it does not require high DA concentrations that cause complete cessation of firing. Furthermore, this desensitization may occur more robustly if there is a gradual increase in DA concentration (see Figure 1).

Adaptation of DA VTA neurons to administration of cocaine has been observed after repeated cocaine administration (Chen et al., 2008). Cocaine-induced long-term potentiation has been observed in brain slices, suggesting a local mechanism of LTP induction (Argilli et al., 2008). Cocaine increases the availability of extracellular DA in the VTA (Brodie and Dunwiddie, 1990). Sustained DA in the VTA (such as might be caused by cocaine administration) may increase excitability in the VTA by reducing D2 receptor-mediated autoinhibition. This increase in excitability may increase the effect of spontaneously-released glutamate as an increased response to AMPA and NMDA receptor stimulation, ultimately leading to a form of LTP. Additional studies will be needed to determine interactions between glutamatergic and dopaminergic systems under the conditions necessary to produce dopamine inhibition reversal.

Numerous drugs of abuse increase DA neurotransmission from the VTA (Di Chiara et al., 2004) in a sustained manner, and sustained increase in DA concentration in the VTA neurons may be factor common to all drugs of abuse. Extended increases in dopamine levels in the VTA could lead to D2 desensitization, which would alter information processing and autoregulation in this important brain area. It has been demonstrated, for example, that intermittent morphine produces a long-lasting desensitization of D2 receptors in nucleus accumbens (Nestby et al., 1995). Although the focus of these studies is often on the target regions of the nucleus accumbens and the prefrontal cortex, as the VTA is the source of pDAergic innervation to these regions as well as other parts of
the extended amygdala, the importance of adaptive changes in neurotransmission in the VTA may be a common feature of drug abuse disorders.

One unique aspect of the present study is the duration of the continuous recordings, which demonstrated the time-dependence and concentration-dependence of dopamine inhibition reversal. While there is a vast literature on the physiology and pharmacology of dopamine systems, previous electrophysiological studies generally examined shorter time courses of responses. For example, in a study of the effects of cocaine on neurons of the VTA in vivo (Einhorn et al., 1988), observed the effects of systemically administered dopaminergic agents and cocaine over periods lasting from 10-20 minutes, and did not report inhibition reversal. Those shorter duration recordings mostly tested doses of agents producing less than 50% inhibition. We found that longer duration application and concentrations reducing the firing rate by 50% or more are needed to observe dopamine inhibition reversal.

There are other pathological states that are associated with an increase in dopamine in the central nervous system. For example, the DA hypothesis of schizophrenia posits that increased DA levels are an important facet of this illness (Howes and Kapur, 2009). The increased DA in this pathological state may result in reduced autoregulation of dopaminergic neurotransmission. Knowledge of the mechanisms of D2 desensitization may lead to better understanding of the pathology and treatment of schizophrenia.
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Address for correspondence:

Mark S. Brodie, Ph.D.
Department of Physiology and Biophysics
University of Illinois at Chicago
835 S. Wolcott, Room E-202, M/C 901
Chicago, IL 60612-7342
E-mail: mbrodie@uic.edu
Legends for Figures:

Figure 1: Dopamine concentration-response curves using step-wise application of a range of dopamine concentrations. A. Ratemeter graph of the effect of acute application of a high DA concentration. Vertical bars indicate the firing rate over 5 sec intervals. Horizontal bars indicate the duration of application of DA (100 µM). Administration of DA produced complete cessation of firing, and the firing resumed after washout of the dopamine from the superfusate. B. Ratemeter graph of the effect of stepwise application of a series of DA concentrations (0.5-100 µM). Vertical bars indicate the firing rate over 5 sec intervals. Horizontal bars indicate the duration of application of DA (concentration in µM indicated above bar). Note that even though the concentration of dopamine was increased every 5 min, inhibition only increased up to 10 µM dopamine, and as concentrations were increased above 10 µM, the firing rate was less inhibited, and eventually firing increased above the pre-dopamine baseline rate. C. Change in spontaneous firing rate (mean ± S.E.M) in the presence of dopamine is plotted as a function of dopamine concentration (log scale). Dopamine was applied in five min steps over a range of concentrations (0.5 to 100 µM, n=63). There was a significant effect of concentration on inhibition with concentrations of 2-10 µM and 50 - 100 µM significantly different from control firing rate.

Figure 2: Dopamine concentration-response curves using long duration application of single dopamine concentrations. Change in firing rate (mean ± S.E.M.) produced by dopamine is plotted as a function of time. A. Effect of dopamine on spontaneous firing rate is plotted as a function of time. Concentrations of dopamine that produced 10, 25, 65, 75 or 100 % inhibition in the first five minutes were applied for 40 min. Choice of the concentration based on initial effect of dopamine controlled for sensitivity of the neurons to the inhibitory effect of dopamine. For concentrations that initially produced 10% (n=7) or 25% (n=7) inhibition in firing rate, there was no significant change in the
effect of dopamine over time. For concentrations that initially produced 65% (n=12), 75% (n=7) or 100% (n=11) inhibition in firing rate, there was a significant reduction in the inhibitory effect of dopamine over time, with the last three time points significantly different from the 5 min time point.

B. Effect of dopamine on spontaneous firing rate is plotted as a function of time for two groups of neurons in which dopamine produced 50% inhibition of firing. In more sensitive cells which were inhibited by 50% by low concentrations of dopamine ([DA] < 1.25 μM, n=13), there was no significant change in the effect of dopamine over time. In less sensitive cells which were inhibited by 50% by higher concentrations of dopamine ([DA] > 1.25 μM, n=8), there was a significant reversal of dopamine inhibition with time with the last three time points significantly different from the 5 min time point.

Figure 3: Mean ratemeter graphs of the effect of long duration dopamine exposure on response to shorter dopamine administration. Mean firing rate over 5 sec intervals is plotted as a function of time for cells exposed to dopamine for 5 min intervals before and after a 40 min exposure to 10 μM dopamine. Administration of low concentrations of dopamine (0.42 ± 0.12 μM) for 5 min produced inhibition of firing (28.1 ± 3.8%, n=6). After these test applications, 10 μM dopamine was applied for 40 min. Inhibition reversal was observed for this 40 min exposure to 10 μM dopamine. Following washout of 10 μM dopamine, the effect of brief 5 min exposures to lower concentrations of dopamine (same concentrations for each cell as tested before long term dopamine exposure) were significantly reduced at 30 min (10.0 ± 4.5%), but was not significantly different at 60 min (17.0 ± 1.7%) or 90 min (18.2 ± 1.6%) min after the long term exposure (one-way ANOVA, F=5.53, p<0.01; Tukey post-hoc test, P<0.05 for significance).
Figure 4: Inhibitory effects of long duration treatment with quinpirole and SKF38393. Long duration applications to VTA dopamine neurons of D2 agonist quinpirole, D1/D5 agonist SKF38393 and the combination were performed. Quinpirole (43.5 ± 3.4 nM, n=10) alone produced significant inhibition (one-way repeated measures ANOVA, P< 0.05, n=10) that reached a peak at about 15 minutes and the firing rate remained inhibited for the duration of quinpirole application. SKF38393 (10 µM) alone (n=6) had a small but significant excitatory effect on firing rate. In the presence of 10 µM SKF38393, quinpirole (102 ± 20 nM) inhibition partially reversed, so that the inhibition at 40 min was significantly less than that at 5 or 10 min (n=10).

Figure 5: Effect of quinpirole after induction of dopamine inhibition reversal. A. Ratemeter graph from a single putative dopaminergic neuron. Vertical bars indicate the firing rate averaged over a 5 sec interval; horizontal bars indicate the duration of application of either 25 nM quinpirole (Q-25) or 10 µM dopamine (DA-10). Note that the inhibitory effect of quinpirole after the 40 min dopamine treatment is much less than that observed before the dopamine treatment. Initially, quinpirole produced inhibition of about 90%; after dopamine treatment, the quinpirole-induced inhibition (tested at 30 min intervals) was 25%, 45% and 44% respectively. B. Effect of quinpirole before and after dopamine in the population of cells tested. Nine cells were tested in a protocol similar to that illustrated in part A above, and comparison was made between responses to quinpirole before and after dopamine treatment. The mean inhibition produced by quinpirole before dopamine was 78.6 ± 7.3%; 30 min after washout of dopamine, the quinpirole-induced inhibition was 49.1 ± 8.6% (t-test, P<0.02, n=9). C. Effect of quinpirole before and after 3 µM quinpirole. Thirteen cells were tested in a protocol similar to that illustrated in part A above, except that 3 µM quinpirole was applied for 25 min instead of the 40 min dopamine application. Comparison was made between responses to low concentrations of quinpirole before (light bar) and after (dark bar) 3 µM quinpirole treatment. The
mean inhibition produced by low quinpirole (mean concentration = 36 ± 13 nM) before the 3µM application was 44.8 ± 2.8%; within one hour of the washout of 3 µM quinpirole, the same concentration of quinpirole induced an inhibition of only 14.8 ± 4.7% (t-test, P<0.005, n=6). In the presence of 10 µM SCH-39166, the mean inhibition produced by low quinpirole (mean concentration = 285 ± 77 nM) before the 3µM application was 42.7 ± 7.8%; within one hour of the washout of 3 µM quinpirole, the same concentration of quinpirole induced an inhibition of 35.1 ± 7.9% that was not significantly different from the value before 3 µM quinpirole treatment (t-test, P>0.05, n=7).

Figure 6: Effects of D1/D5 antagonists on dopamine inhibition reversal. Antagonists applied 10 min before dopamine (A and B): A concentration of dopamine sufficient to cause inhibition of 60% or greater was applied for 40 min in the presence of either (A) 10 μM SCH39166 (dopamine concentration = 2.4 ± 0.4 μM, n=10) or (B) 10 μM SCH23390 (dopamine concentration = 3.9 ± 1.4 μM, n=8). No significant reduction in dopamine-induced inhibition was observed in the presence of either antagonist. On both A and B, the response to a concentration of dopamine that produced 65% inhibition (from Figure 2) is shown for reference (open symbols and dashed line). Antagonists applied after dopamine (C): A concentration of dopamine sufficient to cause inhibition of 60% or greater was applied for 40 min, and then dopamine was continued for an additional 40 min, either alone (n=6, mean dopamine concentration= 5.1 ± 0.9 μM) or in the presence of 10 µM SCH23390 (n=4, dopamine concentration= 4.0 ±1.4 µM). There was a significant reduction in the effect of dopamine over the 80 minute period in both groups (two-way ANOVA, F=7.37, p < 0.001) and a statistically significant difference in the effect of dopamine overall between the cells treated with dopamine alone (n=6) or with dopamine plus SCH23390 (n=4) (two-way ANOVA, p < 0.02). Note that there was no antagonism of the dopamine inhibition reversal with the addition of SCH23390.
Figure 2

A

Change in firing rate produced by dopamine (%)

% Inhibition at the first 5 min
- 10%
- 25%
- 65%
- 75%
- 100%

Time (min)

B

Change in firing rate produced by dopamine (%)

Mean [DA] = 1.25 ± 0.2 uM
- [DA] < 1.25 ± 0.2 uM (n = 13)
- [DA] > 1.25 ± 0.2 uM (n = 8)

Time (min)
Figure 5

A

![Graph showing firing rate over time](image)

B

![Bar graph showing change in firing rate](image)

C

![Bar graph showing change in firing rate produced by low concentration of quinpirole](image)
Figure 6

(A) Change in firing rate (%) vs. Time (min)

(B) Change in firing rate (%) vs. Time (min)

(C) Change in firing rate (%) vs. Time (min)

Legend:
- ■ Dopamine alone
- ▼ Dopamine + SCH23390