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Dopamine D2 receptor desensitization by dopamine or corticotropin releasing factor in ventral tegmental area neurons is associated with increased glutamate release

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Abstract

Neurons of the ventral tegmental area (VTA) are the source of dopaminergic (DAergic) input to important brain regions related to addiction. Prolonged exposure of these VTA neurons to moderate concentrations of dopamine (DA) causes a time-dependent decrease in DA-induced inhibition, a complex desensitization called DA inhibition reversal (DIR). DIR is mediated by conventional protein kinase C (cPKC) through concurrent stimulation of D2 and D1-like DA receptors, or by D2 stimulation concurrent with activation of some Gq-linked receptors. Corticotropin releasing factor (CRF) acts via Gq, and can modulate glutamatergic neurotransmission in the VTA. In the present study, we used brain slice electrophysiology to characterize the interaction of DA, glutamate antagonists, and CRF agonists in the induction and maintenance of DIR in the VTA. Glutamate receptor antagonists blocked induction but not maintenance of DIR. Putative blockers of neurotransmitter release and store-operated calcium channels blocked and reversed DIR. CRF and the CRF agonist urocortin reversed inhibition produced by the D2 agonist quinpirole, consistent with our earlier work indicating that Gq activation reverses quinpirole-mediated inhibition. In whole cell recordings, the combination of urocortin and quinpirole, but not either agent alone, increased spontaneous excitatory postsynaptic

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currents (sEPSCs) in VTA neurons. Likewise, the combination of a D1-like receptor agonist and quinpirole, but not either agent alone, increased sEPSCs in VTA neurons. In summary, desensitization of D2 receptors induced by dopamine or CRF on DAergic VTA neurons is associated with increased glutamatergic signaling in the VTA.

Keywords

Desensitization; Dopamine D2 receptor; glutamate; urocortin; quinpirole; sEPSC

1. Introduction

Several regions of the mesocorticolimbic system receive dopaminergic (DAergic) input from the ventral tegmental area (VTA), including the nucleus accumbens, prefrontal cortex, and amygdala (Koob, 2003; Oades and Halliday, 1987). Activity of the VTA is increased by salient and motivational stimuli, and the resulting DAergic output is important for the rewarding and reinforcing effect of numerous drugs of abuse (Di Chiara and Imperato, 1988; Wise, 1996; Mirenowicz and Schultz, 1996). Mesencephalic DAergic neurons possess D2 receptors that act as autoreceptors to inhibit spontaneous firing when activated by extracellular dopamine (DA) (Aghajanian and Bunney, 1977). While short-term increases in DA concentration reduce the excitability of DAergic neurons of the VTA, long-term increases of DA concentration result in desensitization of the D2 receptor to DA (Nimitvilai and Brodie, 2010). In addition, other receptors could also be affected by long-term increases in DA; for example, elevated DA increases glutamatergic receptor expression in prefrontal cortex (Gao and Wolf, 2008; Sun, et al., 2008).

DAergic neurons of the VTA possess high densities of D2 (Bouthenet, et al., 1991; Sesack, et al., 1994) and D5 receptors (Ciliax, et al., 2000; Khan, et al., 2000), but low levels of D3 receptors (Bouthenet, et al., 1991; Diaz, et al., 1995; Gurevich and Joyce, 1999). D1 and D4 receptors are quite sparse or are not detectable on DAergic VTA neurons (Meador-Woodruff, et al., 1992; Mengod, et al., 1992; Rivera, et al., 2008). However, D1 receptors are present on glutamatergic terminals projecting to the VTA (Caillé, et al., 1996).

Spontaneous firing of action potentials by DAergic VTA neurons is inhibited by the action of DA at D2 autoreceptors (Lacey, et al., 1987; Brodie, et al., 1990). Prolonged application of DA results in a time- and concentration-dependent decrease in the magnitude of DA-induced inhibition, a phenomenon called “DA inhibition reversal” or DIR (Nimitvilai and Brodie, 2010). DIR is mediated by extended (10–30 min) and concurrent stimulation of D2 and D1-like receptors and produces desensitization to D2 inhibition lasting up to 90 min (Nimitvilai and Brodie, 2010). DIR requires stimulation of phosphatidylinositol (PI) turnover, the activation of phospholipase C (PLC) and conventional protein kinase C (cPKC), and does not involve adenylyl cyclase, cAMP or protein kinase A (Nimitvilai, et al., 2012a). Some, but not all, Gq-coupled receptors that stimulate the PLC and PKC pathways can mediate the reversal of D2 agonist inhibition (Nimitvilai, et al., 2012d). One putative neurotransmitter that was not tested in that previous study was corticotropin releasing factor (CRF), which is coupled to Gq in some systems (Gutknecht, et al., 2009) and which is present in the VTA (Tagliaferro and Morales, 2008; Wang and Morales, 2008).

The characterization of DIR has established parallels between DIR and conventional desensitization of the D2 receptor. Involvement of PKC in phosphorylation and internalization of D2 receptors has been reported in many systems such as HEK293 cells, striatal, hippocampal and VTA neurons (Nimitvilai, et al., 2013; Namkung and Sibley, 2004; Bofill-Cardona, et al., 2000; Thibault, et al., 2011). Other actions of dopamine in the VTA may play a role in DIR. For example, increased activity of excitatory glutamatergic inputs during sustained dopamine administration could counteract ongoing D2-mediated inhibition and would appear as a reversal of inhibition.

Release of CRF in the CNS is linked to stress, and CRF release in the VTA may be a factor in reinstatement of abused substances. The role of CRF in the VTA is not fully understood, but as CRF is Gq-linked in some systems, it may have a role in DIR like other Gq-linked receptors (Nimitvilai, et al., 2012d). Furthermore, any link between glutamate and DIR has not been explored. In the present study, we examine the possible contribution of glutamatergic and the CRF mechanisms in the phenomenon of DIR.

2. Methods

2.1 Animals

Male Fischer 344 (F344; adult rats, 4–6 weeks old, 90 – 150 g) or Wistar rats (90–195 g) used in these studies were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Patch-clamp studies employed Wistar rats, and extracellular studies employed both strains of rats. 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 and by the Institutional Animal Care and Use Committee of the Scripps Research Institute.

2.2 Preparation of brain slices

Brain slices containing the ventral tegmental area (VTA) were prepared as previously described (Brodie, et al., 1999a). Briefly, following brief isoflurane anesthesia and 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 to cut coronal sections (400 μ m). An individual slice was placed onto a mesh platform in the recording chamber and was totally submerged in artificial cerebrospinal fluid (aCSF) maintained at a flow rate of 2 ml/min; the temperature in the recording chamber was kept at 35° 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. 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 of recovery was allowed before electrodes were placed in the tissue.

2.3 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. Putative DAergic (pDAergic) neurons have been shown to have distinctive electrophysiological characteristics (Grace and Bunney, 1984; Lacey, et al., 1989). Only those neurons that were anatomically located within the VTA and that 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., 2006a; Margolis, et al., 2003). Additional characterization, such as determining the neurotransmitter content or projection target of our cells of study (Margolis, et al., 2008; Margolis, et al., 2006b; Chieng, et al., 2011), would have been difficult as we used extracellular recording to insure high quality, long duration recordings. The long-duration, low frequency action potentials which characterized the cells from which we recorded are associated with DA-sensitive, DA-containing neurons projecting to the nucleus accumbens, and DA sensitivity also is associated with DA VTA neurons projecting to prefrontal cortex (Margolis, et al., 2008).

2.4 Drug Administration

Drugs were added either to the aCSF or to the microelectrode filling solution (0.9% NaCl). Addition of drugs to the aCSF was performed using stock solutions 100 to 1000 times the desired final concentrations. Knowing the flow rate of the superfusion medium (2 ml/min), we used calibrated syringe pumps to infuse drugs at rates between 0.02 ml/min (1% of the aCSF flow rate) and 0.002 ml/min (0.1% of the aCSF flow rate) into the flowing medium, resulting in dilution of the stock solutions to the desired concentrations. Infusion of these concentrated solutions into the aCSF flow was performed prior to the solution flowing through tubing coils which permitted the drug solutions 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.

When drugs were added to the microelectrode filling solution (0.9% NaCl), a concentration about 10 times greater than the concentration used in the extracellular medium was needed. In all of our previous studies in which agonists and antagonists were delivered via the recording pipette (Nimitvilai, et al., 2012a), the effective concentration of drugs were ten-fold higher than the effective concentration used in the extracellular medium. The concentrations of drugs used in the present study were likewise ten-fold higher than the concentrations reported in the literature for selective action. To allow time for the drug to diffuse from the pipette to the cell, the effects of bath-applied drugs were tested no less than 20 min after initiating the recording; this pipette-application method has produced comparable results to the administration of drugs through the extracellular medium in the cases in which both methods were tested, with the advantage of more localized application

and reduced expense. Such local delivery of drugs through recording pipettes has been used by our lab and others (Nimitvilai, et al., 2012a; Pesavento, et al., 2000). One disadvantage of this method is that the exact concentration of drug received by the neurons from which we recorded is unknown.

DA hydrochloride, quinpirole, and most of the salts used to prepare the extracellular media were purchased from Sigma (St. Louis, MO). DL-AP5, CNQX, MCPG, lamotrigine, riluzole, SKF96365, bicuculline, CGP35348, CRF, urocortin, antisauvagine-30, CGP55845A and NBI27914 were purchased from R & D Systems/Tocris Bioscience (Minneapolis, MN).

2.5 Extracellular recording

Extracellular recording was chosen for these studies as this method permits the recordings to be of long duration and allows us to assess the effects of extended exposure (>60 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 without disrupting the internal milieu. Extracellular recording electrodes were made from 1.5 mm diameter glass tubing with filament and were filled with 0.9% NaCl (or 0.9% NaCl-based drug solutions). Tip resistance of the microelectrodes ranged from 2 – 5 MΩ. An extracellular amplifier was used in conjunction with an IBM-PC-based data acquisition system (ADInstruments Inc., Colorado Springs, CO). Offline analysis was used to calculate, display and store the frequency of firing in 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 $((FRD - FRC) / FRC) \times 100$, 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 that may occur over time. This formula was used to calculate both excitatory and inhibitory drug effects. Peak excitation produced by the drug was defined as the peak increase in firing rate over pre-drug baseline. Inhibition was defined as the lowest firing rate below the pre-drug baseline. Inhibition reversal was identified as a statistically significant reduction in the inhibition induced by dopamine agonists over time.

2.6 Whole-cell recordings

VTA neurons were visualized using infrared differential interference contrast (IR-DIC) optics and an EXi Aqua camera (QImaging, Surrey, BC, Canada). A 60x magnification water immersion objective (Olympus) was used for identifying and approaching neurons. Whole-cell (voltage-clamp and current-clamp) recordings were made with patch pipettes (4–6 MΩ; Warner Instruments) coupled to a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA), low-pass filtered at 2–5 kHz, digitized (Digidata 1440A; Axon Instruments), and stored on a computer using pClamp 10 software (Axon Instruments). Series resistance was typically <10 MΩ and was continuously monitored with a 10 mV

pulse. The intracellular solution used for voltage clamp recordings was composed of (in mM): potassium gluconate 145; EGTA 5; MgCl₂ 5; HEPES 10; Na-ATP 2; Na-GTP 0.2. To isolate only the currents mediated by glutamate receptors, recordings ($V_{\text{hold}} = -60\text{mV}$) were performed in the presence of the GABA_A receptor antagonist bicuculline (30 μM) and the GABA_B receptor antagonist CGP55845A (1 μM). Frequency, amplitude and decay of spontaneous excitatory postsynaptic currents (sEPSCs) were analyzed and visually confirmed using a semi-automated threshold based mini detection software (Mini Analysis, Synaptosoft Inc.). Averages of sEPSC characteristics were determined from baseline and experimental drug conditions containing a minimum of 60 events (time period of analysis varied as a product of individual event frequency) and decay kinetics were determined using exponential curve fittings and reported as decay time (ms). All detected events were used for event frequency analysis, but superimposed events were eliminated for amplitude and decay kinetic analysis.

2.7 Statistical analysis

Averaged numerical values were expressed as the mean $\pm$ the standard error of the mean (S.E.M.). Data in mean response graphs are shown as percentage change in firing rate compared to the pre-drug baseline. Effects of inhibitors alone on firing rate were assessed using a paired t-test. To address the question of whether there is a change in the magnitude of inhibition by dopamine agonists over time, the differences among firing rates during the long drug administration intervals in these studies was assessed with one-way repeated measures ANOVA; degrees of freedom and statistical error terms are shown as subscripts to F in the text (Kenakin, 1987). Comparisons of degree of reversal of inhibition were not made, as there are a variety of factors that may contribute to different degree of reversal, including concentration of agonist (Nimitvilai and Brodie, 2010). Statistical analyses were performed with OriginPro 8.5 (OriginLab Corp. Northampton, MA). Voltage clamp recordings of sEPSCs were analyzed for independent significance using a one-sample t-test and compared using a two-tailed t-test for independent samples, a paired two-tailed t-test for comparisons made within the same recording, and a one-way ANOVA with a Bonferroni *post hoc* analysis for comparisons made between 3 or more groups. The difference in sEPSC characteristics over time was assessed by one-way repeated measures ANOVA between all time-points or paired t-test between specific time-points. All statistical analysis was performed using Prism 5.02 (GraphPad, San Diego, CA).

3. Results

3.1 EXTRACELLULAR STUDIES

3.1.1 VTA neuron characteristics—A total of 157 VTA neurons were tested to examine the effects of various agents on dopamine or quinpirole inhibition. Their firing rate in normal extracellular medium ranged from 0.59 to 4.59 Hz, with a mean of 1.78 ± 0.07 Hz. All neurons had regular firing rates and were inhibited by dopamine agonists. In most experiments, sensitivity to dopamine (0.5 – 5 μM) was initially assessed by administering the agonist for five minutes, and then washing it out until the firing rate recovered to at least 70% of the baseline firing rate; quinpirole (25–150 nM) was administered for 5 min, and the concentration was increased if inhibition greater than 50% was not achieved. The

concentrations of agonist were adjusted for each neuron so that inhibition exceeded 50%, as inhibition that was less than 50% was not reliably reversed (Nimitvilai and Brodie, 2010). This method of adjusting the concentration of DAergic agonist controlled for differences in sensitivity between neurons, but also sometimes resulted in the mean concentrations of dopamine or quinpirole slightly differing between groups. Overall, for pDAergic VTA neurons from adult rats subjected to this protocol, the concentration of dopamine used was $4.33 \pm 0.41 \mu\text{M}$ ($n = 63$) which produced a mean change in firing rate of $-66.5 \pm 1.6\%$ after 5 min of exposure; the concentration of quinpirole used in these experiments was $48.9 \pm 4.2 \text{ nM}$ ($n = 59$) which produced a mean change in firing rate of $-70.8 \pm 2.3 \%$ after 5 min of exposure.

In a few experiments, the concentration of quinpirole administered was set at $0.5 \mu\text{M}$; in these experiments, the mean change in firing rate after 5 min exposure was $-97.0 \pm 0.9 \%$ ($n=29$).

There were no significant differences in the concentration of DAergic agonists (when concentration was variable) or in the percentage inhibition among the groups (Table 1; One-way ANOVA, $p > 0.05$). In the absence of DA transporter blockers, dopamine produces inhibitory effects at concentrations ranging from 0.5 to $100 \mu\text{M}$, 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 at least 70% of their 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 90.1 ± 1.06 minutes, with a range of 70 to 110 minutes.

The effects of various agents tested in this study on the baseline firing frequency of VTA neurons is shown in Table 2. When agents were included in the recording pipette, the firing rate upon beginning of the recording was compared with the rate after 20 min. Only lamotrigine and riluzole significantly altered the baseline frequency; in both cases the mean change in firing was less than 12%. We have previously reported that SKF96365 had no significant effect on baseline firing (Nimitvilai, et al., 2012a). In the case of CNQX and DL-AP5, some cells that were subsequently tested with dopamine were acquired in the presence of the antagonist, so no baseline was possible; for this reason, additional recordings were made to assess the effect of CNQX ($n=5$) and DL-AP5 ($n=7$) on baseline firing rate after 20 min of administration, analogous to the time points for the effects of agents prior to DIR testing; these additional data were averaged with the available data from cells subsequently tested with dopamine ($n=3$ for CNQX, $n=2$ for DL-AP5). These averages are shown in Table 2.

3.1.2 Effects of glutamate receptor antagonists on induction and maintenance of DIR—

As noted above, increases in glutamate transmission produced by increased extracellular dopamine during the development of DIR could counteract D2-mediated inhibition; if this were the case, blockade of glutamate receptors on DA VTA neurons would antagonize the induction and maintenance of DIR. To test for the involvement of glutamate in the induction of DIR, DA was applied for 40 min in the presence of ionotropic and metabotropic glutamate receptor antagonists. With administration of dopamine alone, there

was a gradual decrease in dopamine-induced inhibition, indicative of DIR (Figure 1A and 1C); in the presence of the NMDA antagonist DL-AP5 (10 μ M), there was no DIR over the 40 min DA administration (Figure 1B and 1C). Mean data in Figure 1(C, D and E) represent changes in firing rate as a percentage of the baseline firing rate. The mean change in firing rate for all groups produced by dopamine at the 5 min time point is shown in Table 1. Concentrations of DA that produced greater than 50% inhibition within 5 min were used (see Table 1); under control conditions, the firing rate gradually increased over the 40 min application, indicating a reduced inhibitory effect of DA over time. In contrast, in the presence of an antagonist either of glutamatergic NMDA receptors (DL-AP5, 10 μ M, Figure 1C) or of glutamatergic AMPA receptors, (CNQX, 10 μ M, Figure 1D), there was a gradual further reduction of the firing rate over the 40 min application period. In the presence of the metabotropic glutamate receptor antagonist MCPG (10 μ M, Figure 1E), there was no change in dopamine-induced inhibition over time. These results indicate an involvement of all three glutamate receptor subtypes in the induction of DIR.

We also tested whether glutamate antagonists that could block the induction of DIR could also reverse DIR once it was established. Dopamine was applied for 40 min which produced DIR (one-way repeated measures ANOVA, $F_{(15,90)} = 7.78$, $p < 0.05$ comparison of 5 min and 40 min time points) before a combination of DL-AP5, CNQX and MCPG (10 μ M each) were added to the superfusate, and then these glutamate receptor antagonists and DA were continued for an additional 40 min, for a total of 80 min administration of DA. Figure 1F illustrates a representative recording of a single DA VTA neuron subjected to this protocol. Mean changes in firing rate as compared to the baseline firing rate are shown with (Figure 1G) or without (Figure 1H) the addition of glutamate receptor antagonists. In the absence of antagonists, DA produced inhibition at 5 min, and that inhibition subsided over time, reaching a peak at 30 min that was sustained over the remaining 50 min (Figure 1G). The mean results presented in Figure 1H indicate that no significant change from the time-course of the response to DA (i.e., no reduction of DIR) was observed in the presence of a combination of the three glutamate antagonists, indicating that the glutamate receptor blockers did not antagonize DIR once it had been established (one-way repeated measures ANOVA, Tukey post-hoc test, $p > 0.05$ comparisons between 40 and 80 min).

3.1.3 Effects of riluzole, lamotrigine, and SKF96365 on induction and maintenance of DIR—

As the results illustrated in Figure 1 indicate involvement of glutamate in induction of DIR, we also determined whether blocking glutamate release could block the induction of DIR. Lamotrigine and riluzole have been reported to prevent synaptic release of glutamate (Martin, et al., 1993; Teoh, et al., 1995), and have been shown to block sodium currents (Spadoni, et al., 2002; Stefani, et al., 1997), calcium currents (Stefani, et al., 1997) and ryanodine receptors (Neumann and Copello, 2011). Therefore, we used them to test the possibility that glutamate release was part of the mechanism undermined by glutamate antagonists to interfere with DIR. A representative experiment with riluzole is shown in Figure 2A (for comparison see Figure 1A). In the presence of either riluzole (10 μ M, Figure 2A and 2B) or lamotrigine (25 μ M, Figure 2C), DIR did not develop, and the inhibition produced by dopamine was sustained throughout the 40 min

application period. This result supports the idea that the induction of DIR is dependent on neurotransmitter release, possibly glutamate release.

As blockade of glutamate receptors after induction of DIR did not restore DA sensitivity, we tested whether lamotrigine and riluzole could reverse DIR once it was induced. For these experiments, DA was applied for 40 min, DIR was established, and then in the continued presence of DA, either lamotrigine (25 μ M, Figure 2E) or riluzole (10 μ M, Figure 2F) were applied for an additional 40 min. Figure 2D illustrates a representative recording of a single DA VTA neuron subjected to lamotrigine in this protocol; note that after lamotrigine was added, there was a reduction in firing rate and that the firing rate returned to baseline after washout of both agents. Figure 2E illustrates the mean change in firing rate occurring when lamotrigine (25 μ M) was added to the superfusate at the 40 min time point and maintained throughout the remainder of the experiment; lamotrigine antagonized DIR, and dopamine inhibition was significantly greater at the 80 min time point than at the 40 min time point (one-way repeated measures ANOVA, $F_{(15,120)} = 16.65$, $p < 0.05$). Similarly, when riluzole was added at the 40 min time point, it antagonized DIR (Figure 2F), so that DA-induced inhibition was increased significantly at the 80 min time point compared to the inhibition at the 40 min time point (one-way repeated measures ANOVA, $F_{(15,75)} = 8.48$, $p < 0.05$). In contrast with the experiment using glutamate antagonists, these results suggest the possibility that maintenance of DIR requires either synaptic release of other neurotransmitters, or the functioning of ion channels that are sensitive to lamotrigine and riluzole.

As both riluzole and lamotrigine have effects on calcium currents, we also tested whether interfering with store-operated calcium entry (SOCE) could reverse DIR. We have shown previously that the SOCE antagonist SKF96365 blocks the induction of DIR (Nimitvilai, et al., 2012a). Here we show that when SKF96365 was added at the 40 min time point, it reversed DIR, so that the inhibitory effect of dopamine was greater at 80 min than at the 40 min time point (one-way repeated measures ANOVA, $F_{(15,60)} = 10.69$, $p < 0.05$; Figure 2G). While this does not prove that the effect of riluzole or lamotrigine on calcium channels was the mechanism by which they antagonized DIR, this supports the idea that ongoing calcium channel activity is necessary for the maintenance of DIR.

3.1.4 Interaction of CRF with D2 receptor activation—Unlike DA, inhibition produced by moderate concentrations of the D2 agonist quinpirole does not show reversal (Nimitvilai and Brodie, 2010). Reversal of D2-mediated inhibition requires concurrent activation of D2 and D1-like receptors (Nimitvilai and Brodie, 2010) or concurrent activation of D2 and specific Gq-linked receptors (Nimitvilai, et al., 2012d). Activation of CRF receptors has been linked to Gq activation (Ungless, et al., 2003; Hillhouse, et al., 2002; Ulisse, et al., 1990) and CRF is among the neurotransmitters that interact with glutamate in the VTA (Ungless, et al., 2003), so we tested whether CRF could reverse quinpirole-induced inhibition. As shown in our previous studies (Nimitvilai, et al., 2012d), administration of concentrations of quinpirole that produce about 50% inhibition at 5 min (50 ± 3.2 nM) results in inhibition that peaks at 15 min and is sustained over the remainder of the 40 min test period (Figure 3A). There was a significant decrease in mean firing rate from the 5 min to all other time periods, but no difference among the time periods from 10–

40 min (one-way repeated measures ANOVA, $F_{(7,28)} = 3.84$, $p < 0.01$, Tukey post-hoc test, $p < 0.05$ for significant differences; Figure 3A). With CRF (1 μM) in the recording pipette, a concentration of quinpirole that produced about 50% inhibition at 5 min (22.3 ± 2.3 nM) was administered for 40 min. There was a significant reduction in inhibition produced by quinpirole at 40 min compared to the peak inhibition, but not to the inhibition at 5 min (one-way repeated measures ANOVA, $F_{(7,28)} = 2.85$, $p < 0.05$; Tukey post-hoc test, $p < 0.05$ for significant differences; Figure 3A).

We also assessed the effect of another CRF agonist urocortin (Figure 3B). With 1 μM urocortin in the pipette, quinpirole (26.8 ± 4.0 nM) produced inhibition that reversed over time, so that quinpirole-induced inhibition was significantly reduced at the 35 min time point compared to the peak inhibition (one-way repeated measures ANOVA, $F_{(7,70)} = 2.83$, $p < 0.02$; Tukey post-hoc test, $p < 0.05$ for significant differences; Figure 3B). Repeating this protocol in the presence of the CRF2 receptor antagonist anti-sauvagine-30 (1 μM , ASG) resulted in a significant decrease firing rate from the 5 min time point to later time points but no reversal of quinpirole-induced inhibition (one-way repeated measures ANOVA, $F_{(7,70)} = 3.30$, $p < 0.01$; Tukey post-hoc test, $p < 0.05$ for significant differences; Figure 3B).

Administration of drugs via the recording pipette is a method we have used extensively in the past (Nimitvilai, et al., 2013; Nimitvilai, et al., 2012d). As confirmation of the effects of pipette application, we tested the effects of bath application of either ASG (100 nM) or the CRF1 receptor antagonist NBI27914 (100 nM, NBI) on the action of bath-applied urocortin on quinpirole inhibition. In these experiments, CRF antagonists were added to the superfusate 10 min prior to quinpirole administration. Quinpirole was then applied for 15 min to reach peak inhibition before urocortin (100 nM) was added to the superfusate and quinpirole was continued in the presence of the CRF antagonist for 40 min. Figures 3C and 3D illustrate typical experiments without antagonist (Figure 3C) and with NBI27914 (Figure 3D). As shown in Figure 3F, bath-applied urocortin reversed quinpirole inhibition so that there was a significant reduction of quinpirole-induced inhibition at all points compared to the 5 min time point (one-way repeated measures ANOVA, $F_{(7,35)} = 13.83$, $p < 0.005$; Tukey post-hoc test, $p < 0.05$ for significant differences). In the presence of ASG, there was no significant change in quinpirole-induced inhibition over time between time 5 min and 40 min (One-way repeated measures ANOVA, $F_{7,28} = 0.953$, $p > .05$, $n = 5$). Similarly, in the presence of NBI, there was no significant change in quinpirole-induced inhibition over time between time 5 min and 40 min (One-way repeated measures ANOVA, $F_{7,49} = 0.31$, $p > .05$, $n = 8$). These results indicate that both CRF1 and CRF2 receptors are involved in the urocortin-induced reversal of quinpirole inhibition.

The urocortin experiments were repeated in the presence of antagonists of GABA receptors in order to determine whether there was a contribution of GABA to the reversal of quinpirole-induced inhibition (Supplementary Figure S1A). The effects of GABA antagonists alone or in the presence of urocortin on firing rate is shown in Supplementary Table S1. Administration of quinpirole in the presence of the GABA_A receptor antagonist bicuculline and GABA_B receptor antagonist CGP35348 (10 μM each) produced a reduction in the firing rate with no reversal; there was a significant increase in inhibition at all points

compared to the 5 min time point (one-way repeated measures ANOVA, $F_{(7,42)} = 4.20$, $p < 0.002$; Tukey post-hoc test, $p < 0.05$ for significant differences). When urocortin was included in the pipette and bicuculline and CGP35348 were present in the extracellular medium, the change in firing rate over the 40 min period was similar to that for urocortin and quinpirole without GABA antagonists (Supplementary Figure S1A) (one-way repeated measures ANOVA, $F_{(7,28)} = 2.39$, $p = 0.047$; Tukey post-hoc test, $p > 0.05$ for comparison between time points).

Note that with a shorter administration of a higher concentration of quinpirole, reversal of inhibition was not observed. The results illustrated in Supplementary Figure S1B show that administration of 500 nM quinpirole for 25 min in the presence of GABA antagonists produced decreases in firing rate that persisted over the 25 min period; there was a significant increase in inhibition at all points compared to the 5 min time point (one-way repeated measures ANOVA, $F_{(7,42)} = 4.20$, $p < 0.002$; Tukey post-hoc test, $p < 0.05$ for significant differences). When urocortin (1 μ M) was included in the recording pipette, there was a significant decrease in inhibition at the 25 min time point compared to all other time points (one-way repeated measures ANOVA, $F_{(4,20)} = 5.91$, $p < 0.005$; Tukey post-hoc test, $p < 0.05$ for significant differences).

We also tested whether urocortin could reverse quinpirole-induced inhibition in DAergic VTA neurons in slices from male Wistar rats, a rat strain commonly used in alcohol research (Supplementary Figure S1C). In the presence of GABA antagonists, administration of 500 nM quinpirole produced a decrease in firing rate over 25 min time period with no reversal; there was no significant change in inhibition over the 25 min period (one-way repeated measures ANOVA, $F_{(4,32)} = 0.80$, $p > 0.05$). In the presence of urocortin (1 μ M) in the recording pipette and GABA antagonists in the extracellular medium, a significant reversal of quinpirole-induced inhibition was observed; there was a significant decrease in inhibition at all points compared to the 5 min time point (one-way repeated measures ANOVA, $F_{(4,28)} = 10.19$, $p < 0.001$; Tukey post-hoc test, $p < 0.05$ for significant differences).

3.2 WHOLE CELL RECORDINGS

3.2.1 Effects of urocortin and quinpirole on glutamate transmission in VTA DA neurons—Data from extracellular recordings strongly suggested that changes in glutamate release were involved in the development of DIR, however one limitation of this technique is the inability to directly examine synaptic currents. To determine if synaptic changes in glutamate release contributes to DIR, we performed whole-cell voltage clamp recordings of pharmacologically isolated glutamatergic spontaneous excitatory postsynaptic currents (sEPSCs) in VTA DA neurons. As the reversal of inhibition with quinpirole and urocortin observed in Fisher rats is also observed in extracellular studies conducted in Wistar rats (Supplementary Figure S1C), we performed experiments with the D2 agonist quinpirole and the CRF agonist urocortin in DA VTA neurons obtained from male Wistar rats. A total of 25 neurons were examined. In recordings with a baseline sEPSC frequency of 0.8 ± 0.2 Hz, superfusion of 500 nM quinpirole for 25 min (a concentration and time course previously shown to inhibit DA firing with no reversal of inhibition (Supplementary Figure 1C)), produced no significant change in sEPSC frequency (0.6 ± 0.2 Hz) or amplitude (Figure 4A,

4C and 4F). In recordings with a baseline sEPSC frequency of 1.1 ± 0.3 Hz, superfusion of the CRF receptor agonist urocortin (100 nM) for 25 min also produced no significant change in sEPSC frequency (1.1 ± 0.4 Hz) or amplitude (Figure 4A, 4C and 4F). Although neither quinpirole nor urocortin alone altered sEPSCs, in recordings with a baseline sEPSC frequency of 0.6 ± 0.2 Hz, co-application of quinpirole and urocortin for 25 min produced a significant increase in sEPSC frequency (1.7 ± 0.8 Hz; 212 ± 40.8 % of control); and no change in sEPSC amplitude (97.7 ± 6.6 % of control), rise (100.2 ± 6.0 % of control) or decay (112.1 ± 4.1 % of control; Figure 4A, 4C and 4F). The time course of the change in sEPSC frequency (as % of control) is shown in Figure 4E. Note that there was a time-dependent increase in the sEPSC frequency in the cells exposed to quinpirole and urocortin, but not in those exposed to either agent alone (Figure 4E).

3.2.2 Effects of SKF38393 and quinpirole on glutamate transmission in VTA

DA neurons—DIR is also observed with co-activation of D1-like and D2 receptors. To determine if the same changes in synaptic glutamate release underlie the reversal of inhibition observed with concurrent D1-like/D2 activation, we performed experiments with the D1-like agonist SKF38393 and the D2 agonist quinpirole. Consistent with what was observed with superfusion of quinpirole alone, in recordings with a baseline sEPSC frequency of 0.4 ± 0.2 Hz, superfusion of SKF38393 (10 μ M) for 25 min produced no significant change in sEPSC frequency (0.5 ± 0.2 Hz) or amplitude (Figure 4B, 4C and 4F). However, in recordings with a baseline sEPSC frequency of 1.0 ± 0.4 Hz, co-application of quinpirole and SKF38393 for 25 min produced a significant increase in sEPSC frequency (1.7 ± 0.8 Hz; 193 ± 24.3 % of control) and no change in sEPSC amplitude (93.0 ± 4.0 % of control), rise (140.2 ± 20.2 % of control), or decay (101.3 ± 7.5 % of control; Figure 4B, 4D and 4F). The time course of the change in sEPSC frequency (as % of control) is shown in Figure 4E. Note that there was a time-dependent increase in the sEPSC frequency in the cells exposed to quinpirole and SKF38393, but not in those exposed to either agent alone (Figure 4E). Collectively, these data suggest that the reversal of inhibition observed with either concurrent D2/CRF activation or concurrent D1-like/D2 activation is accompanied by an increase in glutamate release.

4. Discussion

The desensitization of D2 receptors, as observed as a reversal of the inhibition produced by D2 agonists dopamine and quinpirole, is a fundamental physiological response to increased D2 receptor activation that has been described in a number of published reports (Nimitvilai and Brodie, 2010; Nimitvilai, et al., 2012a; Nimitvilai, et al., 2013; Nimitvilai, et al., 2012c; Nimitvilai, et al., 2012b; Nimitvilai, et al., 2012d). In the present study our examination of the contribution of glutamatergic mechanisms to DIR and the role of the CRF system in the reversal of D2-mediated inhibition resulted in several novel observations that further characterized the mechanisms underlying DIR. First, antagonists of NMDA, AMPA or metabotropic glutamate receptors blocked the induction but not the maintenance of DIR. Second, two agents that block ion channels and neurotransmitter release, lamotrigine and riluzole, antagonized both the induction and the maintenance of DIR. Third, we show here that CRF or the CRF agonist urocortin can induce the reversal of quinpirole-induced

inhibition. Adding to these observations, we show that glutamate release was increased under conditions that produce reversal of quinpirole inhibition.

While glutamatergic sEPSCs are increased under conditions that produce DIR and antagonism of glutamate receptors can block the induction of DIR, maintenance of DIR does not appear to require glutamate receptor activation. In contrast, treatments that block calcium entry can interfere with DIR induction (Nimitvilai, et al., 2012a) or maintenance (Figure 2G). What emerges from these results is a picture of complex interaction of factors in the VTA in addition to D2 receptors that contribute to the development and maintenance of the desensitization. Other studies have shown an interaction between dopamine and glutamate in the VTA. Glutamate neurons from a large number of brain areas, including prefrontal cortex (Carr and Sesack, 2000), basolateral nucleus of the amygdala, lateral hypothalamic area, dorsal and median raphe (Geisler, et al., 2007) and the bed nucleus of the stria terminalis (Georges and Aston-Jones, 2002), send projections to the VTA. Dopamine is known to modulate release of glutamate from prefrontal cortical projections (Nicola and Malenka, 1997). The results presented here suggest that the presence of glutamate, or at least activation of glutamatergic receptors, is a necessary factor in reversal of D2-mediated inhibition of VTA neurons. Activation of dopamine receptors increases glutamatergic sEPSCs (Figure 4), which may facilitate the interaction between these receptors and permit desensitization of the D2 receptors.

Lamotrigine and riluzole antagonized the initiation and the maintenance of DIR (Figure 2). Both lamotrigine and riluzole have been shown to block fast-inactivating (Stefani, et al., 1997) and slowly-inactivating (Spadoni, et al., 2002) sodium currents in cortical pyramidal neurons of rat, and both agents block high voltage activated Ca^{2+} current (Stefani, et al., 1997). As lamotrigine also has been shown to block calcium flow through ryanodine receptors (Neumann and Copello, 2011), which have been shown to be important for DIR (Nimitvilai, et al., 2012a), there may be actions of these agents that are responsible for the antagonism of DIR that are unrelated to blockade of glutamate release. We have previously demonstrated that intracellular calcium release and entry of calcium from the extracellular medium is necessary for DIR (Nimitvilai, et al., 2012a). As the SOCE antagonist SKF96365 also blocks the initiation of DIR (Nimitvilai, et al., 2012a) and antagonizes the maintenance of DIR (Figure 2G), it is likely that increased intracellular calcium levels are critical to DIR initiation and maintenance. If the increased calcium influx is disrupted, there could be a rapid re-activation of dopamine sensitivity, probably through increased trafficking of D2 receptors to the membrane. Alternatively, blockade of SOCE could antagonize DIR through a mechanism different from lamotrigine and riluzole. Both of the latter agents could block the synaptic release of all neurotransmitters, especially those that are Gq-linked (Nimitvilai, et al., 2012d), which in turn could eliminate polysynaptic processes necessary for DIR. Any involvement of Na^{+} channels that are blocked by riluzole or lamotrigine and might contribute to their effects on DIR, would require additional studies; note that significant blockade of Na^{+} channels with tetrodotoxin eliminates spontaneous spiking (Grace, 1987).

The results presented here elaborate on the mechanism of desensitization of dopamine D2 receptors in the VTA. We suggest that there are two phases of desensitization: induction and maintenance. Figure 5 presents a diagram integrating the results of the present study and

summarizing a possible underlying mechanism. The overall picture that has developed from these studies indicates a complex interaction of DIR with synaptic release of glutamate and with intracellular calcium concentration. Evidence from our laboratory indicates that DIR operates in many ways like conventional homologous desensitization of neurotransmitter receptors, requiring dynamin and G-protein coupled receptor kinase to achieve a decrease in DA sensitivity of DA VTA neurons (Nimitvilai, et al., 2013). During induction, increased extracellular dopamine causes increased glutamate release, resulting in increased entry of calcium through glutamate receptor ion channels (presumably primarily AMPA receptors). Either pre- or postsynaptic activation of CRF receptors (or D1-like receptors (Nimitvilai and Brodie, 2010)) facilitates the process by which D2 receptors desensitize. Activation of either CRF or D1/D5 receptors could increase glutamate release presynaptically (Wang, et al., 2012), or promote activation of protein kinase C postsynaptically (Nimitvilai, et al., 2012a). A presynaptic site of action is suggested by our results demonstrating an increase in sEPSCs produced by conditions favoring D2 receptor desensitization; a postsynaptic action could result in release of a trans-synaptic substance that increases glutamate release, such as endocannabinoids (Kortleven, et al., 2011; Haj-Dahmane and Shen, 2010; Andre, et al., 2010). While there may be complex interactions between glutamate release and induction of DIR, a simple explanation may involve increases in calcium entry, which would facilitate activation of conventional protein kinase C (cPKC) necessary for DIR (Nimitvilai, et al., 2012a). It is known that glutamate can increase intracellular calcium by activating ionotropic glutamate receptors (Jang, et al., 2011) and by increasing release from intracellular stores via metabotropic glutamate receptors (Morikawa, et al., 2000). Any or all of these processes may be necessary under normal conditions to increase intracellular calcium sufficiently to activate PKC and promote the induction of DIR (Figure 5).

During maintenance of DIR, however, glutamate receptor activation is not needed but calcium entry is required; this suggests that other processes take over the role played by glutamate receptors for increasing intracellular calcium concentration during DIR maintenance (Figure 5). Increased sensitivity of intracellular stores to calcium release, or increased capability for calcium entry into the cell could be induced by some of the same mechanisms that cause DIR. For example, ryanodine receptors can be upregulated by dopamine D1 activation (Kurokawa, et al., 2012). Intrinsic excitability of prefrontal cortical neurons is subject to a long-term increase that can be induced by dopamine acting at D1-like receptors; this increase in intrinsic excitability is due to an increase in calcium influx via non-L-type calcium channels, and is dependent on protein kinase C (Chen, et al., 2007). Numerous additional studies would be necessary to determine the specific calcium-related mechanisms involved in the maintenance of DIR. Molecular studies could provide complementary information regarding the biochemical pathways and molecular differences between induction and maintenance of DIR.

CRF in the central nervous system may be a critical link between stress and addiction. The increased release of CRF that is associated with behavioral paradigms related to anxiety and stress (Merlo, et al., 1995; Lee, et al., 2008) may also play a role in reinstatement of drug-seeking behavior. Injection of CRF into the VTA produces reinstatement of cocaine self-administration in rats (Blacktop, et al., 2011), suggesting that CRF has a role in the action of drugs of abuse in the VTA. Stress, such as mild foot shock, triggers reinstatement of cocaine

or heroin self-administration (Shaham, et al., 2000), and increases release of CRF in the VTA (Wang, et al., 2005). The action of CRF on CRF2 receptors appears critical for footshock reinstatement of drug self-administration (Wang, et al., 2007). Future behavioral studies may demonstrate a link between reinstatement and DIR in processes involved in drug-seeking behaviors.

Desensitization of D2 receptors is likely to take place under conditions of elevated extracellular DA concentration. Cocaine produces a blockade of DA reuptake in the VTA (Brodie and Dunwiddie, 1990), and, in vivo, this action may alter the physiology of DA VTA neurons. Studies examining the actions of cocaine on the VTA have demonstrated alteration of glutamatergic signaling following cocaine exposure. Even a single exposure to cocaine can induce long-term potentiation of glutamatergic signaling (Ungless, et al., 2001). A single injection of cocaine is sufficient to induce behavioral sensitization and produces an increase in GluR1 mRNA in the VTA (Grignaschi, et al., 2004). Upregulation of AMPA receptor subunits GluR1 and GluR2 in the VTA of rats has been shown to increase the motivation for cocaine self-administration (Choi, et al., 2011). Cocaine-seeking is reinstated by mild footshock (which causes release of CRF in the VTA) or by CRF injection into the VTA (Wise and Morales, 2010). We show here that DIR increases glutamate release in the VTA, and CRF promotes DIR. In the presence of cocaine, DIR is achieved with much lower concentrations of dopamine (Nimitvilai, et al., 2012a). Interactions of glutamate and CRF in the VTA become pronounced after cocaine treatment, and appear to be important in compulsive cocaine seeking (Wise and Morales, 2010). The interactions of CRF and DIR may result in alteration of glutamatergic signaling relevant to reward and reinforcement in the VTA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-Standard abbreviations

pDAergic	putative dopaminergic
DAergic	dopaminergic
VTA	ventral tegmental area
DA	dopamine
aCSF	artificial cerebrospinal fluid
DL-AP5	DL-2-Amino-5-phosphonopentanoic acid
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
MCPG	(RS)- α -Methyl-4-carboxyphenylglycine

SKF96365	1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]-1H-imidazole hydrochloride
CGP35348	(3-Aminopropyl)(diethoxymethyl) phosphinic acid
CGP55845A	(2S)-3-[[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl) phosphinic acid hydrochloride
CRF	corticotropin releasing factor
ASG-30	antisauvagine-30
NBI27914	5-Chloro-N-(cyclopropylmethyl)-2-methyl-N-propyl-N'-(2,4,6-trichlorophenyl)-4,6-pyrimidinediamine hydrochloride

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Highlights

- Dopamine inhibition reversal (DIR) is the process by which D2 receptors desensitize
- Glutamate receptor antagonists blocked induction but not maintenance of DIR
- Like D1 agonists, CRF and the CRF agonist urocortin promoted D2 desensitization
- Urocortin or D1 agonists increased sEPSCs only in presence of D2 agonist quinpirole
- Glutamate release in the VTA increases under the same conditions that produce DIR

Figure 1. Effect of glutamate antagonists on induction of DIR

Extracellularly-recorded firing rate of DA VTA neurons was monitored, and the change in firing rate produced by dopamine (as percentage change in firing rate compared to the pre-drug baseline) was calculated at 5 min intervals.

A, B. Examples of firing rate plotted as a function of time. Vertical bars indicate the firing rate over a 5 sec interval; horizontal bars indicate the duration of administration of dopamine at the indicated concentrations. Dopamine concentrations were raised in a stepwise manner until inhibition was 50% or greater. A. 40 min administration of 3 μ M dopamine resulted in a gradual reduction of dopamine-induced inhibition of spontaneous firing. B. In the presence of DL-AP5 (10 μ M), inhibition of firing produced by 1 μ M dopamine was sustained for the 40 min duration of dopamine administration; firing rate recovered upon washout of dopamine. The effects of CNQX and MCPG on dopamine inhibition were similar to the effect of DL-AP5 (data not shown).

Mean results from these experiments were calculated for DA VTA neurons in the absence or presence of NMDA antagonist DL-AP5 (C), AMPA antagonist CNQX (D) or metabotropic glutamate receptor antagonist MCPG (E). In the absence of antagonist ($\square$), there was a time-dependent increase in firing rate from the peak inhibition produced by dopamine. In the presence of either DL-AP5 (C, 10 μ M, $\blacktriangledown$) or CNQX (D, 10 μ M, $\blacktriangledown$), there was a time-dependent decrease in firing rate from the peak inhibition produced by dopamine. In the presence of MCPG (E, 10 μ M, $\blacktriangledown$), dopamine produced a sustained inhibition in firing rate with no reversal. F. Firing rate of a single neuron plotted as a function of time. Vertical bars indicate the firing rate over a 5 sec interval; horizontal bars indicate the duration of administration of agents at the indicated concentrations. Dopamine concentration was raised in a stepwise manner from 1 to 5 to 10 μ M and that final dopamine concentration was

maintained for 80 min. After 40 min, glutamate receptor antagonists DL-AP5, CNQX and MCPG (10 μ M each) were added to the superfusate for an additional 40 min, at which point all drugs were washed out.

G, H. Mean results from experiments similar to that shown in part F. Extracellularly-recorded firing rate of DA VTA neurons was monitored, and the change in firing rate produced by dopamine (as percentage change in firing rate compared to the pre-drug baseline) was calculated at 5 min intervals. Dopamine was administered for 40 min, and then administration continued for an additional 40 min without (G) or with (H) the addition of glutamate receptor antagonists DL-AP5, CNQX and MCPG (10 μ M each). In both cases, 40 min of dopamine application resulted in a significant reversal of inhibition compared to the peak inhibitory effect of dopamine. Addition of glutamate receptor antagonists (H) did not antagonize the reversal of dopamine-induced inhibition, similar to what was observed in the absence of glutamate receptor antagonists (G).

Figure 2. Effect of riluzole, lamotrigine, and SKF96365 on development and maintenance of DIR
 Extracellularly-recorded firing rate of DA VTA neurons was monitored, and the change in firing rate produced by dopamine (as percentage change in firing rate compared to the pre-drug baseline) was calculated at 5 min intervals.

A. Example of firing rate plotted as a function of time. Vertical bars indicate the firing rate over a 5 sec interval; horizontal bars indicate the duration of administration of dopamine at the indicated concentrations. Dopamine concentrations were raised in a stepwise manner until inhibition was 50% or greater. In the presence of riluzole (10 μ M), inhibition produced by 7.5 μ M dopamine did not reverse but was sustained for the 40 min duration of dopamine administration (compare to control conditions shown in Figure 1A).

B. and C. Mean results from experiments similar to that shown in part A. Extracellularly-recorded firing rate of DA VTA neurons was monitored, and the change in firing rate produced by dopamine (as percentage change in firing rate compared to the pre-drug baseline) was calculated at 5 min intervals. Mean results from these experiments were calculated for DA VTA neurons in the absence or presence of blockers of glutamate release riluzole (B) or lamotrigine (C). In the presence of either riluzole (B, 10 μ M, $\blacktriangledown$) or lamotrigine (C, 25 μ M, $\blacktriangledown$), there was a time-dependent decrease in firing rate from the peak inhibition produced by dopamine. The effect of dopamine in the absence of release blockers ($\square$) is the same data as shown in Figure 1, and is shown here for reference.

D. Example of firing rate of a single neuron plotted as a function of time. Vertical bars indicate the firing rate over a 5 sec interval; horizontal bars indicate the duration of

administration of dopamine at the indicated concentrations. Dopamine concentration was raised in a stepwise manner from 1 to 1.5 μM , at which point inhibition greater than 50% was observed. Dopamine concentration (1.5 μM) was maintained for 80 min. After 40 min, lamotrigine (25 μM) was added to the superfusate for an additional 40 min, at which point all drugs were washed out. Note that after lamotrigine was added, there was resumption of the magnitude of inhibition observed 10 to 15 min after 1.5 μM dopamine was initially added; upon washout of dopamine and lamotrigine, the firing rate returns to the pre-drug baseline. E–G. Mean results from experiments similar to that shown in part D.

Extracellularly-recorded firing rate of DA VTA neurons was monitored, and the change in firing rate produced by dopamine (as percentage change in firing rate compared to the pre-drug baseline) was calculated at 5 min intervals. Dopamine was administered for 40 min, and then administration continued for an additional 40 min with the addition of lamotrigine (E, 25 μM), riluzole (F, 10 μM) or SKF96365 (G, 10 μM) to the superfusate. In all cases, 40 min of dopamine application resulted in a significant increase in the firing rate compared to the peak effect of dopamine (5 min time point). With lamotrigine (E), riluzole (F) or SKF96365 (G), there was a significant reduction in firing rate that reached a level not significantly different from the 5 min time point. Control responses in a similar dopamine protocol without addition of channel antagonists is shown in Figure 1G.

Figure 3. Effect of CRF agonists on quinpirole-induced inhibition

Extracellularly-recorded firing rate of DA VTA neurons was monitored, and the change in firing rate produced by D2 agonist quinpirole (as percentage change in firing rate compared to the pre-drug baseline) was calculated at 5 min intervals. Quinpirole typically does not produce peak inhibition until 15 min. The protocol for these experiments was analogous to that used in experiments illustrated in Figure 1A.

A. With vehicle in the pipette, quinpirole inhibition reached its greatest magnitude at 15 min and did not reverse over the 40 min application period. With 1 μ M CRF included in the recording pipette, there was a significant increase in the firing rate of DA VTA neurons at the 40 min time point compared to the 15 min time point.

B. With 1 μ M urocortin included in the recording pipette, there was a significant increase in the firing rate over the 40 min of quinpirole administration compared to the 5 min time point. When 1 μ M urocortin and anti-sauvagine-30 (1 μ M) was present during the quinpirole administration, there was no increase in firing rate over time compared to the 5 or 15 min time point.

C and D. Examples of firing rate plotted as a function of time. Vertical bars indicate the firing rate over a 5 sec interval; horizontal bars indicate the duration of administration of dopamine at the indicated concentrations. C. Control (DMSO vehicle) or D. NBI27914, were added 10 min before the recordings. Quinpirole concentrations were raised in a stepwise manner until inhibition was 50% or greater. After 15 min of quinpirole, urocortin was added to the superfusate and the recording was continued for an additional 40 min. Note that the inhibition produced by quinpirole was reversed when urocortin was added in the absence (C.) but not in the presence (D.) of NBI27914.

E. Mean data from experiments similar to those shown in Parts C and D above. Either DMSO vehicle (●), NBI (100nM, △), or ASG-30 (100 nM, □) was added to the superfusate and remained on during the remainder of the experiment. Vertical line indicates the time point 5 min after urocortin was added to the superfusate. In the presence of DMSO vehicle, bath-applied urocortin reversed quinpirole inhibition, producing a significant reduction of quinpirole-induced inhibition (One-way ANOVA, $F_{11,60}=2.10$, $p < .05$, $n = 6$). In the presence of ASG, there was no significant change in quinpirole-induced inhibition over time between time 0 and 40 min (One-way ANOVA, $F_{11,59}=1.21$, $p > .05$, $n = 5$). Likewise, in the presence of NBI, there was no significant change in quinpirole-induced inhibition over time between time 0 and 40 min (One-way ANOVA, $F_{11,95}=0.61$, $p > .05$, $n = 8$).

Figure 4. Effect of D2 and CRF receptor activation and D1/D2 receptor activation on glutamate currents in VTA DA neurons

A) Representative whole-cell voltage clamp recordings of spontaneous excitatory postsynaptic currents (sEPSCs) in VTA DA neurons before and during superfusion of 500 nM quinpirole (top traces), before (control) and during superfusion of 100 nM urocortin (middle traces) and before (control) and during co-application of urocortin + quinpirole (lower traces).

B) Representative whole-cell voltage clamp recordings of sEPSCs in VTA DA neurons before (control) and during superfusion of 10 μ M SKF38393 (top traces) and before (control) and during co-application of SKF38393 + quinpirole (lower traces).

C) Representative scaled average sEPSC traces during control and Quinpirole + Urocortin (Q+U) conditions from the recording depicted in A.

D) Representative scaled average sEPSC traces during control and Quinpirole + SKF38393 (Q+SKF) conditions from the recording depicted in B.

E) Summary of the change in sEPSC frequency over time during application of Quinpirole + Urocortin (Q+U, ○); Quinpirole + SKF38393 (Q+SKF, ◇); Quinpirole (Q, □); Urocortin (U, △); and SKF38393 (SKF, ▽); * $p < 0.05$ by repeated measures one-way ANOVA or paired t-test, $n = 25$.

F) Summary of the changes in sEPSC frequency (left) and amplitude (right) observed in VTA DA neurons with superfusion of quinpirole (500 nM), SKF38393 (10 μ M), urocortin (100nM), quinpirole + urocortin and quinpirole + SKF38393 * $p < 0.05$ by one-sample t-test, $n = 25$.

Figure 5. Model for induction and maintenance of DIR

Proposed model for changes in D2 receptors and calcium currents during induction and maintenance of DIR. DIR requires concurrent activation of D2 receptors and Gq-linked receptors (either D1-like receptors or alternative receptors like CRF receptors). This concurrent activation of receptors results in stimulation of a calcium-dependent cascade involving protein kinase C and other intracellular elements. During induction of DIR, intracellular calcium increases as a result of increased glutamate release and activation of glutamate receptors, and calcium entry via somatic calcium channels, including SOCE and release of calcium from internal stores. An increase in intracellular calcium is necessary for activation of protein kinase C and other processes that have been shown to be necessary for DIR. During maintenance of DIR, there is a reduction of D2 receptor function, and this reduction is maintained by increased intracellular calcium (depicted here by an increase in somatic calcium channels, but other mechanisms (such as increased availability of calcium released from intracellular stores) may account for this increase. Intracellular calcium may continue to be increased by activation of glutamate receptors, but this may not be necessary if intracellular calcium is sufficiently increased by other processes.

TABLE 1

Effects of dopamine agonists in extracellular experiments

Condition	DA (μ M)	Change in firing rate at 5 min (%)
Figure 1		
C. DA (control)	7.2 ± 2.19	-57.97 ± 1.97
DA + DL-AP5	5.63 ± 1.27	-61.01 ± 3.25
D. DA + CNQX	5.08 ± 1.26	-60.23 ± 3.93
E. DA + MCPG	5.25 ± 1.11	-65.47 ± 4.51
G. DA 80 min	5.7 ± 1.5	-71.26 ± 6.88
H. DA + DL-AP5 + CNQX + MCPG	7.21 ± 1.34	-62.54 ± 4.95
Figure 2		
B. DA + Riluzole	3.08 ± 0.9	-79.59 ± 4.32
C. DA + Lamotrigine	3.44 ± 0.52	-59.42 ± 1.51
E. DA + Lamotrigine	3.44 ± 0.67	-66.53 ± 3.3
F. DA + Riluzole	5.58 ± 1.53	-78.3 ± 5.67
G. DA + SKF96365	6.8 ± 1.15	-70.76 ± 7.02
Condition	Quinpirole (nM)	Change in firing rate at 5 min (%)
Figure 3		
A. Quinpirole (control)	50 ± 3.16	-79.44 ± 4.14
Quinpirole + CRF	22.33 ± 2.33	-60.69 ± 2.91
B. Quinpirole + Urocortin	26.82 ± 3.95	-69.46 ± 4.83
Quinpirole + Urocortin + ASG-30	75.71 ± 23.16	-60.88 ± 3.44
E. Quinpirole + Urocortin	52.5 ± 15.6	-56.28 ± 10.48
Quinpirole + Urocortin + ASG-30	50.0 ± 12.65	-59.72 ± 8.46
Quinpirole + Urocortin + NBI	56.2 ± 4.98	-68.75 ± 5.80

TABLE 2

Effects of agents on baseline firing frequency of VTA neurons

Condition	Baseline firing rate (Hz)	Firing rate in drug (Hz)	Change in firing rate (%)	P < 0.05?
Figure 1				
C. DL-AP5	1.69 ± 0.21	1.64 ± 0.20	-2.88 ± 2.92	N
D. CNQX	2.12 ± 0.13	2.02 ± 0.14	-4.32 ± 3.54	N
E. MCPG	1.37 ± 0.18	1.32 ± 0.19	-3.37 ± 4.53	N
Figure 2				
B. Riluzole	1.57 ± 0.28	1.38 ± 0.24	-11.58 ± 2.76	Y
C. Lamotrigine	2.08 ± 0.20	1.87 ± 0.18	-9.45 ± 2.46	Y
Figure 3				
A. CRF	2.52 ± 0.26	2.37 ± 0.23	-5.31 ± 4.61	N
B. Urocortin	2.59 ± 0.31	2.58 ± 0.29	0.80 ± 2.58	N
B. Urocortin + ASG-30	2.85 ± 0.45	2.91 ± 0.41	3.00 ± 2.54	N