Title Page

Reversal of dopamine D2 agonist-induced inhibition of ventral tegmental area neurons by Gq-linked neurotransmitters is dependent on protein kinase C, G protein-coupled receptor kinase and dynamin

Sudarat Nimitvilai, Maureen A. McElvain, 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
Abstract

Dopaminergic neurons of the ventral tegmental area are important components of brain pathways related to addiction. Prolonged exposure of these neurons to moderate concentrations of dopamine (DA) decreases their sensitivity to inhibition by DA, a process called DA inhibition reversal (DIR). DIR is mediated by phospholipase C and conventional subtype of protein kinase C (cPKC) through concurrent stimulation of D2 and D1-like DA receptors, or by D2 stimulation concurrent with activation of 5-HT₂ or neurotensin receptors. In the present study, we further characterized this phenomenon using extracellular recordings in brain slices to examine whether DIR is linked to G protein-coupled receptor kinase-2 (GRK2) or dynamin by assessing DIR in the presence of antagonists of these enzymes. DIR was blocked by β-ARK1 inhibitor, which inhibits GRK2, and by dynasore, which blocks dynamin. Reversal of inhibition by D2 agonist quinpirole was produced by serotonin (50 µM) and by neurotensin (5-10 nM). Serotonin- or neurotensin-induced reversal was blocked by β-ARK1 inhibitor, dynasore or cPKC antagonist Gö6976. This further characterization of DIR indicates that cPKC, GRK2 and dynamin play important roles in desensitization of D2 receptors. As drugs of abuse produce persistent increases in dopamine concentration in the VTA, reduction of D2 receptor sensitivity as a result of drug abuse may be a critical factor in the processes of addiction.
Introduction

Dopaminergic (DAergic) neurons in the ventral tegmental area (VTA) project to several regions of the mesocorticolimbic system including the nucleus accumbens, prefrontal cortex, and amygdala (Koob, 2003; Oades and Halliday, 1987). Increases in DAergic neurotransmission are caused by salient and motivational stimuli, and are important for reward and reinforcement by numerous drugs of abuse (Di Chiara and Imperato, 1988; Wise, 1996; Mirenowicz and Schultz, 1996). Prolonged increases in dopamine concentrations in the VTA may affect the excitability of DAergic neurons of the VTA and may 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).

Five classes of dopamine receptors have been identified: two “D1-like” receptors (D1 and D5) and three “D2-like” receptors (D2, D3, and D4) (Sibley, et al., 1993; Neve, et al., 2004; Ciliax, et al., 2000; Khan, et al., 2000). The 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 in the DAergic VTA neurons (Meador-Woodruff, et al., 1992; Mengod, et al., 1992; Rivera, et al., 2008). However, the D1 receptors appear to be on presynaptic glutamatergic terminals projecting to the region, not on the DAergic VTA neurons themselves (Caillé, et al., 1996).

pDAergic VTA neurons fire action potentials spontaneously in vivo (Bunney, et al., 1973) and in vitro (Brodie and Dunwiddie, 1987). This spontaneous firing is inhibited
by the action of dopamine at D2 autoreceptors (Lacey, et al., 1987; Brodie, et al., 1990). However, we demonstrated that prolonged application of dopamine results in a time- and concentration-dependent decrease in the magnitude of dopamine-induced inhibition, a phenomenon that we termed “dopamine inhibition reversal or DIR” (Nimitvilai and Brodie, 2010). This DIR is mediated by concurrent stimulation of D2 and D1-like receptors, requires 10-40 min to develop, and persists for up to 90 min (Nimitvilai and Brodie, 2010). Activation of D1/D5 receptor linked to phosphatidylinositide (PI) accumulation, but not those that cause adenylyl cyclase (AC) activation, produced a decrease in sensitivity of D2 receptor to its agonist (Nimitvilai, et al., 2012c). DIR also requires the activation of phospholipase C (PLC) and conventional protein kinase C (cPKC), without involvement of AC, cAMP and protein kinase A (Nimitvilai, et al., 2012a). Recently we have demonstrated that some (e.g., 5HT2 and neurotensin), but not all (e.g., α-1 adrenergic and group I metabotropic glutamate), Gq-coupled receptors that stimulate PLC and PKC pathway can mediate the reversal of D2 agonist inhibition (Nimitvilai, et al., 2012c).

An involvement of PKC in phosphorylation and internalization of D2 receptors has been reported in many systems such as HEK293 cells, striatal and hippocampal neurons (Namkung and Sibley, 2004; Bofill-Cardona, et al., 2000; Thibault, et al., 2011). Phosphorylation and internalization of D2 receptors may contribute to the reversal of dopamine inhibition found in the pDAergic VTA neurons. In the present study, therefore, we extended our investigation of DIR to examine elements shown to be involved in phosphorylation and internalization of the D2 receptors. We also examined whether
activation of either serotonin or neurotensin receptors requires similar phosphorylation and internalization processes to mediate the reversal of D2 agonist-induced inhibition.
Methods

Animals: Male Fischer 344 (F344; adult rats, 4-6 weeks old, 90 - 150 g) 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 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 \( \mu \text{m} \) thick). An individual slice was placed onto a mesh platform in the recording chamber and 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, Na2HPO4 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 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. 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., 2006; Chieng, et al., 2011).

Additional characterization, such as determining the projection target of our cells of study (Margolis, et al., 2008) would have been difficult as we have 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). One consequence of differential initial sensitivity to dopamine inhibition among groups of neurons projecting to different brain areas (Margolis, et al., 2008; Lammel, et al., 2008) would be different amounts of dopamine inhibition reversal (Nimitvilai and Brodie, 2010), resulting in a greater relative change in neurons more sensitive to dopamine inhibition.
**Drug Administration:** Drugs were added either to the aCSF or to the microelectrode filling solution (0.9% NaCl). Application of drugs to the aCSF by means of a calibrated infusion pump from stock solutions 100 to 1000 times the desired final concentrations 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 300 μl) 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 that which would have been 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., 2012b), 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 (data not shown), 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 (Pesavento, et al., 2000; Nimitvilai, et al., 2012a). 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, serotonin, neurotensin, and most of the salts used to prepare the extracellular media were purchased from Sigma (St. Louis, MO). Gö6976 (5,6,7,13-tetrahydro-13-methyl-5-oxo-12H-indolo[2,3-a]pyrrolo[3,4c]carbozole-12-propanenitrile) and dynasore were purchased from Tocris (Ellisville, MO). β-ARK1 inhibitor (Methyl 5-[2-(5-nitro-2-furyl)vinyl]-2-furoate) was purchased from Calbiochem (Gibbstown, NJ). MiTMAB™ (Tetradecyltrimethylammonium bromide) was purchased from Abcam® (Cambridge, MA).

**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. Tip resistance of the microelectrodes ranged from 2 – 5 MΩ. A Fintronics amplifier was used in conjunction with an IBM-PC-based data acquisition system (ADI Instruments 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 \((\text{FRD} - \text{FRC}) / \text{FRC} \times 100\), 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 (e.g., DA) 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.

**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 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.). Mean response graphs are shown as relative change in firing rate normalized to the inhibition observed in the first five minute interval; in these cases, the mean percentage inhibition as a function of baseline firing rate is indicated in the text. 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).
Results

VTA neuron characteristics

A total of 121 VTA neurons were examined. Their firing rate in normal extracellular medium ranged from 0.6 to 4.87 Hz, with a mean of 2.24 ± 0.08 Hz. All neurons had regular firing rates and were inhibited by dopamine agonists. 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, the concentration of dopamine used was 5.66 ± 0.67 μM (n = 31) which produced a mean change in firing rate of -67.55 ± 2.28% after 5 min of exposure; the concentration of quinpirole used was 84.19 ± 5.99 nM (n = 80) which produced a mean change in firing rate of -64.65 ± 1.59% after 5 min of exposure. There were no significant differences in the concentration of DAergic agonists 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 μM, although in dissociated DA VTA neurons, concentrations as low as 50 nM can completely inhibit spontaneous action potential firing.
Cells which did not return to at least 70% of their pre-DA firing rate during this washout were not used; eight out of 129 cells did not return to 70% of their pre-agonist baseline firing rate. Table 1 also lists the effects on spontaneous firing rate of the various inhibitors used in the experiments described below; none of the inhibitors produced a significant change in firing rate (paired t-test, p<0.05). 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 95.58 ± 0.66 minutes, with a range of 90 to 105 minutes.

Dopamine inhibition reversal did not occur when either G protein-coupled receptor kinase-2 or dynamin GTPase was suppressed

Time-dependent reversal of dopamine inhibition occurs with moderate concentrations of dopamine alone or D2 agonist quinpirole in the presence of D1-like receptor agonist (Nimitvilai and Brodie, 2010; Nimitvilai, et al., 2012a). This phenomenon is dependent on calcium and is mediated by activation of phospholipase C (PLC) and conventional protein kinase C (cPKC) pathway (Nimitvilai, et al., 2012a). D1/D5 agonists linked to the PI/PLC, but not to the AC/cAMP, pathway also induce the reversal of quinpirole-induced inhibition (Nimitvilai, et al., 2012c). There is evidence that agonist-induced D2 receptor desensitization and internalization is dependent on G protein-coupled receptor kinase-2 (GRK2) and endocytotic GTPase dynamin (Ito, et al., 1999; Iwata, et al., 1999; Thibault, et al., 2011).

In the present study, therefore, we examined whether dopamine inhibition reversal is inhibited by blockers of GRK2 or dynamin (Figure 1 and 2). Figures 1A-1D illustrate...
data from single neurons, and, for clarity, the pooled data in Figure 2 are presented normalized to the firing rate 5 min after dopamine was superfused; increases in relative firing rate indicate reversal of inhibition, decreases in relative firing rate indicate more inhibition with time. The selective inhibitor of GRK2 called β-ARK1 inhibitor (300 μM) (Iino, et al., 2002), the dynamin inhibitor dynasore (800 μM) (Macia, et al., 2006), or the dynamin inhibitor MiTMAB™ (400 μM) (Quan, et al., 2007) was dissolved in saline. Saline alone or saline containing one of these drugs was used to fill the recording electrodes that were used to make extracellular recordings of single pDAergic VTA neurons. After initiating recording of pDAergic neurons and allowing the drug in the pipettes to act locally for at least 20 min, concentrations of dopamine were applied in the superfusate in a step-wise fashion, in which each concentration was added for 5 min, and increased until inhibition of firing of 50% or greater was achieved; this concentration was applied for 40 min. As in our previous studies (Nimitvilai, et al., 2012c; Nimitvilai and Brodie, 2010), dopamine alone (Figure 1A and 2) produced an inhibition in firing rate at 5 min of 72.78 ± 4.83%, and this inhibition partially reversed with time so that at 40 min, there was a significant reduction in the dopamine-induced inhibition.

In Figure 2A, DIR is illustrated as a relative increase in firing rate (%) compared to the 5 min time point (■, [DA] = 4.45 ± 1.25 μM, n = 10) (one-way repeated measures ANOVA, F(7,63) = 5.96, p < 0.05). In the presence of β-ARK1 inhibitor (Figure 1B and 2A), however, dopamine produced a significant reduction in firing rate, with no reversal (●, [DA] = 5.2 ± 1.2 μM, n = 9) (one-way repeated measures ANOVA, F(7,56) = 3.23, p < 0.05). In the presence of dynasore (Figure 1C and 2B), no significant reversal of dopamine inhibition was observed (▼, [DA] = 6.64 ± 1.58 μM, n = 7) (one-way repeated
measures ANOVA, $F_{(7,42)} = 1.29, p > 0.05$). Likewise, in the presence of dynamin inhibitor MiTMAB™ (Figure 1D and 2B), dopamine produced a significant inhibition in firing rate, with no reversal (●, [DA] = 7.5 ± 1.12 µM, n = 5) (one-way repeated measures ANOVA, $F_{(7,28)} = 5.46, p < 0.05$). These results suggest that dopamine inhibition reversal is mediated by GRK-2 phosphorylation and dynamin-dependent internalization of D2 receptors.

**Activation of neurotensin receptors reversed quinpirole-induced inhibition through conventional protein kinase C, G protein-coupled receptor kinase-2 and dynamin-dependent processes**

We have demonstrated previously that some, but not all, Gq-coupled receptors produce a decrease in sensitivity of D2 receptors to D2 agonist quinpirole (Nimitvilai, et al., 2012c). Since activation of neurotensin receptors produce the reversal of quinpirole-induced inhibition, we explored whether this phenomenon is also mediated by GRK2 and dynamin-dependent processes (Figure 3). The saline alone or saline containing either GRK2 inhibitor β-ARK1 inhibitor (300 µM) or dynamin inhibitor dynasore (800 µM) was used to fill the recording pipettes, and these pipettes were used to measure changes in firing rate of pDAergic VTA neurons over time. After obtaining the recording of pDAergic VTA neurons, the firing rate was measured for at least 20 min to allow the drug in the pipettes to act locally on the neurons. Then neurotensin (10 nM) was added to the superfusate for 15 min, producing an increase in firing rate of 72.0 ± 29.2% and this new firing rate was used as a new baseline for measuring the effect of quinpirole over 40 min time course. Concentrations of quinpirole were applied in a step-wise fashion, in
which each concentration was added for 5 min and increased until inhibition of 50% or greater was achieved. This concentration of quinpirole was sustained for 40 min. As shown previously (Nimitvilai, et al., 2012c), in the presence of neurotensin (○, n = 9), quinpirole (100 ± 19.54 nM) produced a significant inhibition in firing rate with a maximum inhibition of -71.74 ± 5.85% at 10 min, and this inhibition partially reversed with time, there was a significant difference between the last four time points compared with the 10 min time point (one-way repeated measures ANOVA, F(7,35) = 4.0, p < 0.05). Without neurotensin, quinpirole (54 ± 8.5 nM) alone significantly inhibited the firing rate, and this inhibition did not reverse with time (□, n = 10) (one-way repeated measures ANOVA, F(7,63) = 11.2, p < 0.05) (Figure 3A). In control experiments, when neurotensin alone was applied for 60 min, firing rate significantly increased by 78.8 ± 20.7% within 15 min and there was no significant change in firing rate from 15 to 60 min of neurotensin administration (One-way repeated measures ANOVA, F(11,55) = 4.8, P < 0.05) (data not shown). This result indicates that the apparent reversal of inhibition was not due to a gradual increase in neurotensin-mediated excitation over time, but is more likely to be due to reduction of the quinpirole-induced inhibition.

With β-ARK1 inhibitor (300 µM) in the recording pipettes, when neurotensin was applied in the superfusate (●, n = 6), no reversal of quinpirole inhibition was observed ([quinpirole] = 125 ± 8.26 nM); however, unlike under control conditions, quinpirole did not produce significant additional inhibition in firing rate over time (one-way repeated measures ANOVA, F(7,35) = 3.33, p > 0.05) (Figure 3A). As the effect of the β-ARK1 inhibitor could have been overwhelmed by the effect of the high neurotensin concentration, and as the β-ARK1 inhibitor has limited solubility, we tested whether a
lower concentration of neurotensin could mediate the reversal of quinpirole inhibition, and whether β-ARK1 inhibitor would be able to block quinpirole inhibition reversal induced by this lower neurotensin concentration.

Neurotensin (1 and 5 nM) was added to the superfusate for 15 min. Then concentrations of quinpirole that produced at least 50% inhibition during the first 5 min were applied for 40 min with the continued administration of neurotensin. In the presence of 5 nM neurotensin, quinpirole (60.7 ± 9.2 nM, ▼, n = 7) inhibited firing rate by 61.65 ± 6.2% at 5 min. Unlike the effect of quinpirole alone, however, quinpirole in the presence of 5 nM neurotensin did not significantly produce more inhibition over time; partial reversal of quinpirole inhibition was observed (One-way repeated measures ANOVA, $F_{(7,42)} = 1.25$, $p > 0.05$) (Figure 3B). Neurotensin (1 nM) did not mediate quinpirole inhibition reversal; there was a significant increase in inhibition over the time course of quinpirole administration in the presence of 1 nM neurotensin (One-way repeated measures, $F_{(7,21)} = 5.03$, $p < 0.05$) (data not shown). Therefore, 5 nM neurotensin was used to test whether β-ARK1 inhibitor could block neurotensin-induced quinpirole inhibition reversal.

With β-ARK1 inhibitor (300 µM) in the recording pipettes and neurotensin (5 nM) in the superfusate, quinpirole (45.8 ± 3.7 nM, ▼, n = 8) produced a significant inhibition in firing rate with no reversal (one-way repeated measures ANOVA, $F_{(7,49)} = 4.2$, $p < 0.05$), and this inhibition was similar to that produced by quinpirole alone (Figure 3B). This result suggests that there is a competitive dose effect in the interaction between neurotensin and β-ARK1 inhibitor; greater activation of neurotensin receptors can
overcome the interference by β-ARK1 inhibitor with the mechanism of reversal of quinpirole inhibition.

There is evidence that neurotensin activation of PKC can phosphorylate D2 receptors in HEK293 cells, resulting in phosphorylation and desensitization of the receptors (Thibault, et al., 2011). Therefore, we examined whether inhibition of cPKC can interfere with neurotensin reversal of quinpirole-induced inhibition. In the presence of cPKC inhibitor Gö6976 (10 µM) in the recording pipette and neurotensin (10 nM) in the superfusate (♂, n = 5), quinpirole significantly inhibited firing rate, with no reversal ([quinpirole] = 64 ± 34.07 nM) (one-way repeated measures ANOVA, F(7,28)= 87.9, p < 0.05) (Figure 3C), suggesting that neurotensin activation of cPKC may phosphorylate GRK2 that further phosphorylate and desensitize D2 receptors.

Then we tested whether neurotensin reversal of quinpirole inhibition is dependent on a dynamin-dependent process. In the presence of neurotensin (10 nM) in the superfusate and dynasore (800 µM) in the recording electrode (▼, n = 7), no reversal of quinpirole-induced inhibition was observed; quinpirole (75 ± 12.2 nM) produced a significant inhibition in firing rate over time (one-way repeated measures ANOVA, F(7,42) = 6.91, p < 0.05) (Figure 3D). This result suggests that desensitization of D2 receptors induced by activation of neurotensin receptors is also dependent on a dynamin-dependent process, such as internalization of the receptor.

**Activation of serotonin receptors reversed quinpirole-induced inhibition through conventional protein kinase C, G protein-coupled receptor kinase-2 and dynamin-dependent processes**
We also examined whether prolonged activation of serotonin receptor, that has been reported to reverse quinpirole inhibition (Nimitvilai, et al., 2012c), is dependent on phosphorylation and internalization induced by GRK2 and dynamin, respectively (Figure 3). As in our previous report (Nimitvilai, et al., 2012c), when serotonin (50 µM) was added to the superfusate (■, n = 9), quinpirole (115 ± 16.96 nM) produced an inhibition in firing rate initially with the maximum inhibition of 69.23 ± 5.85% at 10 min, and this inhibition partially reversed with time; there was a significant difference between firing rate at the last three time points and the firing rate at the 10 min time point (one-way repeated measures ANOVA, F(7,56) = 5.18, p < 0.05) (Figure 4A). Serotonin (50 µM) alone did not produce a significant change in firing rate over the 60 min time course (One-way repeated measures ANOVA, F(11,33) = 2.3, P > 0.05) (data not shown).

In the presence of serotonin in the superfusate and β-ARK1 inhibitor (300 µM) in the recording electrode (●, n = 7), quinpirole (72.86 ± 8.01 nM) produced an inhibition in firing rate, and this inhibition did not significantly reverse over the duration of drug application (one-way repeated measures ANOVA, F(7,42) = 1.49, p > 0.05) (Figure 4A).

We also tested whether blocking cPKC could interfere with serotonin reversal of quinpirole inhibition. A similar suppression of serotonin reversal of quinpirole inhibition was observed when the cPKC inhibitor Gö6976 (10 µM) was present in the recording pipettes (●, n = 6); in this case, quinpirole (102.5 ± 25.3 nM) significantly inhibited the firing rate over the duration of drug application (one-way repeated measures ANOVA, F(7,35) = 2.8, p < 0.05) (Figure 4B). These results suggest that serotonin activation of cPKC may directly phosphorylate GRK2, and this activated GRK2 further phosphorylates D2 receptor.
When serotonin was added in the superfusate with dynasore (800 µM) in the recording electrode (▼, n = 6), quinpirole (125 ± 38.7 nM) produced a significant inhibition in firing rate, with no reversal (one-way repeated measures ANOVA, $F_{(7,35)} = 4.0, p < 0.05$) (Figure 4C). This result suggests that serotonin reversal of quinpirole inhibition requires internalization of D2 receptors induced by the endocytic GTPase dynamin.
Discussion

We have previously reported a phenomenon of dopamine inhibition reversal (DIR) that is induced by extended periods of exposure to moderate concentrations of dopamine; DIR persists for up to 90 min, and requires the concurrent stimulation of D1/D5 and D2 dopamine receptors (Nimitvilai and Brodie, 2010). We have also demonstrated that DIR is mediated by PLC and cPKC, and is dependent on both extracellular calcium influx and intracellular calcium release (Nimitvilai, et al., 2012a). In addition, agonists of either 5HT2 or neurotensin receptors, both of which are linked to PLC activation, produced a decrease in sensitivity of D2 receptor to D2 agonist quinpirole (Nimitvilai, et al., 2012c), suggesting that this phenomenon is mediated through Gq/PLC/PKC pathway. In the present study, we extended our examination of reversal of dopamine or quinpirole inhibition to show that enzymes involved in phosphorylation of D2 receptors are required; phosphorylation as a result of dopamine, serotonin, or neurotensin receptor stimulation is mediated by G-protein receptor kinase-2 (GRK2) and internalization is mediated by the endocytic GTPase dynamin. Of course, we are basing our interpretation on our pharmacological results, but biochemical studies would be needed to confirm this interpretation of our findings. As we did not specifically examine phosphorylation of D2 receptors, we cannot be certain that it is indeed the D2 receptor that is the phosphorylation target.

Functional efficiencies of G-protein coupled receptors (GPCRs) are not static, but are dynamic and dependent on a memory of prior receptor activation (Hausdorff, et al., 1990). Prolonged or repeated activation of a receptor results in a reduced response to a subsequent receptor stimulation, a process called desensitization. Desensitization can be
homologous or heterologous; homologous desensitization is due to a decrease in response of a receptor as a result of binding its agonist, while heterologous desensitization is due to a decrease in response to a receptor as a result of agonist binding to a different receptor.

We found that dopamine inhibition reversal of DAergic VTA neurons is neither homologous- nor heterologous- desensitization since it requires concurrent stimulation of D2 and D1/D5 receptors (Nimitvilai and Brodie, 2010). To our knowledge, the D2 receptor is unique in that no other G-protein-coupled receptor requires activation of two receptors to stimulate the processes necessary for desensitization of that receptor. G-protein receptor kinases (GRKs) and β-arrestins are the two major cytoplasmic components responsible for desensitizing GPCR signaling (Premont, et al., 1995; Sterne-Marr and Benovic, 1995). Binding of a ligand as well as the release of Gα and Gβγ induce the conformational change of the GPCR, resulting in a recruitment of GRK to the serine/threonine phosphorylation site of the intracellular loops or the COOH terminus of the receptor (Pitcher, et al., 1998). GRK phosphorylation of the GPCR increases the affinity of that GPCR for β-arrestin. Once β-arrestin binds to GPCR, it prevents the reformation of functional GPCR so that a ligand cannot bind and activate the receptor. The β-arrestin also recruits clathrin and the adaptor protein AP2 to the phosphorylated GPCR, resulting in the formation of clathrin-coated vesicle. Dynamin GTPase then pinches off the clathrin-coated pit from the cell surface, internalizing the receptor (McMahon and Boucrot, 2011).

Dopamine receptors, like other GPCRs, can be regulated in a number of ways. Dopamine receptors contain phosphorylation sites for GRK, PKC, and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) on their third intracellular loop and their C-
terminal region (Namkung and Sibley, 2004; Bofill-Cardona, et al., 2000). Phosphorylation and desensitization of D2 receptors by PKC and CaMKII has also been studied in HEK293 cells, striatal and hippocampal neurons (Rogue, et al., 1990; Bofill-Cardona, et al., 2000; Namkung and Sibley, 2004; Thibault, et al., 2011). Second messenger-dependent protein kinases can either directly phosphorylate and desensitize the GPCR (Bofill-Cardona, et al., 2000; Namkung and Sibley, 2004), or phosphorylate GRKs (Pronin and Benovic, 1997; Pronin, et al., 1997; Chuang, et al., 1996; Chuang, et al., 1995; Winstel, et al., 1996). Second-messenger protein kinase phosphorylation of GRK can either activate or inhibit GRK activity. For example, PKC phosphorylation can activate GRK2 (Chuang, et al., 1995; Winstel, et al., 1996), but inhibit GRK5 (Pronin and Benovic, 1997) activity in β-adrenergic receptors. Calmodulin inhibits GRK activity with a higher specificity of GRK5 (IC50 ~ 50 nM) over GRK2 (IC50 ~ 2 µM) (Chuang, et al., 1996; Pronin, et al., 1997). Phosphorylation and desensitization of dopamine D2 receptors by GRK2 and GRK5 have been reported (Ito, et al., 1999).

At present, seven subtypes of GRKs (GRK1-7) have been identified. GRK1 and GRK7 are expressed in the photoreceptor rhodopsin, and GRK4 is found predominantly in the male germ line. GRK2, 3, and 6 are widely distributed in the rat brain, in which GRK2 is present in high levels in most brain areas, and is also expressed in VTA neurons (Erdtmann-Vourliotis, et al., 2001). In this study, we demonstrated that desensitization of D2 receptor induced by dopamine, serotonin, or neurotensin was not observed when GRK2 was suppressed, suggesting that GRK2 is involved in the desensitization of D2 receptors induced by dopamine, serotonin, or neurotensin. We only used one antagonist (β-ARK1 inhibitor) to interfere with GRK2, as it is somewhat unique in having properties...
suitable for use with the methods used in other experiments in this study; more complex studies using other methods (e.g., siRNA or knock-out of GRK2) would be needed to more definitively establish the importance of GRK2 in the desensitization mechanism. Since cPKC is required to produce D2 desensitization (Nimitvilai, et al., 2012a) and the present study showed that blocking of cPKC by Gö6976 also inhibited serotonin- or neurotensin-induced reversal of quinpirole inhibition, it is possible that activation of cPKC by either dopamine, serotonin, or neurotensin results in its binding to and phosphorylation of GRK2.

Of seven subtypes of serotonin receptors, 5-HT2 is the G protein-coupled receptor that is linked to Gq, and 5-HT2 inhibitor ketanserin is able to block serotonin-induced quinpirole inhibition reversal (Nimitvilai, et al., 2012c). For the neurotensin receptors, NTS1 is the likely subtype of the neurotensin receptor present in the VTA (Palacios and Kuhar, 1981;Quirion, et al., 1985). Therefore, our model proposes that dopamine, serotonin or neurotensin activate a cPKC that binds to and phosphorylates GRK2, increasing the functional activity of GRK2 in DAergic VTA neurons (Figure 5). The functional GRK2 may further phosphorylate D2 receptor, resulting in the desensitization of the receptor. However, there is also evidence of the ability of GRK2 to constitutively attenuate D2 receptor function through a mechanism independent of receptor phosphorylation (Namkung, et al., 2009). Whether GRK2 desensitization of D2 receptors in DAergic VTA neurons requires receptor phosphorylation is not known. It is unlikely that GRK5, which is also involved in phosphorylation and desensitization of D2 receptors in HEK293 and COS7 cells (Ito, et al., 1999), is involved in DIR since it is strongly inhibited by both Ca²⁺/calmodulin and PKC (Pronin, et al., 1997;Chuang, et al., 1996),
and overexpression of GRK5 fails to mediate desensitization of several GPCRs (Diviani, et al., 1996; Rockman, et al., 1996). In addition, GRK5 and GRK6, when co-expressed with D2 receptors in HEK293 cells, have no impact on agonist-induced D2 signaling (Namkung, et al., 2009). Other possibilities, such as whether GRK3 or other proteins sensitive to β-ARK1 inhibitor are involved in desensitization of D2 receptor in DAergic VTA neurons will be a subject for future study.

We also demonstrated that inhibition of dynamin GTPase suppressed dopamine inhibition reversal, and quinpirole inhibition reversal produced by either serotonin or neurotensin. These results suggest that once D2 receptor has been desensitized, it will be internalized into the endosome. The endocytosed receptor may be dephosphorylated before returning to the cell surface or it may be degraded to lysosomes. We have shown previously that the effect of DIR is long-lasting, persisting for up to 90 min after washout of dopamine (Nimitvilai and Brodie, 2010). However, whether the reoccurrence of inhibition in firing rate produced by D2 agonist is the result of recycling of D2 receptors that had been initially desensitized, or is the result of activation of newly synthesized D2 receptors is not known.

Exposure to drugs of abuse causes a sustained increase in DAergic neurotransmission in the reward system; desensitization of the inhibitory D2 autoreceptors in the VTA neurons through the PI/PLC/cPKC pathway may increase the excitability of these neurons. The present study further defines the mechanism of that desensitization induced by dopamine, serotonin, or neurotensin, with the involvement of phosphorylation and internalization of D2 receptors. The action of drugs of abuse on DAergic VTA neurons to reduce D2 autoreceptor inhibition, resulting in an increase in
DAergic neurotransmission in the reward/reinforcement system, may be a key event in the development of addiction. Understanding molecular mechanisms underlying the reversal of dopamine inhibition in the VTA may contribute to medication discovery for more effective treatment of addiction disorders.
Authorship contributions

Participated in research design: Nimitvilai and Brodie

Conducted experiments: Nimitvilai and McElvain

Performed data analysis: Nimitvilai and Brodie

Wrote or contributed to the writing of the manuscript: Nimitvilai and Brodie
Reference List


Lacey MG, Mercuri NB and North RA (1987) Dopamine acts on D2 receptors to increase potassium conductance in neurones of the rat substantia nigra zona compacta. *J Physiol (Lond)* **392**:397-416.


Nimitvilai S, Arora D and Brodie M (2012a) Reversal of dopamine inhibition of dopaminergic neurons of the ventral tegmental area is mediated by protein kinase C. *Neuropsychopharmacology* **37**:543-556.

Nimitvilai S, Arora DS and Brodie MS (2012b) Reversal of Dopamine Inhibition of Dopaminergic Neurons of the Ventral Tegmental Area is Mediated by Protein Kinase C. *Neuropsychopharmacology* **37**:543-556.


Nimitvilai S, McElvain MA, Arora DS and Brodie MS (2012c) Reversal of quinpirole inhibition of ventral tegmental area neurons is linked to the phosphatidylinositol system and is induced by agonists linked to Gq. *J Neurophysiol In Press*.


Footnotes

The authors gratefully acknowledge support for this work from the National Institutes of Health, National Institute on Alcohol Abuse and Alcoholism, [PHS Grant AA05846 and AA09125].

Reprint requests should be sent to:

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
E-mail: mbrodie@uic.edu
Legends for Figures:

Figure 1 Mean ratemeter graphs of the effects of long duration application of dopamine in the presence or absence of either GRK2 inhibitor or dynamin inhibitor in single neurons. Vertical bars indicate the firing rate over 5 sec intervals; dashed vertical line indicates the end of the dopamine administration for clarity. Horizontal bars indicate the duration of drug application (concentrations indicated above bar). (A) Dopamine alone initially produced a decrease in firing rate which subsided over time in the continued presence of dopamine. (B) In the presence of 300 μM β-ARK1 inhibitor in the recording pipette, dopamine produced an inhibition in firing rate, and this inhibition did not reverse with time. (C) In the presence of 800 μM dynasore in the recording pipette, dopamine produced an inhibition in firing rate, with no reversal during dopamine administration. (D) In the presence of 400 μM MitMAB™ in the recording pipette, dopamine produced a significant inhibition in firing rate over the time course of administration indicating blockade of reversal.

Figure 2 Effect of inhibitors of GRK2 and dynamin on long-duration dopamine application. Relative change in firing rate (mean ± S.E.M.) is plotted as a function of time. In experiments similar to those shown in Figure 1, the effect of dopamine at each time point was normalized by subtracting the change in firing rate (%) at the 5 min time point. (A) Effect of β-ARK1 inhibitor on long-duration application of dopamine. A concentration of dopamine that produced more than 50% inhibition at 5 min time point was applied for 40 min. Dopamine (■, [DA] = 4.45 ± 1.25 μM, n = 10) alone initially inhibited the firing rate, and this inhibition significantly reversed with time (one-way
repeated measures ANOVA, $F_{(7,63)} = 5.96, p < 0.05$). In the presence of β-ARK1 inhibitor (300 μM), no reversal of dopamine inhibition was observed; dopamine significantly inhibited the firing rate over time (●, $[DA] = 5.2 \pm 1.2 \mu M, n = 9$) (one-way repeated measures ANOVA, $F_{(7,56)} = 3.23, p < 0.05$).

(B) Effect of dynamin inhibitors dynasore or MiTMAB™ on long-duration application of dopamine. The effect of dopamine alone (■ and dashed line) from Figure 2A is shown for comparison. In the presence of dynasore (800 μM) in the recording pipette, dopamine did not produce the inhibition reversal (▼, $[DA] = 6.64 \pm 1.58 \mu M, n = 7$) (one-way repeated measures ANOVA, $F_{(7,42)} = 1.29, p > 0.05$). In the presence of MiTMAB™ (400 μM) in the recording pipette, dopamine produced a significant inhibition in firing rate, with no reversal (●, $[DA] = 7.5 \pm 1.12 \mu M, n = 5$) (one-way repeated measures ANOVA, $F_{(7,28)} = 5.46, p < 0.05$).

Figure 3 Effect of inhibitors of GRK2, cPKC and dynamin on long-duration application of neurotensin and quinpirole Relative change in firing rate (mean ± S.E.M.) is plotted as a function of time. (A) Effect of β-ARK1 inhibitor on long-duration application of quinpirole and neurotensin (10 nM). Quinpirole (54 ± 8.5 nM) alone produced a sustained significant inhibition in firing rate over the duration of quinpirole application (□, $n = 10$) (one-way repeated measures ANOVA, $F_{(7,63)} = 11.2, p < 0.05$). With 10 nM neurotensin in the superfusate (○, $n = 9$), there was a significant difference in the magnitude of quinpirole inhibition at the 25-40 min time points compared to the 10 min time point, indicating reversal of inhibition (one-way repeated measures ANOVA, $F_{(7,35)} = 4.0, p < 0.05$). In the presence of 10 nM neurotensin in the superfusate and 300 μM β-
ARK1 inhibitor in the recording pipette (●, n = 6), no reversal and no significant increase in inhibition in firing rate produced by quinpirole were observed ([quinpirole] = 125 ± 8.26 nM) (one-way repeated measures ANOVA, $F_{(7,35)} = 3.33, p > 0.05$).

(B) Effect of β-ARK1 inhibitor on long-duration application of quinpirole and neurotensin (5 nM). The effect of quinpirole alone (□ and dashed line) from Figure 3A is shown for comparison. With 5 nM neurotensin in the superfusate (∇, n = 7), no significant inhibition produced by quinpirole (60.7 ± 9.2 nM) was observed (one-way repeated measures ANOVA, $F_{(7,42)} = 1.25, p > 0.05$); there was a partial reversal in the magnitude of quinpirole inhibition over time. In the presence of 5 nM neurotensin in the superfusate and 300 µM β-ARK1 inhibitor in the recording pipette (▼, n = 8), quinpirole (45.83 ± 3.71 nM) produced a significant inhibition in firing rate over the time course (one-way repeated measures ANOVA, $F_{(7,49)} = 4.16, p < 0.05$).

(C) Effect of Gö6976 on long-duration application of quinpirole and neurotensin (10 nM). The effect of quinpirole alone (□ and dashed line) and quinpirole in the presence of 10 nM neurotensin (■ and dashed line) from Figure 3A are shown for comparison. In the presence of 10 µM Gö6976 in the recording pipette and neurotensin in the superfusate (●, n = 5), no significant quinpirole inhibition reversal was observed ([quinpirole] = 64 ± 34.1 nM) (one-way repeated measures ANOVA, $F_{(7,28)} = 87.9, p < 0.05$).

(D) Effect of dynasore on long-duration application of quinpirole and neurotensin. The effect of quinpirole alone (□ and dashed line) and quinpirole in the presence of 10 nM neurotensin (■ and dashed line) from Figure 3A are shown for comparison. In the presence of 10 nM neurotensin in the superfusate and 800 µM dynasore in the recording
electrode (▼, n = 7), no reversal of quinpirole-induced inhibition was observed; quinpirole (75 ± 12.2 nM) produced a significant inhibition in firing rate over time (one-way repeated measures ANOVA, F(7,42) = 6.91, p < 0.05).

Figure 4 Effect of inhibitors of GRK2, cPKC and dynamin on long-duration application of serotonin and quinpirole

Relative change in firing rate (mean ± S.E.M.) is plotted as a function of time. (A) Effect of β-ARK1 inhibitor on long-duration application of quinpirole and serotonin. The effect of quinpirole alone (□ and dashed line) from Figure 3A is shown for comparison. With 50 μM serotonin in the superfusate (■, n =9), there was a significant difference in the magnitude of quinpirole inhibition at 30-40 min time points compared to the 10 min time point, indicating reversal of inhibition. In the presence of 50 μM serotonin in the superfusate and 300 μM β-ARK1 inhibitor in the recording pipette (●, n = 7), no reversal and no significant inhibition in firing rate produced by quinpirole were observed ([quinpirole] = 72.9 ± 8.0 nM) (one-way repeated measures ANOVA, F(7,42)= 1.49, p > 0.05).

(B) Effect of Gö6976 on long-duration application of quinpirole and serotonin. The effect of quinpirole alone (□ and dashed line) from Figure 3A and quinpirole in the presence of 50 μM serotonin (■ and dashed line) from Figure 4A are shown for comparison. In the presence of 10 μM Gö6976 in the recording pipette and serotonin in the superfusate (●, n = 6), no significant quinpirole inhibition reversal was observed ([quinpirole] = 102.5 ± 25.3 nM) (one-way repeated measures ANOVA, F(7,35)= 92.8, p < 0.05).
(C) Effect of dynasore on long-duration application of quinpirole and serotonin. The effect of quinpirole alone (□ and dashed line) from Figure 3A and quinpirole in the presence of serotonin (■ and dashed line) from Figure 4A are shown for comparison. In the presence of 50 µM serotonin in the superfusate and 800 µM dynasore in the recording electrode (▼, n = 6), no reversal of quinpirole-induced inhibition was observed; quinpirole (125 ± 38.7 nM) produced a significant inhibition in firing rate over time (one-way repeated measures ANOVA, \( F_{7,35} = 4.0, p < 0.05 \)).

Figure 5. Model of the action of different Gq-coupled receptors on cPKC and D2 receptor sensitivity. Diagram illustrates an hypothetical interaction of 5HT₂, neurotensin (NTS1), and dopamine (D2, D1-like) receptors on cPKC in DA VTA neurons, based on the results of the present study. The G-protein coupled inwardly rectifying potassium channel (GIRK) that is activated by \( G\beta\gamma \) and that causes the hyperpolarization resulting in a decrease in firing rate is shown. Activation of dopamine receptors (D2 plus D1/D5), serotonin 5HT₂, or neurotensin NTS1 receptors activates cPKC, which phosphorylates and stimulates GRK2. The stimulated GRK2 then phosphorylates the D2 receptor, resulting in the desensitization of the receptor.
Table 1 – Firing rates of VTA neurons: Effects of inhibitors and quinpirole

<table>
<thead>
<tr>
<th>Figures</th>
<th>Change by inhibitors</th>
<th>Pre-quinpirole or DA baseline FR (Hz)</th>
<th>Percentage change by DA or quinpirole at 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Control (no inhibitor)</td>
<td>-</td>
<td>2.07 ± 0.17</td>
<td>-72.78 ± 4.83</td>
</tr>
<tr>
<td>- β-ARK1 inhibitor</td>
<td>-2.71 ± 3.25</td>
<td>2.45 ± 0.27</td>
<td>-64.47 ± 3.42</td>
</tr>
<tr>
<td>- Dynasore</td>
<td>-2.51 ± 2.49</td>
<td>2.35 ± 0.24</td>
<td>-62.99 ± 3.0</td>
</tr>
<tr>
<td>Figure 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Control (no inhibitor)</td>
<td>-</td>
<td>2.55 ± 0.34</td>
<td>-68.27 ± 4.94</td>
</tr>
<tr>
<td>- Neurotensin (10 nM)</td>
<td>-</td>
<td>3.63 ± 0.38</td>
<td>-65.5 ± 4.16</td>
</tr>
<tr>
<td>- Neurotensin (5 nM)</td>
<td>-</td>
<td>3.15 ± 0.58</td>
<td>-61.65 ± 6.2</td>
</tr>
<tr>
<td>- β-ARK1 inhibitor</td>
<td>-7.75 ± 3.53</td>
<td>1.8 ± 0.45</td>
<td>-68.21 ± 6.85</td>
</tr>
<tr>
<td>- Gö6976</td>
<td>0.88 ± 3.0</td>
<td>1.38 ± 0.22</td>
<td>-64.54 ± 6.24</td>
</tr>
<tr>
<td>- Dynasore</td>
<td>-6.79 ± 3.9</td>
<td>1.75 ± 0.15</td>
<td>-64.31 ± 6.35</td>
</tr>
<tr>
<td>Figure 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Serotonin</td>
<td>-</td>
<td>2.53 ± 0.21</td>
<td>-64.02 ± 5.38</td>
</tr>
<tr>
<td>- β-ARK1 inhibitor</td>
<td>-2.95 ± 1.94</td>
<td>2.23 ± 0.32</td>
<td>-73.68 ± 5.93</td>
</tr>
<tr>
<td>- Gö6976</td>
<td>-9.04 ± 3.63</td>
<td>1.41 ± 0.28</td>
<td>-58.3 ± 2.75</td>
</tr>
<tr>
<td>- Dynasore</td>
<td>-2.03 ± 2.63</td>
<td>2.59 ± 0.3</td>
<td>-62.26 ± 6.57</td>
</tr>
</tbody>
</table>