Published in final edited form as:

Eur J Neurosci. 2012 May ; 35(9): 1406–1415. doi:10.1111/j.1460-9568.2012.08029.x.

Presynaptic Inhibition of Glutamate Transmission by Alpha-2 Receptors in the VTA

Carlos A. Jiménez-Rivera, Johnny Figueroa, Rafael Vázquez, María Vélez, David Schwarz, María C. Velásquez-Martínez, and Francisco Arencibia-Albite

Department of Physiology, University of Puerto Rico, Medical Sciences Campus, San Juan, Puerto Rico, 00936-50 67

Abstract

The ventral tegmental area (VTA) forms part of the mesocorticolimbic system and plays a pivotal role in reward and reinforcing actions of drugs of abuse. Glutamate transmission within the VTA controls important aspects of goal-directed behavior and motivation. Noradrenergic receptors also present in the VTA have important functions in the modulation of neuronal activity. Here we studied the effects of alpha-2 noradrenergic receptor activation in the alteration of glutamate neurotransmission in VTA dopaminergic neurons from male Sprague-Dawley rats. We used whole cell patch clamp recordings from putative VTA dopaminergic neurons and measured excitatory postsynaptic currents. Clonidine (40 μ M) and UK 13,408 (40 μ M), both alpha-2 receptor agonists, reduced (~ 40%) the amplitude of glutamate-induced excitatory postsynaptic currents. After clonidine administration, there was a dose-dependent reduction over the concentration range of 15–40 μ M. Using yohimbine (20 μ M) and two other alpha-2 adrenergic receptor antagonists, idazoxan (40 μ M) and atipemazole (20 μ M), we demonstrated that the inhibitory action is specifically mediated by alpha-2 receptors. Moreover, by inhibiting protein kinases with H-7 (75 μ M), Rp-adenosine 3',5'-cyclic (11 μ M) and chelerythrine (1 μ M) it was shown that the clonidine-induced inhibition seems to involve a selective activation of the protein kinase C intracellular pathway. An increased paired-pulse ratios and changes in spontaneous and miniature excitatory postsynaptic currents frequencies but not amplitudes indicated that the alpha-2 agonist's effect was presynaptically mediated. It is suggested that the suppression of glutamate excitatory inputs onto VTA dopaminergic neurons might be relevant in the regulation of reward and drug seeking behaviors.

Keywords

rats; clonidine; addiction; dopamine; patch clamp

Introduction

The Ventral Tegmental Area (VTA) is the source of dopaminergic (DA) projections to structures in the ventral and cortical areas giving rise to the mesocorticolimbic system (Dahlstrom and Fuxe, 1964; Ungerstedt, 1971). The VTA plays a pivotal role in motivated behavior and reinforcing actions of drug of abuse (Schultz, 2002; Grace et al., 2007).

Glutamate in the VTA is one of the most important neurotransmitters involved in reward and addictive processes (see reviews by Zellner and Rinaldi, 2010). Glutamatergic synaptic inputs onto VTA neurons arise from several brain areas, among them the medial prefrontal

cortex (PFC), bed nucleus of stria terminalis and laterodorsal tegmentum (LDT) (Carr and Sesack, 2000; Chou et al. 2001; Georges and Aston-Jones, 2002; Omelchenko and Sesack, 2007). The convergence of all these glutamatergic inputs onto the VTA indicates an exceptional integrative capacity of this area that might be used in guiding reward and motivational behavioral responses (Geisler and Zahm, 2005).

The physiological activity of VTA neurons is profoundly altered by glutamate neurotransmission. There are two main patterns of activity of VTA DA neurons, pacemaker or bursting activity (Cooper, 2002; Kitai et al., 1999; Overton and Clark, 1997). Glutamatergic afferents provide a potent excitatory drive essential for burst firing of VTA DA neurons (Floresco et al., 2003; Lodge and Grace, 2006). Changes from single action potentials to bursting firing enhance neurotransmitter release and promote activity-dependent synaptic plasticity (Cooper, 2002). Burst firing in VTA DA cells signals reward and modulate goal-directed behavior (Schultz, 1998; Grace, 1991; Phillips et al. 2003; Lodge and Grace, 2006). The LDT plays a permissive gating action enabling bursting in VTA DA neurons whereas the pedunculopontine tegmentum directly regulates the bursting pattern in these cells (Lodge and Grace, 2006). Therefore, modulatory changes in glutamate neurotransmission at the VTA can induce dramatic alterations in firing activity of DA and non-DA neurons that will eventually alter complex physiological behaviors.

The VTA receives noradrenergic (NE) innervation arising mainly from the A1, A2, A5 and locus coeruleus (Mejias-Aponte et al., 2009). These afferent inputs make synaptic contacts onto midbrain DA neurons (Liprando et al., 2004). Several subtypes of NE receptors are present in the VTA, which suggest that NE inputs play an important role in the modulation of VTA DA neuronal activity (Lee et al., 1998).

In many brain regions the activation of NE receptors can modulate glutamate transmission. In the BST, presynaptic activation of alpha-2A receptors decreases glutamate transmission (Shields et al., 2009; Egli et al., 2005). Similarly, in sympathetic preganglionic, bulbospinal, hippocampal, spinal cord and brainstem neurons presynaptic activation of alpha-2A and in some cases alpha-2D receptors, inhibit glutamate release (Miyazaki et al., 1998; Abdallah and Guyenet, 1999; Boem, 1999; Pan et al., 2002; Bertolino et al., 1997). As a result, we asked if activation of alpha-2 receptors in putative VTA DA cells from rat midbrain could also produce presynaptic inhibition of glutamate transmission. Here we report that presynaptic activation of alpha-2 receptors inhibits glutamate release onto VTA DA cells.

Methods

Animals

All experimental procedures were performed according to the US Public Health Service publication "Guide for the Care and Use of Laboratory Animals" and were approved by the Animal Care and Use Committee at the University of Puerto Rico Medical Sciences Campus. Sprague-Dawley male rats (26–40 days postnatal) were used as the experimental subjects. Animals were housed two per cage and maintained at constant temperature and humidity with a 12-hr light/dark cycle. Water and food was provided ad libitum.

Slice preparation

Male Sprague-Dawley rats between 26 and 40 postnatal days were anesthetized by an intraperitoneal injection of 90-mg/kg chloral hydrate (Sigma, St. Louis, MO, USA) and their brains were rapidly removed. Horizontal slices (220 μ m) containing the ventral tegmental area were prepared using a vibratome (VT1000S, Leica, Germany). Slices were cut in an ice-cold oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 127 NaCl; 2.5 KCl; 1.25 NaH_2PO_4 ; 25 NaHCO_3 ; 2 CaCl_2 ; 1 MgCl_2 ; 25 D(+)-glucose, saturated with

95% O₂–5% CO₂ gas mixture to a pH=7.4. Slices were transferred to an intermediate chamber and were incubated at 32°C in the same solution for 45-min before being used for electrophysiological recordings. MK-801 (10 μM) was added to the incubation solutions to block *N*-methyl-D-aspartate (NMDA)-mediated excitotoxicity.

Electrophysiological recordings

For recording, slices were placed in a recording chamber (500 μL) superfused with ACSF at 2.5–3 ml/min. Picrotoxin (100 μM) was added to the ACSF during recording to block GABA_A receptor-mediated IPSC's. Whole cell voltage clamp recordings were obtained from visually identified neurons in the VTA using an infrared microscope with DIC, (BX51WI Olympus, Japan). Recordings were acquired through data acquisition software (pClamp 9 and 10, Axon Instruments). All recordings were performed in putative DA neurons identified by the presence of a large hyperpolarization-activated cation current (*I_h* >200 pA), evoked by a 1-s hyperpolarizing step from –60 to –130 mV. According to Sarti et al., 2007, *I_h* is present in about 84% VTA DA cells and VTA GABA neurons do not express this conductance (Margolis et al., 2006). Thus, the contribution of non-dopaminergic cells to the experimental recordings performed in this study is likely to be not significant. All whole-cell voltage clamp recordings were made at a holding potential of –70 mV. Borosilicate glass patch pipettes (O.D. 1.5 mm, I.D. 1.0 mm WPI, Sarasota, FL) were pulled to the final resistance of 3–6 MΩ when filled with (in mM): 115 KCH₃SO₄ (Methyl potassium sulfate); 20 KCl; 1.5 MgCl₂; 5 HEPES; 1 EGTA; 2 ATP; 0.2 GTP; 10 Creatine Phosphate (CP); pH=7.25, 290 mOsm. (Na) GTP, (Mg) ATP and (Na) CP were added fresh daily. Data were collected through a Multiclamp 700B amplifier (Axon Instruments, Foster City, CA, USA), filtered at 1 kHz, digitized at 5 kHz and stored in a PC computer and analyzed off line using GraphPad Prism 4.5 (GraphPad Software, Inc) software. Liquid junction potential of the pipette was offset compensated using standard Multiclamp 700B circuitry. The seal quality used was typically 4–6 GΩ. The series resistances were checked during the experiments and data was discarded if changes more than 15% occurred.

For miniature AMPA EPSCs (mEPSCs) experiments, tetrodotoxin (TTX, 0.5 μM) was added to the ACSF. For a given cell, mEPSCs were collected (1 sweep for each condition, 3 min/sweep) for a control and clonidine period. The recorded mEPSCs were analyzed afterwards using Mini Analysis program 6.0.7 (Synaptosoft Inc.). Detection criteria were set at >6 pA, <1.3 ms rise time, and <0.1 ms decay time. The choice of these cutoff amplitudes for acceptance of mEPSCs was made to obtain a high signal-to-noise ratio. Then, each event was also visually inspected to prevent noise disturbance of the analysis.

Drugs

Pharmacological agents used in this study were: Clonidine hydrochloride (2(2,6-Dichloroanilino)-2-imidazoline hydrochloride), Yohimbine hydrochloride (17-Hydroxy-yohimban-16-carboxylic acid methyl ester hydrochloride), UK 14,304 (5-Bromo-N-(2-imidazolin-2-yl)-6-quinoxalinamine), Rp (Rp-adenosine 3',5'-cyclic monophosphorothioate triethylammonium salt hydrate), Chelerythrine chloride (1,2-dimethoxy-12-methyl[1,3]benzodioxolo[5,6-c]phenanthridinium chloride), H-7 ((±)-1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride), Idaxozan (2-(1,4-Benzodioxan-2-imidazoline hydrochloride), Picrotoxin (1R,3R,5S,8S,13R,14S)-1-hydroxy-14-(2-hydroxypropan-2-yl)-13-methyl-4,7,10-trioxapentacyclo[6.4.1.1^{9,12}.0^{3,5}.0^{4,13}]-tetradecane-6,11-dione; (1R,5S,8S,13R,14R)-1-hydroxy-13-methyl-14-(prop-1-en-2-yl)-4,7,10-trioxapentacyclo[6.4.1.1^{9,12}.0^{3,5}.0^{4,13}]-tetradecane-6,11-dione, (+)-MK 801 (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate, Atipamezole (5-(2-Ethyl-2,3-dihydro-1H-indene-2-yl)-1H-imidazole hydrochloride, TTX (Octahydro-12-(hydroxymethyl)-2-

imino-5,9:7,10a-dimethano-10aH-{1,3}dioxocino[6,5-d]pyrimidine-4,7,10,11,12-pentol, NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f] quinoxaline-7-sulfonamide. All drugs were freshly made daily, dissolved in Ringer and applied to bath perfusion as needed. Picrotoxin and UK 14,304 were diluted in DMSO. All chemicals were obtained from Tocris (Ballwin, MO) or Sigma Chemical (St. Louis, MO).

Stimulus and Data Analysis

A bipolar stainless steel stimulating electrode (FHC, Inc, Bowdoin, ME) was placed approximately 100 μ m rostral to the recording electrode and was used to stimulate at 0.1 Hz by applying a brief (400 μ s; low pass filter 1 KHz, digitized 5 KHz) electrical pulse (100–300 μ A). AMPA-mediated Excitatory Post-Synaptic Currents (EPSC's) were recorded at –70 mV. All EPSCs shown in figures are averages of 5 current traces for the treatment under inspection. The amplitudes of the AMPA EPSC's were calculated by taking a 1 ms window around the peak of the EPSC and comparing this to a 5 ms window immediate before the stimulation artifact. Peak EPSCs amplitudes were average during control recordings. Then, this value was used to normalize control and treatment recordings. This procedure allowed expressing data as percentages of the control condition permitting in turn appropriate statistical comparisons.

Pair stimuli were given with an interstimulus interval of 50 ms. Pair Pulse Ratio or PPR was calculated as the ratio of the first and second EPSC's. Averaged data from different experiments were presented as mean $\pm$ SEM. Data statistical significance ($p < 0.05$) was assessed with a Student's *t*-test or One-Way Anova as appropriate, except when examining the significance of horizontal shifts to the cumulative probability distribution plots obtained from single cell recordings. For the latter case we used the Kolmogorov–Smirnov (K-S) test.

GABA_A inhibitory post synaptic currents (IPSCs) recordings were made with whole cell electrodes containing the following (mM) 128 KCL; 20 NaCL; 2 MgCl 2; 10 HEPES; 0.1 EGTA; 0.3 CaCL2; 2 ATP; 0.2 GTP. Due to the composition of the internal solution, IPSCs were inward at this membrane potential, and were blocked completely by a GABA_A receptor antagonist picrotoxin (100 μ M, $n=3$, data not shown). All GABA_A mediated currents were recorded in a constant presence of 2-amino-5 phosphonopentanoic acid (AP5; 50 μ M) 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline (CNQX; 10 μ M) and eticlopride (100 nM) to block NMDA, AMPA and dopamine D2-mediated synaptic currents, respectively.

Results

EPSCs were evoked and studied in putative VTA DA neurons using whole cell patch clamp recordings in slice preparations. We confirmed that this evoked current was due to AMPA receptor activation by blocking the response with the potent and selective AMPA receptor antagonist NBQX (30 μ M, data not shown). Similar to previous studies (Arencibia-Albite et al., 2007; Inyushin et al., 2010) recorded cells were located lateral to the fasciculus retroflexus and medial to the medial terminal nucleus of the accessory optic tract (Paxinos and Watson, 2009). Cells exhibited a pronounced I_h current, slow spontaneous activity and relatively regular interspike intervals, which are common accepted electrophysiological features for DA neurons in this area (Aghajanian and Bunney, 1973; Grace and Bunney, 1983; Grace and Onn, 1989).

Effect of alpha-2 receptor activation on glutamate-induced EPSCs

We use the alpha-2 receptor agonist clonidine to address the question of whether alpha-2 receptor activation can modulate glutamate transmission. Fig. 1A shows a representative trace where clonidine (40 μ M) superfusion induced a significant reduction in the AMPA

EPSC amplitude in a putative VTA DA cell held at -70 mV. The effect was reversible since it returned to near control levels after a washout period. As illustrated in figure 1B, clonidine's actions lasted throughout the period of drug administration (10 min), rapidly returning to pretreatment levels during the washout period (20 min). Figure 1C depicts a population summary of clonidine's action on EPSCs in all VTA DA cells tested ($n = 10$). As seen in this figure clonidine application resulted in a $\sim 40\%$ reduction in EPSCs amplitudes (EPSCs amplitudes were $60 \pm 5\%$ of control) which was statistically significant (control $99 \pm 1\%$ vs. clonidine $60 \pm 5\%$, $p = 6 \times 10^{-5}$, paired t-test, $n = 10$). No significant difference was observed between washout and control periods (control $99 \pm 1\%$ vs. washout $95 \pm 8\%$, $p = 0.58$, paired t-test, $n = 10$). This inhibitory action of clonidine was dose-dependent over the concentration range of 15 – 40 μM (Fig. 1D), was mimicked by the α -2 receptor agonist UK 13,408 (40 μM , data not shown) and was specific to EPSCs as clonidine application did not alter the amplitudes of IPSCs (Fig. 1C, control: $105 \pm 4\%$ vs. clonidine $103 \pm 7\%$, $p = 0.82$, $n = 6$).

We next determined the receptor specificity of clonidine-induced inhibition on AMPA EPSCs by using yohimbine (20 μM) an α -2 receptor antagonist. A 10 minute bath application of yohimbine alone did not alter EPSC amplitude when compare to control values (Fig. 2A, 2B and 2C; Control $101 \pm 1.0\%$ vs. Yoh $96 \pm 3\%$, $p = 0.54$, paired t-test, $n = 8$). However, when yohimbine and clonidine were co-superfuse for an additional 10 minutes the latter was unable to exert its typical inhibitory action (Fig 2D; Yoh $96 \pm 3\%$ vs. Yoh + Clon $98 \pm 5\%$, $p = 0.16$, paired t-test, $n = 8$). Thus, the clonidine-evoked inhibition of glutamatergic EPSCs in VTA DA cells seems to be the result of α -2 receptor activation. Indeed, α -2 antagonists idaxozan (40 μM , data not shown) and atipamezole (20 μM , Fig 2D) were also capable of completely abolishing clonidine's effect (atipamezole $102 \pm 5\%$ vs. atipamezole + Clon $98 \pm 9\%$, $p = 0.13$, paired t-test, $n = 7$; idaxozan $101 \pm 10\%$ vs. idaxozan + Clon $95 \pm 11\%$, $p = 0.52$, paired t-test, $n = 7$).

Studies have demonstrated that α -2 receptors are coupled with $G_{i/o}$ proteins (Docherty, 1998; Wurch et al., 2001). Two of the critical steps in the α -2 receptor intracellular cascade are the inhibition of protein kinase A (PKA) or the activation of protein kinase C (PKC) (Boron and Boulpaep, 2009; Boehm et al., 1996). To test which of these pathways might mediate the clonidine-induced reduction of EPSCs we first used the non-specific protein kinase inhibitor H-7 which inhibits both enzymes (Hidaka et al., 1984). If the α -2 mediated EPSC inhibition is the result of PKA inactivation then application of H-7 alone should imitate clonidine's action since H-7 inhibits PKA activity. Nonetheless, there was no change in the EPSC amplitude after superfusing H-7 (Fig. 3B, 75 μM ; control $99 \pm 1\%$ vs. H7 $99 \pm 1\%$, $p = 0.22$, paired t-test, $n = 6$). This finding makes it unlikely that the α -2 mediated EPSC reduction is evoked by PKA inhibition. In the presence of H-7, however, clonidine did not alter EPSC amplitude (H7 $99 \pm 1\%$ vs. H7 + Clon $96 \pm 6\%$, $p = 0.15$, paired t-test, $n = 6$) suggesting that clonidine's action at least involves the activation of PKC. Consistent with this claim, bath application of chelerythrine (1 μM , Fig 3A and 3C), a potent PKC inhibitor, was able to completely block clonidine-induced inhibition of EPSCs (chelerythrine $98 \pm 5\%$ vs. chelerythrine + Clon $109 \pm 6\%$, $p = 0.14$, paired t-test, $n = 6$). Superfusion of 11 μM Rp, an specific inhibitor of protein kinase A (PKA) (Wang et al., 1991) did not block the action of the α -2 agonist (Fig. 3D; Rp $84 \pm 6\%$ vs Rp + Clon $62 \pm 7\%$, $p = 0.009$, paired t-test, $n = 6$), further supporting the specific involvement of the PKC intracellular system in clonidine's effect.

To determine the synaptic site of action of clonidine-induced inhibition of EPSCs we first examined changes in paired-pulse ratio (PPR) in the presence of clonidine's administration. Figure 4A illustrates representative pair-pulse traces of AMPA EPSCs before (control) and after clonidine application while Fig. 4B summarizes the change in PPR of 6 experiments as

the one depicted in Fig. 4A. Analysis of the paired-pulse ratio in this set of cells (Fig. 4C) indicated a significant paired-pulse facilitation in response to clonidine (40 μ M) application (from 0.7 ± 0.04 before and 0.84 ± 0.03 after clonidine, $p=0.002$, paired t-test, $n=6$). This action was specific to EPSCs as clonidine application did not alter IPSCs' PPR (Fig. 4V, control: 0.73 ± 0.09 vs. clonidine: 0.74 ± 0.1 , $p=0.79$, paired t-test, $n=6$). As expected clonidine's effect on PPR was blocked by yohimbine administration (Fig. 4D; $F(2,15) = 1.33$, $p=0.29$, $n=6$). Consequently, it seems that clonidine acts through alpha 2 receptors at the presynaptic site decreasing glutamate liberation.

To further establish the presynaptic action of clonidine in the inhibition of AMPA EPSCs, we investigated possible changes in spontaneous EPSCs (sEPSCs) before and after clonidine's (40 μ M) administration. Sample recordings before (control) and after the agonist's application (Fig. 5A) showed that there was a reduction in the frequency of sEPSCs but not the amplitude. Clonidine reduced the frequency of sEPSCs from 1.5 ± 0.4 to 0.4 ± 0.1 Hz ($p=0.026$, paired t-test, $n=7$; Fig 5D) while the mean amplitude of sEPSCs before and during clonidine application was 23.2 ± 2.1 and 26.1 ± 3.3 pA, respectively ($p=0.45$, paired t-test, $n=7$; Fig. 5D). These findings were further supported by their respective cumulative probability distributions. In particular, a positive shift was observed in frequency distribution but no shift was seen in the amplitude distribution implying longer interevent intervals and no change in amplitude, respectively (Fig. 5B and 5C). Consequently, it appears that clonidine reduced glutamatergic transmission onto VTA DA neurons by an alpha2-mediated presynaptic mechanism.

In order to test whether clonidine effects were only due to an action-potential dependent mechanism we measured changes in miniature EPSCs (mEPSCs) in the presence of TTX (0.5 μ M). A sample recording in the presence of TTX, illustrates that clonidine application significantly decreases the frequency of mEPSCs (Fig. 6A) but not its amplitude. The frequency was decreased from 0.5 ± 0.1 to 0.2 ± 0.3 Hz ($p=0.011$, paired t-test, $n=8$; Fig. 6D). In contrast, changes in amplitude were non-significant, from 24.1 ± 2.0 pA before drug application to 26.6 ± 2.9 pA after clonidine administration ($p=0.31$, paired t-test, $n=8$; Fig. 6D). These results were also confirmed by the inter-event and amplitude cumulative distributions. The inter-event distribution showed a positive shift with clonidine administration (Fig. 6B) while no changes were observed in the amplitude distribution under clonidine treatment (Fig. 6C). It follows then that clonidine seems to act at the presynaptic site by an action-potential independent mechanism.

Discussion

The present study demonstrated that alpha-2 receptor agonists produced a dose response inhibition of glutamate synaptic transmission onto putative VTA DA neurons. Clonidine-induced suppression of glutamatergic EPSCs in putative VTA DA cells seems to be specifically mediated by alpha-2 receptors since yohimbine and atipamezole, two selective alpha-2 receptors antagonists, blocked clonidine's inhibitory actions. By employing inhibitors of protein kinases such as H-7, Rp and chelerythrine we were able to demonstrate that clonidine-induced suppression of glutamate transmission involves a selective activation of a PKC intracellular pathway. Furthermore, an analysis of changes in paired-pulse ratios and sEPSCs and mEPSCs recordings indicated that the inhibitory effect was mediated by activation of heterosynaptic presynaptic receptor mechanisms.

The source of glutamatergic afferents onto VTA DA neurons arises from several brain areas that include the mPFC, bed nucleus stria terminalis and LDT. The glutamatergic inputs from the mPFC synapses mainly onto VTA DA neurons that in turns connect back to the mPFC and to GABAergic cells projecting to the nucleus accumbens (Carr and Sesack, 2000). This

excitatory connection from the cortex might facilitate learning in VTA DA neurons by the communication of future expectations (Schultz, 1997). The reciprocal interaction with the PFC mediates an inhibitory feedback mechanism since DA suppresses PFC cell activity (Sesack and Bunney, 1989). In addition, a powerful excitatory influence onto VTA DA neurons is exerted by glutamatergic projections from the bed nucleus stria terminalis (Georges and Aston-Jones, 2002). This connection has been linked to reward-directed behaviors such as cocaine self-administration and drug seeking (Dumont et al., 2005; Aston-Jones and Harris, 2004). The LDT also provides a strong input onto VTA neurons (Omelchenko and Sesack, 2005). LDT innervation selectively targets the mesoaccumbens DA neurons whose activation is involved in drug abuse. All these excitatory inputs converging onto the VTA highlight the exceptional integrative capacity of this area. The glutamate-induced activation of these DA neurons appears to be closely related to the detection of rewards, novelty and experience dependent plasticity associated with the pathophysiology of addiction (Wise, 2008; Legault and Wise, 2001; Kauer and Malenka, 2006).

Noradrenergic afferents emerging from the A1, A2, A5 and locus coeruleus innervate the VTA (Mejias-Aponte et al., 2009). Many subtypes of NE receptors are present in this area but the alpha-2C receptor moiety is the most abundant variant (Lee et al., 1998; Rosin et al., 1996). Alpha-2 receptors belong to the $G_{i/o}$ -protein coupled receptor family that mediates primarily inhibitory effects of the catecholamines (Knaus et al., 2007). When activated they trigger an intracellular cascade that results in either a decrease in cAMP that leads to PKA inhibition or the activation PKC signaling (Dowary 1998; Warch et al., 2001; Baron and Boulpaep 2009; Bohen et al., 1996).

Few studies have looked at the physiological role of alpha-2 receptor activation in VTA DA cells. Investigations have shown that clonidine administration regularized VTA activity (Greenhoff and Svensson, 1989) or inhibited neuronal firing (Guiard et al., 2008) in the presence of a small depolarization of membrane potential (Inyushin et al., 2010). These effects were mainly postsynaptic in nature. The present study is the first, to our knowledge, demonstrating an alpha-2 receptor action at the presynaptic site of putative DA cells within the VTA. In addition, it also suggests that these alpha-2 receptors might be heterosynaptically localized at glutamatergic fibers terminating onto VTA DA neurons.

In the present study we were able to assess the alpha-2 receptor response specificity using pharmacological tools. The selective alpha-2 agonist, UK 14304 gave similar results as clonidine whereas the selective non-subtype specific alpha-2 antagonists blocked clonidine-induced inhibition. These results clearly suggest that clonidine's inhibition of glutamate transmission is specifically mediated by alpha-2 receptor activation.

Recent studies from our laboratory demonstrated that alpha-2 receptor activation in VTA DA neurons inhibited the hyperpolarization-activated cation current (Inyushin et al., 2010). Analysis of the second messenger system associated with the alpha-2 receptor revealed that the induced-inhibition was mediated by the activation of a PKC signaling pathway. To explore if a similar mechanism can mediate clonidine's induced suppression of EPSCs in putative VTA DA cells, we first employed the non-specific protein kinase blocker H-7. A clear blockade of the effect was observed with H-7, suggesting that clonidine's-induced suppression involves, at least, the activation of protein kinases. Moreover, superfusion of Rp, an specific inhibitor of PKA, was unable to block clonidine's inhibitory action. Similar to our previous studies, after inhibiting PKC with the selective antagonist chelerythrine, there was a strong blockade indicating that clonidine's effect was mediated by the activation of this intracellular cascade. We therefore concluded that clonidine reduces glutamate EPSCs via an alpha-2 receptor that activates a PKC intracellular pathway. These results

partially coincide with other studies demonstrating that activation of alpha-2 receptors in the CNS are related to the stimulation of phospholipase C with the subsequent activation of PKC (Carr et al., 2007; Overland et al., 2009).

We initially assessed the synaptic site of clonidine's action on glutamatergic transmission onto VTA DA neurons using a PPR protocol. PPR is a form of short-term plasticity that indicates a change in presynaptic release probability. An increase in PPR typically indicates a decrease in the probability of release (Zucker, 1989). The increased PPR suggested a presynaptic action of the agonist. Clonidine's presynaptic effect was further confirmed by the finding that it also reduced the frequency of both sEPSCs and mEPSCs without affecting their mean amplitude.

The findings in the present study are consistent with several investigations demonstrating a role of heterosynaptic activation of alpha-2 receptor in the modulation of glutamate transmission in other CNS areas (Shields et al., 2009; Miyazaki et al., 1998; Abdallah and Guyenet, 1999; Boem, 1999; Pan et al., 2002; Bertolino et al., 1997). Our results add to this group suggesting that the alpha-2 heterosynaptic inhibition of glutamate transmission might be a common mechanism rather than the exception in nerve cells that express this receptor moiety. However, it is still not known if alpha-2 receptors are expressed in ventral tegmental terminals of glutamatergic projections. Further studies should be carried out to address this possibility.

Given that the VTA is a brain structure central to reward processing and addiction the alpha-2 induced inhibition of glutamate release onto VTA DA neurons may be highly relevant to reward and drug abuse. Studies have demonstrated that cocaine seeking in animals can be induced by glutamate release in the VTA dopamine system (You et al., 2007; Harris and Aston-Jones, 2003). Inhibition of ionotropic glutamate receptors in this area can reduce the rewarding effects of intravenous cocaine injections. Thus, activation of alpha-2 receptors in the VTA should tend to block these addictive behaviors associated with glutamate release within this structure. An investigation of the role of alpha-adrenergic receptors in the initiation and expression of cocaine sensitization demonstrated that systemic clonidine administration attenuated the acute response and retarded the increased locomotor activity during the initiation of behavioral sensitization (Jimenez-Rivera et al., 2007). However, clonidine was unable to fully suppress the development but it blocked the expression of sensitization. Recent preliminary studies using clonidine microinjections via intra-VTA cannulas provided similar results as the investigation with systemic injections (Ramos-Acevedo et al., 2009). There was a delay in the initiation but the animals developed behavioral sensitization. Moreover, clonidine microinjections significantly reduced the expression of sensitization. Although we do not know if these results could be attributable to clonidine's action on glutamate release onto VTA DA neurons it might be argued that the inhibition of the liberation of this excitatory transmitter can play an important part in the process.

NE is a neurotransmitter closely related to the stress response in mammals (see review by Morilak et al., 2005). In drug addiction an important factor to relapse in humans is stress (Kreek and Koob, 1998). Animal studies of stress-induced relapse have shown that mild intermittent footshock stress reliably reinstates cocaine and alcohol seeking behavior (Erb et al., 2000; Le et al., 2005). Pretreatment with alpha-2 agonists blocked the stress but not cocaine-induced reinstatement of drug seeking after extinction. Similarly, alpha-2 agonists and antagonists attenuated or reinstated alcohol seeking and alcohol self-administration, respectively. Furthermore, it has been shown that mild footshock stress induces VTA glutamate release together with dopaminergic activation (Wang et al., 2005). Dopamine release was inhibited by the local application of ionotropic glutamate receptor blockers.

Therefore, it may be possible that the inhibitory role of the alpha-2 receptor activation observed on the reinstatement of cocaine and alcohol seeking can be due to the active suppression of the presynaptic glutamate release demonstrated here.

Acknowledgments

This work was supported by grants from NIGMS, GMSO6 50695, GM084854 and MBRS-RISE R25-GM061838 to CAJR.

Abbreviations

DA	dopamine
EPSCs	excitatory postsynaptic currents
LDT	laterodorsal tegmentum
sEPSCs	spontaneous excitatory postsynaptic currents
mEPSCs	miniature excitatory postsynaptic currents
mPFC	medial prefrontal cortex
NE	norepinephrine
PPR	paired-pulse ratio
VTA	Ventral Tegmental Area

References

- Arencibia-Albite F, Paladini C, Williams JT, Jimenez-Rivera CA. Noradrenergic modulation of the hyperpolarization-activated cation current (I_h) in dopamine neurons of the ventral tegmental area. *Neuroscience*. 2007; 149:303–314. [PubMed: 17884297]
- Aston-Jones G, Harris GC. Brain substrates for increased drug seeking during protracted withdrawal. *Neuropharmacology*. 2004; 47(Suppl 1):167–179. [PubMed: 15464135]
- Bertolino M, Vicini S, Gillis R, Travagli A. Presynaptic alpha2-adrenoceptors inhibit excitatory synaptic transmission in rat brain stem. *Am J Physiol*. 1997; 272:G654–661. [PubMed: 9124588]
- Boehm S, Huck S, Freissmuth M. Involvement of a phorbol ester-insensitive protein kinase C in the alpha2-adrenergic inhibition of voltage-gated calcium current in chick sympathetic neurons. *J Neurosci*. 1996; 16:4596–4603. [PubMed: 8764648]
- Boron, WF.; Boulpaep, EL. Medical physiology: a cellular and molecular approach. Saunders/Elsevier; Philadelphia, PA: 2009.
- Bunney BS, Aghajanian GK, Roth RH. Comparison of effects of L-dopa, amphetamine and apomorphine on firing rate of rat dopaminergic neurones. *Nat New Biol*. 1973; 245:123–125. [PubMed: 4518113]
- Carr DB, Andrews GD, Glen WB, Lavin A. alpha2-Noradrenergic receptors activation enhances excitability and synaptic integration in rat prefrontal cortex pyramidal neurons via inhibition of HCN currents. *J Physiol*. 2007; 584:437–450. [PubMed: 17702809]
- Carr DB, Sesack SR. Projections from the rat prefrontal cortex to the ventral tegmental area: target specificity in the synaptic associations with mesoaccumbens and mesocortical neurons. *J Neurosci*. 2000; 20:3864–3873. [PubMed: 10804226]
- Cooper DC. The significance of action potential bursting in the brain reward circuit. *Neurochem Int*. 2002; 41:333–340. [PubMed: 12176075]
- Dahlstrom A, Fuxe K. Localization of monoamines in the lower brain stem. *Experientia*. 1964; 20:398–399. [PubMed: 5856530]
- Docherty JR. Subtypes of functional alpha1- and alpha2-adrenoceptors. *Eur J Pharmacol*. 1998; 361:1–15. [PubMed: 9851536]

- Dumont EC, Mark GP, Mader S, Williams JT. Self-administration enhances excitatory synaptic transmission in the bed nucleus of the stria terminalis. *Nat Neurosci.* 2005; 8:413–414. [PubMed: 15735642]
- Egli RE, Kash TL, Choo K, Savchenko V, Matthews RT, Blakely RD, Winder DG. Norepinephrine modulates glutamatergic transmission in the bed nucleus of the stria terminalis. *Neuropsychopharmacology.* 2005; 30:657–668. [PubMed: 15602500]
- Erb S, Hitchcott PK, Rajabi H, Mueller D, Shaham Y, Stewart J. Alpha-2 adrenergic receptor agonists block stress-induced reinstatement of cocaine seeking. *Neuropsychopharmacology.* 2000; 23:138–150. [PubMed: 10882840]
- Floresco SB, West AR, Ash B, Moore H, Grace AA. Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. *Nat Neurosci.* 2003; 6:968–973. [PubMed: 12897785]
- Geisler S, Zahm DS. Afferents of the ventral tegmental area in the rat-anatomical substratum for integrative functions. *J Comp Neurol.* 2005; 490:270–294. [PubMed: 16082674]
- Georges F, Aston-Jones G. Activation of ventral tegmental area cells by the bed nucleus of the stria terminalis: a novel excitatory amino acid input to midbrain dopamine neurons. *J Neurosci.* 2002; 22:5173–5187. [PubMed: 12077212]
- Grace AA. Phasic versus tonic dopamine release and the modulation of dopamine system responsivity: a hypothesis for the etiology of schizophrenia. *Neuroscience.* 1991; 41:1–24. [PubMed: 1676137]
- Grace AA, Bunney BS. Intracellular and extracellular electrophysiology of nigral dopaminergic neurons--3. Evidence for electrotonic coupling. *Neuroscience.* 1983; 10:333–348. [PubMed: 6633865]
- Grace AA, Floresco SB, Goto Y, Lodge DJ. Regulation of firing of dopaminergic neurons and control of goal-directed behaviors. *Trends Neurosci.* 2007; 30:220–227. [PubMed: 17400299]
- Grace AA, Onn SP. Morphology and electrophysiological properties of immunocytochemically identified rat dopamine neurons recorded in vitro. *J Neurosci.* 1989; 9:3463–3481. [PubMed: 2795134]
- Grenhoff J, Svensson TH. Clonidine modulates dopamine cell firing in rat ventral tegmental area. *Eur J Pharmacol.* 1989; 165:11–18. [PubMed: 2569981]
- Guiard BP, El Mansari M, Blier P. Cross-talk between dopaminergic and noradrenergic systems in the rat ventral tegmental area, locus ceruleus, and dorsal hippocampus. *Mol Pharmacol.* 2008; 74:1463–1475. [PubMed: 18703671]
- Harris GC, Aston-Jones G. Critical role for ventral tegmental glutamate in preference for a cocaine-conditioned environment. *Neuropsychopharmacology.* 2003; 28:73–76. [PubMed: 12496942]
- Hayar A, Guyenet PG. Alpha2-adrenoceptor-mediated presynaptic inhibition in bulbospinal neurons of rostral ventrolateral medulla. *Am J Physiol.* 1999; 277:H1069–1080. [PubMed: 10484430]
- Hidaka H, Inagaki M, Kawamoto S, Sasaki Y. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry.* 1984; 23:5036–5041. [PubMed: 6238627]
- Inyushin MU, Arencibia-Albite F, Vazquez-Torres R, Velez-Hernandez ME, Jimenez-Rivera CA. Alpha-2 noradrenergic receptor activation inhibits the hyperpolarization-activated cation current (I_h) in neurons of the ventral tegmental area. *Neuroscience.* 2010; 167:287–297. [PubMed: 20122999]
- Jimenez-Rivera CA, Feliu-Mojer M, Vazquez-Torres R. Alpha-noradrenergic receptors modulate the development and expression of cocaine sensitization. *Ann N Y Acad Sci.* 2006; 1074:390–402. [PubMed: 17105937]
- Kauer JA, Malenka RC. LTP: AMPA receptors trading places. *Nat Neurosci.* 2006; 9:593–594. [PubMed: 16639403]
- Kitai ST, Shepard PD, Callaway JC, Scroggs R. Afferent modulation of dopamine neuron firing patterns. *Curr Opin Neurobiol.* 1999; 9:690–697. [PubMed: 10607649]
- Knaus AE, Muthig V, Schickinger S, Moura E, Beetz N, Gilsbach R, Hein L. Alpha2-adrenoceptor subtypes--unexpected functions for receptors and ligands derived from gene-targeted mouse models. *Neurochem Int.* 2007; 51:277–281. [PubMed: 17664025]

- Koga E, Momiyama T. Presynaptic dopamine D2-like receptors inhibit excitatory transmission onto rat ventral tegmental dopaminergic neurones. *J Physiol.* 2000; 523(Pt 1):163–173. [PubMed: 10673553]
- Kreek MJ, Koob GF. Drug dependence: stress and dysregulation of brain reward pathways. *Drug Alcohol Depend.* 1998; 51:23–47. [PubMed: 9716928]
- Le AD, Harding S, Juzysch W, Funk D, Shaham Y. Role of alpha-2 adrenoceptors in stress-induced reinstatement of alcohol seeking and alcohol self-administration in rats. *Psychopharmacology (Berl).* 2005; 179:366–373. [PubMed: 15551068]
- Lee A, Wissekerke AE, Rosin DL, Lynch KR. Localization of alpha2C-adrenergic receptor immunoreactivity in catecholaminergic neurons in the rat central nervous system. *Neuroscience.* 1998; 84:1085–1096. [PubMed: 9578397]
- Legault M, Wise RA. Novelty-evoked elevations of nucleus accumbens dopamine: dependence on impulse flow from the ventral subiculum and glutamatergic neurotransmission in the ventral tegmental area. *Eur J Neurosci.* 2001; 13:819–828. [PubMed: 11207817]
- Liprando LA, Miner LH, Blakely RD, Lewis DA, Sesack SR. Ultrastructural interactions between terminals expressing the norepinephrine transporter and dopamine neurons in the rat and monkey ventral tegmental area. *Synapse.* 2004; 52:233–244. [PubMed: 15103690]
- Lodge DJ, Grace AA. The laterodorsal tegmentum is essential for burst firing of ventral tegmental area dopamine neurons. *Proc Natl Acad Sci U S A.* 2006; 103:5167–5172. [PubMed: 16549786]
- Margolis EB, Lock H, Hjelmstad GO, Fields HL. The ventral tegmental area revisited: is there an electrophysiological marker for dopaminergic neurons? *J Physiol.* 2006; 577:907–924. [PubMed: 16959856]
- Mejias-Aponte CA, Drouin C, Aston-Jones G. Adrenergic and noradrenergic innervation of the midbrain ventral tegmental area and retrorubral field: prominent inputs from medullary homeostatic centers. *J Neurosci.* 2009; 29:3613–3626. [PubMed: 19295165]
- Miyazaki T, Kobayashi H, Tosaka T. Presynaptic inhibition by noradrenaline of the EPSC evoked in neonatal rat sympathetic preganglionic neurons. *Brain Res.* 1998; 790:170–177. [PubMed: 9593880]
- Morilak DA, Barrera G, Echevarria DJ, Garcia AS, Hernandez A, Ma S, Petre CO. Role of brain norepinephrine in the behavioral response to stress. *Prog Neuropsychopharmacol Biol Psychiatry.* 2005; 29:1214–1224. [PubMed: 16226365]
- Omelchenko N, Sesack SR. Laterodorsal tegmental projections to identified cell populations in the rat ventral tegmental area. *J Comp Neurol.* 2005; 483:217–235. [PubMed: 15678476]
- Omelchenko N, Sesack SR. Glutamate synaptic inputs to ventral tegmental area neurons in the rat derive primarily from subcortical sources. *Neuroscience.* 2007; 146:1259–1274. [PubMed: 17391856]
- Overland AC, Kitto KF, Chabot-Dore AJ, Rothwell PE, Fairbanks CA, Stone LS, Wilcox GL. Protein kinase C mediates the synergistic interaction between agonists acting at alpha2-adrenergic and delta-opioid receptors in spinal cord. *J Neurosci.* 2009; 29:13264–13273. [PubMed: 19846714]
- Overton PG, Clark D. Burst firing in midbrain dopaminergic neurons. *Brain Res Brain Res Rev.* 1997; 25:312–334. [PubMed: 9495561]
- Pan YZ, Li DP, Pan HL. Inhibition of glutamatergic synaptic input to spinal lamina II(o) neurons by presynaptic alpha(2)-adrenergic receptors. *J Neurophysiol.* 2002; 87:1938–1947. [PubMed: 11929913]
- Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. Academic Press/Elsevier; Amsterdam; Boston: 2007.
- Phillips PE, Stuber GD, Heien ML, Wightman RM, Carelli RM. Subsecond dopamine release promotes cocaine seeking. *Nature.* 2003; 422:614–618. [PubMed: 12687000]
- Ramos-Acevedo, JM.; González-Barbosa, SN.; Vazquez-Torres, R.; Santos-Vera, B.; Jiménez, L.; Vélez, ME.; Schwarz, D.; Nieves, J.; Jiménez-Rivera, CA. Program No. 253.13. 2009 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience; 2009. Alpha-2 adrenergic receptor activation in the VTA and PFC modulates the development of cocaine sensitization. Online

- Rosin DL, Talley EM, Lee A, Stornetta RL, Gaylinn BD, Guyenet PG, Lynch KR. Distribution of alpha 2C-adrenergic receptor-like immunoreactivity in the rat central nervous system. *J Comp Neurol*. 1996; 372:135–165. [PubMed: 8841925]
- Sarti F, Borgland SL, Kharazia VN, Bonci A. Acute cocaine exposure alters spine density and long-term potentiation in the ventral tegmental area. *Eur J Neurosci*. 2007; 26:749–756. [PubMed: 17686047]
- Schultz W. Dopamine neurons and their role in reward mechanisms. *Curr Opin Neurobiol*. 1997; 7:191–197. [PubMed: 9142754]
- Schultz W. Predictive reward signal of dopamine neurons. *J Neurophysiol*. 1998; 80:1–27. [PubMed: 9658025]
- Schultz W. Getting formal with dopamine and reward. *Neuron*. 2002; 36:241–263. [PubMed: 12383780]
- Sesack SR, Bunney BS. Pharmacological characterization of the receptor mediating electrophysiological responses to dopamine in the rat medial prefrontal cortex: a microiontophoretic study. *J Pharmacol Exp Ther*. 1989; 248:1323–1333. [PubMed: 2564893]
- Shields AD, Wang Q, Winder DG. alpha2A-adrenergic receptors heterosynaptically regulate glutamatergic transmission in the bed nucleus of the stria terminalis. *Neuroscience*. 2009; 163:339–351. [PubMed: 19527774]
- Ungerstedt U. Stereotaxic mapping of the monoamine pathways in the rat brain. *Acta Physiol Scand Suppl*. 1971; 367:1–48. [PubMed: 4109331]
- Wang B, Shaham Y, Zitzman D, Azari S, Wise RA, You ZB. Cocaine experience establishes control of midbrain glutamate and dopamine by corticotropin-releasing factor: a role in stress-induced relapse to drug seeking. *J Neurosci*. 2005; 25:5389–5396. [PubMed: 15930388]
- Wang LY, Salter MW, MacDonald JF. Regulation of kainate receptors by cAMP-dependent protein kinase and phosphatases. *Science*. 1991; 253:1132–5. [PubMed: 1653455]
- Wise RA. Dopamine and reward: the anhedonia hypothesis 30 years on. *Neurotox Res*. 2008; 14:169–183. [PubMed: 19073424]
- Wurch T, Okuda J, Pauwels PJ. Reciprocal modulation of alpha(2A)-adrenoceptor and G(alpha o) protein states as determined by carboxy-terminal mutagenesis of a G(alpha o) protein. *Mol Pharmacol*. 2001; 60:666–673. [PubMed: 11562427]
- You ZB, Wang B, Zitzman D, Azari S, Wise RA. A role for conditioned ventral tegmental glutamate release in cocaine seeking. *J Neurosci*. 2007; 27:10546–10555. [PubMed: 17898226]
- Zellner MR, Ranaldi R. How conditioned stimuli acquire the ability to activate VTA dopamine cells: a proposed neurobiological component of reward-related learning. *Neurosci Biobehav Rev*. 2010; 34:769–780. [PubMed: 19914285]
- Zucker RS. Short-term synaptic plasticity. *Annu Rev Neurosci*. 1989; 12:13–31. [PubMed: 2648947]

Fig. 1. Bath application of clonidine reduces AMPA EPSCs amplitude in putative VTA DA neurons

A. Representative recordings from the same cell illustrating that clonidine superfusion (40 μM), induces a significant reduction in AMPA EPSCs amplitude in a putative VTA DA cell voltage clamped at -70 mV. The effect was reversible since it returned to near control values after a washout period. **B.** Summary time course of the effect of clonidine bath application on AMPA EPSCs amplitude recorded from 10 putative VTA DA neurons. A 10 min clonidine (40 μM) application causes depression of AMPA EPSCs. There was a rapid return to control levels upon clonidine removal. All recordings were made at 5 minutes intervals. **C.** Bar graph showing that on average clonidine application resulted in a ~40% decrease on AMPA EPSCs amplitude. No significant differences were observed between control ($99.4 \pm 0.5\%$) and washout period ($94.8 \pm 8.2\%$). This effect was specific to AMPA EPSCs as GABAa IPSCs were not affected by clonidine treatment (control: $105 \pm 4\%$ vs. clonidine $103 \pm 7\%$, $p=0.82$). **D.** The clonidine-induced inhibition was dose-dependent over the concentration range of 15 to 40 μM. Numbers in parenthesis indicate the sample size used to test each concentration. *** $p=6 \times 10^{-5}$

Fig. 2. Alpha-2 antagonists block clonidine effect on AMPA EPSCs

A. Representative recordings from the same cell showing that the alpha2-antagonist yohimbine (20 μ M) completely abolishes the clonidine-evoked inhibition of AMPA EPSCs. **B.** Summary time course illustrating that the alpha2-antagonist yohimbine (20 μ M) blocks the clonidine-evoked inhibition of AMPA EPSCs. Note that yohimbine alone has no effect on EPSCs amplitude. At each plotted point $n = 9$. **C.** Superfusing alpha2-antagonists yohimbine (Yoh) or atipamezole (Atip) leaves AMPA EPSCs amplitude unaltered. **D.** Cosuperfusing clonidine (40 μ M) plus yohimbine (20 μ M) or atipamezole (20 μ M) prevents the clonidine-evoked reduction of AMPA EPSCs amplitude.

Fig. 3. The alpha-2 receptor mediated decrease in AMPA EPSCs amplitude results from the activation of PKC signaling

A. Representative recordings from the same cell showing that chelerythrine (1 μ M), a selective PKC inhibitor, completely prevents the clonidine-evoked inhibition of AMPA EPSCs in VTA DA cells. **B.** Bar graph demonstrating that superfusion of the non-specific protein kinase inhibitor H-7 (75 μ M) blocked clonidine's inhibitory effect (40 μ M, 10 min application) on AMPA EPSCs amplitude in VTA DA cells. Note that H7 alone had no effect on AMPA EPSCs suggesting that inhibition of PKA signaling does not affect EPSCs amplitude. **C.** Bar graph showing that application of Chelerythrine (Chelery), a potent PKC inhibitor, completely abolished clonidine's inhibitory action (40 μ M, 10 min application) on

AMPA EPSCs in VTA DA cells. Note that Chelerythrine alone had no effect. **D.** Rp, a specific PKA inhibitor did not block clonidine's effect. ** $p = 0.009$

Fig. 4. Clonidine bath application increases Paired-Pulse Ratio (PPR) in putative VTA DA neurons

A. Representative recordings from the same cell illustrating that clonidine superfusion (40 μ M, 10 min application), induces a significant increase in paired pulse ratio (PPR = EPSC₂/EPSC₁) in a putative VTA DA neuron voltage clamp at -70 mV. Note that the time interval between consecutive EPSCs is 50 ms. **B.** Graph summarizing the change in PPR of 6 cells after a 10 min clonidine (40 μ M) bath application. **C.** Bar graph showing that the clonidine-evoked increase in PPR is statically significant and specific to AMPA EPSCs PPR as no effect was evoked by a similar treatment onto GABA_A IPSCs PPR. **D.** Clonidine's effect on PPR is blocked by yohimbine an alpha-2 antagonist. ** p= 0.002

Fig. 5. Clonidine reduces sEPSCs frequency with no effect on its amplitude

A. Representative recordings from the same cell illustrating that clonidine application (40 μ M) decreases sEPSCs frequency without affecting its amplitude. The cell was voltage clamped at -70 mV during the recordings. **B.** Clonidine superfusion (40 μ M, 10 min. application) results in a positive shift of the inter-event interval cumulative distribution (K-S, $p = 0.01$) implying a decrease in sEPSCs frequency. The plot was constructed from a single cell recording distinct from cell in part A. **C.** Clonidine application does not shift the amplitude cumulative distribution (K-S, $p = 0.43$). Thus, clonidine has no effect on sEPSCs amplitudes. The plot was constructed from the cell used in B. **D.** Summary graph showing that clonidine superfusion decreases sEPSCs frequency without affecting its amplitude.

* $p=0.026$

Fig. 6. Clonidine reduces mEPSCs frequency with no effect on its amplitude

A. Representative recordings from the same cell illustrating that in the presence of TTX (0.5 μ M) clonidine application (40 μ M) decreases mEPSCs frequency without affecting its amplitude. The cell was voltage clamped at -70 mV during the recordings. **B.** Clonidine superfusion (40 μ M, 10 min. application) results in a positive shift of the inter-event interval cumulative distribution (K-S, $p=0.0007$) implying a decrease in mEPSCs frequency. The plot was constructed from a single cell recording distinct from cell in part A. **C.** Clonidine application does not shift the amplitude cumulative distribution (K-S, $p=0.72$). Thus, clonidine has no effect on mEPSCs amplitudes. The plot was constructed from the cell used in B. **D.** Summary graph showing that clonidine superfusion in the presence of TTX decreases mEPSCs frequency without affecting its amplitude. * $p=0.011$