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ALPHA-1 ADRENORECEPTORS MODULATE GABA RELEASE ONTO VENTRAL TEGMENTAL AREA DOPAMINE NEURONS

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Abstract

The ventral tegmental area (VTA) plays an important role in reward and motivational processes involved in drug addiction. Previous studies have shown that alpha1-adrenoreceptors (α1-AR) are primarily found presynaptically at this area. We hypothesized that GABA released onto VTA-dopamine (DA) cells is modulated by presynaptic α1-AR. Recordings were obtained from putative VTA-DA cells of male Sprague-Dawley rats (28–50 days postnatal) using whole-cell voltage clamp technique. Phenylephrine (10μM; α1-AR agonist) decreased the amplitude of $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) evoked by electrical stimulation of afferent fibers (n=7; $p<0.05$). Prazosin (1μM, α1-AR antagonist), blocked this effect. Paired-pulse ratios were increased by phenylephrine application (n=13; $p<0.05$) indicating a presynaptic site of action. Spontaneous IPSCs frequency but not amplitude, were decreased in the presence of phenylephrine (n=7; $p<0.05$). However, frequency or amplitude of miniature IPSCs were not changed (n=9; $p>0.05$). Phenylephrine in low Ca^{2+} (1mM) medium decreased IPSC amplitude (n=7; $p<0.05$). Chelerythrine (a protein kinase C inhibitor) blocked the α1-AR action on IPSC amplitude (n=6; $p<0.05$). Phenylephrine failed to decrease IPSCs amplitude in the presence of paxilline, a BK channel blocker (n=7; $p<0.05$). Taken together, these results demonstrate that α1-ARs at presynaptic terminals can modulate GABA release onto VTA-DA cells. Drug-induced changes in α1-AR could contribute to the modifications occurring in the VTA during the addiction process.

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Keywords

Dopamine neurons; GABA release; alpha1-adrenoreceptor; Ventral Tegmental Area

INTRODUCTION

The mesocorticolimbic system is composed of dopamine (DA) neurons projecting mainly from the ventral tegmental area (VTA) to cortical and ventral forebrain structures (Dahlstrom and Fuxe, 1964, Ungerstedt, 1971, Lammel et al., 2011). Activation of VTA DA neurons has been implicated in motivated behaviors as well as in mediating the reinforcing actions of drugs of abuse (Schultz, 2002, Kauer, 2004, Grace et al., 2007).

VTA DA neurons receive noradrenergic (NE) inputs from locus coeruleus and other pontine structures (Jones et al., 1977, Mejias-Aponte et al., 2009) and tracing studies have shown that NE afferents have extrasynaptic and synaptic connections on VTA DA neurons (Liprando et al., 2004). Moreover, the VTA contains alpha-1 adrenoreceptors (α1-ARs) (Greene et al., 2005) which are located primarily in pre-synaptic elements (Rommelfanger et al., 2009). Noradrenergic (NE) inputs have been shown to facilitate VTA DA neuronal transmission and induce changes in burst firing via α1 adrenergic receptors (α1-ARs) (Grenhoff et al., 1993, Grenhoff and Svensson, 1993, Grenhoff et al., 1995, Paladini and Williams, 2004). Also, α1-ARs participate in the development of stress and anxiety responses, and in addiction-related behaviors (Cecchi et al., 2002, Hague et al., 2003, Jimenez-Rivera et al., 2006, Greenwell et al., 2009).

The VTA receives considerable inhibitory inputs mainly in the form of GABAergic innervation from the mesopontine tegmentum, the lateral habenula via the rostromedial tegmentum (RMTg), nucleus accumbens (NAcc) and the periaqueductal gray (Jhou et al., 2009a, Jhou et al., 2009b). Changes in GABAergic input on VTA DA neurons can control their firing patterns (Paladini and Tepper, 1999, Lobb et al., 2010). For example, decreased GABAergic inhibition contributes to the generation of bursts in DA neurons (Jhou et al., 2009a, Lobb et al., 2011, Morikawa and Paladini, 2011). Since bursting firing has been related to enhance neurotransmitter release (Floresco et al., 2003), modulation of DA neuronal bursting activity is one mechanisms that can modify DA release in VTA reward-related projections.

Pharmacological stimulation of α1-ARs induces changes in GABA-mediated synaptic transmission in different brain areas. This α1-ARs-mediated effect has been evidenced in different brain structures such as the hippocampus, frontal cortex, ventrolateral BNST, cerebellar, pyriform, and enthorrinal cortices, basolateral amygdala (BLA), septal and septohippocampal area (Mouradian et al., 1991, Alreja and Liu, 1996, Bergles et al., 1996, Marek and Aghajanian, 1996, Kawaguchi and Shindou, 1998, Braga et al., 2004, Dumont and Williams, 2004, Herold et al., 2005, Lei et al., 2007, Hillman et al., 2009). The control of GABA neurotransmission onto VTA DA neurons could have important implications, however, up to our knowledge, there is no direct evidence demonstrating such mechanism. The results in this work using brain slices demonstrate the modulatory role of presynaptic α1-ARs activation on the GABA release onto VTA DA neurons.

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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 Universidad Central del Caribe and Medical Sciences Campus - University of Puerto Rico. Electrophysiological experiments were performed with male Sprague-Dawley rats (28 – 50 days postnatal). Animals were housed two per cage and maintained at constant temperature and humidity with a 12-hr hour light/dark cycle. Water and food were provided ad libitum.

Electrophysiology

Sprague-Dawley male rats were anesthetized with a 90 mg/kg i.p. chloral-hydrate injection of (Sigma, St Louis, MO, USA) and their brains rapidly removed. Midbrain horizontal slices (220 μM) containing the VTA were cut using a vibratome (VT1000S, Leica, Germany). The rat midbrain was placed into 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, and saturated with 95% O_2–5% CO_2 gas mixture to a pH=7.4. Slices were transferred to an intermediate chamber and incubated at 32° in the same solution for 45-min before the initiation of electrophysiological recordings. MK-801 (10 μM, Tocris, Ellisville, MO, USA) was added to the incubation solutions to block *N*-methyl-D-aspartate (NMDA)-mediated excitotoxicity (Velasquez-Martinez et al., 2012).

Whole cell voltage clamp recordings were obtained from visually identified neurons in the VTA using an infrared microscope with differential interference contrast (DIC) optics, (BX51WI Olympus, Japan). Recordings were acquired through data acquisition software (pClamp 10, Molecular Devices, Sunnyvale, CA). The VTA was identified as the region lateral to the fasciculus retroflexus and medial to the medial terminal nucleus of the accessory optic tract (MT) (Paxinos and Watson, 2007). Recording from the substantia nigra compacta (SNc), identified as the regions rostral and caudal to the MT, was avoided. 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 1-s hyperpolarizing steps from −60 to −130 mV. I_h is present in about 84% VTA DA neurons (Margolis et al., 2006). Therefore, the contribution of non-dopaminergic neurons to the experimental recording performed in this study is likely to be not significant. Whole-cell voltage clamp recordings were made at a holding potential of −70 mV unless indicated.

$GABA_A$ Inhibitory Postsynaptic Currents (IPSCs) Recordings—VTA slices were totally submerged in a recording chamber (500 μL) with ACSF superfusion at 1–2 ml/min at 32°C. Superfusion medium contained 2-amino-5-ph osphonopentanoic acid (AP5; 100 μM) and either 6-cyano-2,3-dihydroxy-7 nitroquinox saline (CNQX; 10 μM) or 6,7-Dinitroquinoxaline-2,3-dione (DNQX; 10 μM) to block fast NMDA- or AMPA- mediated synaptic potentials respectively. In all experiments, eticlopride (100 μM) were included in the superfusion solution to block any possible effect mediated by the dopamine D2 receptor. Whole-cell voltage-clamp recordings were made using micropipette filled with a solution

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containing (in mM): 70 K-gluconate, 80 KCI, 1 EGTA, 5 HEPES, 2 MgATP, and 0.3 GTP. To verify the nature of the current, at the end of each experiment, picrotoxin (100 μM) was added to abolish all evoked or spontaneous $GABA_A$ mediated postsynaptic currents.

Data were collected through a Multiclamp 700B amplifier (Axon Instruments, Foster City, CA, USA), filtered at 1 kHz, digitized at 5 kHz using Digidata 1440A (Axon Instruments, Foster City, CA, USA), and stored in a PC computer and analyzed off line using pClamp (v. 10) and final graphs were done using GraphPad Prism 5 (GraphPad Software, Inc) software. Pipette's Liquid junction potential was offset compensated using standard Multiclamp 700B circuitry. The seal's qualities used were 4–6 GΩ. Series resistances were not compensated and were monitored during the entire experiment. Data were discarded if changes of series resistance of more than 15% occurred.

Recording of Synaptic Currents

A bipolar stainless steel electrode (FHC Inc, Bowdoin, ME) was placed approximately 100 μm rostral to the recording electrode and used to stimulate afferents at 0.1 Hz by applying a brief electrical pulse (100–300 μA). $GABA_A$ IPSCs were recorded at holding potential of −70 mV. All IPSCs shown in figures were averages of 5 current traces for the treatment under inspection. $GABA_A$ IPSCs amplitudes were calculated by taking a 1 ms window around the peak of the IPSC and comparing this to a 5 ms window immediate before the stimulation artifact. Peak IPSCs amplitudes were averaged during control recordings. This value was used to normalize the following recordings. This procedure allows expressing data as percentages of the control condition for appropriate statistical comparisons. Paired stimuli were given with a 50 ms interstimulus interval. Paired Pulse Ratio (PPR) was calculated as the ratio of the first and second IPSC's. Spontaneous $GABA_A$ IPSCs (sIPSCs) and miniature $GABA_A$ IPSCs (mlPSCs) were recorded. Tetrodotoxin (TTX, 0.5 μM, Alomone Laboratories, Jerusalem, Israel) was added to the ACSF to observe the mlPSCs that are independent on the spontaneous presynaptic action potentials. sIPSCs and mlPSCs were recorded at −70 mV of holding potential, filtered at 1 kHz and digitized at 5 kHz using pCLAMP 10 software (Molecular Devices, Sunnyvale, CA, USA). For a given cell, sIPSCs and mlPSCs were collected (1 sweep for each condition, 3min/sweep) for a control and phenylephrine's period. The recorded sIPSCs and mlPSCs were analyzed afterward using Mini Analysis program 6.0.7 (Synaptosoft Inc. Decatur, GA). Detection criteria were set at >6 pA, <1.3 ms rise time, and <0.1 ms decay time. The choice of this cutoff amplitude for acceptance of sIPSCs and mlPSCs was made to obtain a high signal-to-noise ratio. Then, each event also was visually inspected to prevent noise disturbance of the analysis.

Drugs

Phenylephrine hydrochloride ([R]-[-]-1 -[3-Hydroxyphenyl]-2-methylaminoethanol hydrochloride), methoxamine hydrochloride (α-[1-Aminoethyl]-2,5-dimethoxybenzyl alcohol hydrochloride), prazosin hydrochloride (1-[4-Amino-6,7-dimethoxy-2-quinazolinyl]-4-[2-furanylcarbonyl]piperazine hydrochloride), chelerythrine chloride (1,2-dimethoxy-12-methyl[1,3]benzodioxolo[5,6-c]phenanth ridinium chloride tetrodotoxin citrate) were purchased from Sigma (St Louis, MO, USA). Thapsigargin (3S,3aR,4S,6S, 6AR,7S,8S,9bS)-6- (Acetyloxy)-2,3,3a,4,5,6,6a,7,8,9b–decahydro-3,3a–dihydroxy-3,6,9-

trimethyl-8-[[(2Z)-2-methyl-1-oxo-2-butenyl]oxy]-2-oxo-4-(1-oxobutoxy)azuleno[4,5-b]furan-7-yl octanoate) was purchased from Tocris (Ballwin, MO). Paxilline was donated from Alomone Laboratories (Jerusalem, Israel). All substances were diluted in fresh ACSF until completely mixed, then transferred to separate graduated reservoirs connected to the chamber. The effects on current amplitude were measured within 5 min after the beginning of the flow (1–2 ml/min).

Data Analysis

All data were presented as mean ± SEM. Statistical significance were assessed using Student's paired t-test, One-Way ANOVA with Newman-Keuls as *post hoc* analysis 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. P values were reported throughout the text and significance were set as $p<0.05$.

RESULTS

VTA DA neurons seem to be inhibited by both intrinsic and extrinsic sources of GABA (Johnson and North, 1992a, Matsumoto and Hikosaka, 2007, Jhou et al., 2009a). The presence of α1-ARs has been reported in the VTA area (Greene et al., 2005). α1-ARs were found to be primarily localized at pre-synaptic elements in the VTA region (Rommelfanger et al., 2009). However, these studies do not clearly define the synaptic terminal type where α1-ARs are specifically localized; therefore, the presynaptic elements mentioned could be GABAergic terminals.

In order to assess if the activation of α1-ARs alters GABAergic transmission on VTA DA neurons, whole cell recordings of GABA IPSCs were performed on putative DA neurons identified by the presence of a large Ih (> 200 pA), slow spontaneous activity and relatively regular inter-spike intervals (Grace and Bunney, 1983, Grace and Onn, 1989). We confirmed that this evoked current was due to $GABA_A$ receptor activation by blocking the response with the receptor antagonist, picrotoxin (100 μM, data not shown).

Effect of α1-AR activation on $GABA_A$-induced IPSCs

In order to address the question of whether α1-AR activation can modulate GABAergic transmission, we recorded GABA IPSCs amplitude upon the superfusion of the α1-AR agonist phenylephrine. Fig. 1A shows a representative trace where phenylephrine (10μM) superfusion induced a significant reduction in the GABA IPSCs 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. Similarly, superfusion of methoxamine (40 μM), another α1-AR agonist, significantly decreased GABA IPSCs peak amplitude from 99.46 ± 2.27% to 63.87 ± 8.96% after 10 minutes' superfusion (n=5; ANOVA $F_{2,14} = 10.06$, $p<0.005$; Fig 1B). As seen in figure 1B, phenylephrine application resulted in a ~25% and 30% reduction in IPSCs amplitudes after 5 (77.4 ± 8.6 % of control) and 10 min respectively (69.1 ± 8.3 % of control) which was statistically significant (n=7, ANOVA $F_{2,18}=6.86$, $p<0.05$). As illustrated in figure 1C, phenylephrine's actions lasted throughout the period of drug

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application (10 min), rapidly returning to pretreatment levels during the washout period (10 min). Phenylephrine's inhibitory action was dose-dependent over the concentration of 0.1 and 100 μM (Fig. 1D).

The receptor specificity of phenylephrine-induced inhibition on GABA IPSCs was determined by using prazosin (1 μM), an α1-AR antagonist. Prazosin alone, after 10 minutes bath application; did not alter IPSC amplitude when compared to control values (Fig. 2A, B and C; Control 99.4 ± 1.3 % vs. Prazosin 97.4 ± 15.3 %, n = 7). However, when prazosin and phenylephrine were co-superfused for an additional 10 minutes the latter was unable to exert its typical inhibitory action (Prazosin 97.4 ± 15.3 % vs. Prazosin + Phenylephrine 108.2 ± 9.2 %, ANOVA $F_{4,28}$=0.84, p>0.05, n = 7). Thus, the phenylephrine-evoked inhibition of GABAergic IPSCs in VTA DA cells seems to be the result of α1-AR activation.

Presynaptic α1-AR activation decreases GABA release on VTA DA neurons

To determine the synaptic site of action of phenylephrine-induced inhibition of GABA IPSCs, we examined the paired pulse ratio (PPR=$IPSC_2/IPSC_1$) before and after phenylephrine superfusion. Fig. A shows the increase in PPR sample recordings after 10 μM phenylephrine's superfusion. The PPR increased from 0.99 ± 0.06 to 1.17 ± 0.08 after 10 minutes phenylephrine's application (n=13; paired *t*-test, p<0.01, Figs. 3B *and* C). These results clearly supports that the activation of α1-ARs evokes IPSCs through a presynaptic decrease in the release probability of GABA.

To further confirm if the observed effects were mediated by presynaptic mechanism we examined changes in the stochastic release, the spontaneous IPSCs (sIPSCs) and the miniature IPSCs (mIPSCs) before and after phenylephrine's (10 μM) administration. Sample recordings before (control) and after the agonist's application (Fig. 4A) showed that there was a reduction in the frequency but not in the amplitude of sIPSCs. Phenylephrine decreased the frequency of these events from 0.89 ± 0.12 to 0.53 ± 0.12 Hz (paired t-test, p<0.05, n=7; Fig 4D) while the mean amplitude of sIPSCs population before and during phenylephrine application was 51.68 ± 3.77 and 50.41 ± 3.2 pA, respectively (paired t-test, p=0.68, n=7; Fig 4D). These findings were further supported by their respective cumulative probability distributions. In particular, a right shift was observed in the inter-event interval distribution (reduction in frequency) but no change was seen in the amplitude distribution (Fig. 4B and C). Consequently, the results support a presynaptic modulation of α1-AR-activation reducing GABAergic neurotransmission on VTA DA neurons.

In order to test whether phenylephrine effects were only due to an action-potential dependent mechanism we measured changes in miniature IPSCs (mlPSCs) in the presence of TTX (0.5 μM). Under these conditions, sample recordings before and after 10 minutes phenylephrine's application showed that there was no change in the mIPSC frequency or amplitude (Fig. 5A). The inter-event intervals probability and amplitude distribution demonstrated no significant changes after 10 minutes phenylephrine's application (Figs. 5B and C, respectively). Population analysis illustrate that phenylephrine did not produce significant changes in frequency (from 1.10 ± 0.15 to 1.07 ± 0.19 Hz, n=9, paired *t*-test p=0.69, Fig. 5D), or amplitude (from 34.11 ± 5.36 to 30.94 ± 4.14 pA, n=9, paired *t*-test

p=0.19, Fig. 5D) compared to control recordings. Altogether, these results support the notion that α1-AR effect on GABA release on VTA DA neurons is action potential-dependent.

α1-ARs modulatory effect on GABA release is independent of both intra and extracellular calcium

To test if extracellular calcium was involved in the observed α1-ARs effects, we reduced the calcium concentration of the ACSF from 2.0 mM to 1.0 mM to limit the calcium influx to the presynaptic terminal. Fig. 6A shows sample traces of GABA IPSCs before and after phenylephrine's administration in the presence of a reduced calcium concentration. Phenylephrine, in 1.0 mM calcium, decreased the GABA IPSCs amplitude (75.58 ± 7.68% of control, n=7, ANOVA $F_{2,18} = 5.39$, $p<0.05$, Fig. 6B and C). Therefore, these results suggest that α1-AR effect on GABA release on VTA DA neurons is independent of extracellular calcium concentration.

We also, explored whether intracellular calcium stores participated in the α1-ARs mediated effect. Brain slices were pre-incubated in thapsigargin (10 μM; 30 min) which depletes intracellular Ca^{2+} stores by blocking the ATPase that mediates Ca^{2+} uptake (Thastrup et al., 1990, Mathew and Hablitz, 2008). Thapsigargin did not block phenylephrine's effect on $GABA_A$ IPSCs amplitude (71.31 ± 8.22% of control, n=8, ANOVA $F_{2,23} = 4.24$, $p<0.05$, Fig. 6D). These results suggest that intracellular Ca^{2+} stores are not involved in the α1-AR effect on $GABA_A$ IPSCs in VTA DA neurons.

α1-AR-mediated decrease in GABA release through PKC pathway

Protein Kinase C (PKC) has been shown to be a downstream element in the intracellular signaling pathway of α1-AR activation (Tamura et al., 1993). Therefore, the effect of α1-AR on GABA release could be mediated by direct coupling via PKC. To explore whether PKC is required for the α1-AR mediated presynaptic GABA release, brain slices were superfused with the membrane-permeable PKC inhibitor chelerythrine (1 μM). Phenylephrine's application after 10 minutes of chelerythrine superfusion failed to induce changes on GABA ISPCs peak amplitude (control: 100.42 ± 1.72%; chelerythrine 10 min: 93.43 ± 4.39%; phenylephrine 10 min: 103.22 ± 6.05%; n=6; ANOVA $F_{4,26} = 1.28$, p=0.30, Fig. 7).

α1-AR-mediated decrease in GABA release involves BK channels activation

Activation of BK channels induces a potassium outward current in response to a membrane depolarization (Vandael et al., 2010). Therefore, BK channel activation could induce a membrane hyperpolarization and decrease neurotransmitter release. To explore if BK channels were involved in α1-AR mediated presynaptic GABA release, slices were superfused with paxilline (1 μM), a selective BK channel inhibitor. After 10 minutes of paxilline superfusion (96.76 ± 3.97% of control), phenylephrine failed to induce a decrease on $GABA_A$ IPSCs (100.71 ± 2.17% of control, n=7, ANOVA $F_{4,31} = 0.83$, p=0.51, Fig. 8). These results indicate that BK channel stimulation is required to reduce GABA release on VTA DA neurons caused by α1-AR activation.

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DISCUSSION

GABA RELEASE MODULATION BY α1-AR ONTO VTA DA NEURONS

Our studies demonstrate that α1-ARs modulate GABA neurotransmission onto VTA DA neurons. Phenylephrine-induced suppression of $GABA_A$ IPSCs in VTA DA cells seems to be α1-ARs selective since prazosin (α1-AR antagonist) blocked phenylephrine's inhibitory actions on IPSCs. Furthermore, a paired-pulse ratio analysis and an evaluation of sIPSCs recordings indicated that the α1-ARs' inhibitory effect was mediated by activation of a presynaptic receptor mechanism. Moreover, the α1-AR modulatory effect on GABA transmission requires presynaptic action potentials, the selective activation of a PKC intracellular pathway and recruitment of BK channels. However, it seems that α1-ARs' effect on GABA release does not require the presence of either intra or extracellular calcium since it was still present in experiments with low intra and extracellular calcium. Because any manipulation of external calcium can alter GABA release indirectly, it is difficult to completely rule out a role of calcium on the phenylephrine-induced reduction in GABA transmission.

DA neurons seem to be inhibited by both intrinsic and extrinsic GABA sources (Johnson and North, 1992a, Matsumoto and Hikosaka, 2007, Jhou et al., 2009b). GABAergic synaptic afferents to VTA DA cells have been identified arising from the rostromedial tegmental nucleus (RMTg), nucleus accumbens shell, ventral pallidum, periaqueductal gray (PAG) and laterodorsal tegmentum (LDT) (Kalivas et al., 1993, Geisler and Zahm, 2005, Jhou et al., 2009a, Sesack and Grace, 2010, Morikawa and Paladini, 2011).

Immunoreactivity for GABA terminals forming symmetric synapses with dendritic shafts of VTA neurons has been described (Charara et al., 1996). A considerable majority of RMTg axons in the VTA form synapses with dendrites of DA cells (Balcita-Pedicino et al., 2011). Moreover, neighboring GABA neurons can innervate DA cells through local connections (Johnson and North, 1992b, Nugent and Kauer, 2008, Omelchenko and Sesack, 2009).

α1-ARs modulation of GABA-mediated synaptic transmission have been described in several brain structures. Presynaptic α1-ARs' activation mediates inhibition of GABA-releasing cells in rat hippocampal cultures, auditory cortex, cerebellum and spinal dorsal horn neurons (Mouradian et al., 1991, Croce et al., 2003, Yuan et al., 2009, Salgado et al., 2011).

We initially assessed the synaptic site of phenylephrine's action on GABAergic transmission on VTA DA neurons using the PPR protocol. A variation in PPR is associated with changes in presynaptic release probability. An increase in PPR typically indicates a decrease in the probability of neurotransmitter release (Zucker, 1989). Our results showed that α1-ARs activation increases the PPR thus, they strongly support that α1-ARs are localized presynaptically to VTA DA neurons. In accordance to our data a recent immunohistochemical study showed that α1-ARs are co-localized with GABA-positive terminals in the VTA thus providing the locus for the observed interaction (Mitrano et al., 2012).

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Changes in sIPSC activity induced by α1-ARs activation have been reported in different brain structures. Croce et al (Croce et al., 2003) demonstrated that NE has an inhibitory effect on GABA-releasing cells in the rat's hippocampus. The NE's inhibitory action was due to α1-ARs activation since superfusion of the α1-ARs agonist, cirazoline, reduced the frequency of sIPSCs and mIPSCs recorded from cultured hippocampal cells. Such results suggest that α1-ARs activation decreases GABA neurotransmission via a presynaptic mechanism. Similarly, our results showed that α1-ARs activation decreases $GABA_A$ sIPSCs frequency but not the amplitude in VTA DA cells. Also, our data suggest that the decrease in GABA release induced by α1-ARs activation is dependent on presynaptic action potentials, since in the presence of TTX, changes in mIPSCs were absent. In contrast, NE activation of α1-ARs increases $GABA_A$ -IPSC frequency in vlBNST neurons, cerebellar Purkinje cells, hippocampal CA1 pyramidal cells and interneurons in piriform cortex (Marek and Aghajanian, 1996, Dumont and Williams, 2004, Herold et al., 2005, Hillman et al., 2009). These studies indicate that α1-ARs present on presynaptic terminals, once activated, can also increase GABA release. Altogether, the above data suggest that α1-ARs modulation of GABA release in the CNS is complex.

Protein kinase C (PKC), a phospholipid-dependent serine/threonine kinase, appears to be involved in the signal transduction response activated by many neurotransmitters (Olive and Newton, 2010, Kang et al., 2012). The PKC family is divided into three subcategories according to their structure, calcium-activation dependence and lipid activators. The conventional PKCs (cPKCs, α, βI, (βII, and γ) are activated by calcium and by the lipid signaling intermediate diacylglycerol (DAG). Novel PKCs (nPKCs, δ, ε, η, and θ) are activated by DAG but not by calcium. Atypical PKCs (aPKCs, ζ and λ/1) require neither DAG nor calcium and are instead activated by lipids such as phosphatidic and arachidonic acid (Hirai and Chida, 2003, Olive and Newton, 2010, Zeng et al., 2012). α1-ARs are Gq-coupled receptors that induce PKC activation. Our results showed that chelerythrine superfusion (a specific PKC inhibitor) blocked phenylephrine's inhibitory action on IPSCs amplitude. Thus, α1-ARs modulation of GABA neurotransmission onto VTA DA cells, similarly to α1-ARs presynaptic changes of glutamate neurotransmission, uses a PKC-dependent pathway (Velasquez-Martinez et al., 2012). However, since α1-AR effect on GABA IPSCs is still present in low calcium ACSF and under intracellular calcium depletion using thapsigargin, we suggest that the PKC subtype activated by α1-AR-Gq-mediated pathway is probably the PKCδ subtype. Studies using specific PKCδ blockers need to be carried out to directly test this hypothesis.

The BK channels are large conductance voltage and Ca^{2+} activated K^+ channels. These channels are sensitive to voltage-gated calcium channels (VGCC) and to increases in intracellular calcium (Marrion and Tavalin, 1998, Vergara et al., 1998, Lee and Cui, 2010). BK channel activation induces membrane hyperpolarization by K^+ efflux from the cell and reduces intracellular Ca^{2+} by closing VGCC (Hu et al., 2001, Wang, 2008, Cui, 2010). These BK mechanisms, namely membrane hyperpolarization and reduction of intracellular Ca^{2+}, have been associated to neurotransmitter release modulation in different brain areas (Hu et al., 2001, Raffaelli et al., 2004, Wang, 2008, Martire et al., 2011).

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PKC regulation of BK channels has been previously described. PKC can increase the activity of BK channels reconstituted in lipid bilayers (Reinhart and Levitan, 1995), present in COS7 cells (Kim and Park, 2008) or in the CA3 region of hippocampal slice cultures (Raffaelli et al., 2004). Specifically, PKCδ, has been related to BK channel activation (Kim and Park, 2008). Therefore, α1-ARs could exert their effects by a PKCδ stimulation which in turns phosphorylates and increases BK channel function allowing synaptic terminal hyperpolarization. This hyperpolarization will decrease GABA release. Our results demonstrated that in the presence of paxilline, a BK channel inhibitor, the α1-AR-mediated decrease in GABA IPSCs amplitude was blocked. Therefore, BK channel activation seems to exert an important role in α1-ARs effect on GABA release onto VTA DA neurons.

Stimulation of GABA-RMTg neurons increases the IPSC amplitude in VTA DA cells that project to the NAcc lateral shell (Lammel et al., 2012). The VTA DA – NAcc lateral shell connection is highly associated with reward behavior (Bromberg-Martin et al., 2010, Lammel et al., 2011). Activation of GABA neurons within the VTA has been linked with a disruption of reward (van Zessen et al., 2012). Taken together, these data suggest that activation of GABA-RMTg inputs or local VTA GABA neurons can induce an inhibition of VTA DA cells which in turn will modulate reward-related behaviors. Consequently, a decrease in these two inhibitory sources onto VTA DA cells can facilitate the development of reward behaviors.

In conclusion, the present results demonstrated that α1-ARs activation decreases GABAergic neurotransmission presynaptically onto VTA DA neurons (Fig 9). α1-ARs-mediated modulation of GABA release may play an important role in the development of cocaine addiction by reducing the inhibitory inputs onto VTA DA neurons (Bocklisch et al., 2013). Further evaluation of the mechanism presented here could prove to be relevant for new therapeutic interventions.

Acknowledgments

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Highlights

- α1-ARs activation at the presynaptic site decreases GABA release onto putative DA cells within VTA.
- Presynaptic α1-ARs activation modulates GABAergic inputs that affect VTA DA neurons excitability.
- α1-ARs effect might be heterosynaptically localized at GABAergic fibers terminating onto VTA-DA neurons.

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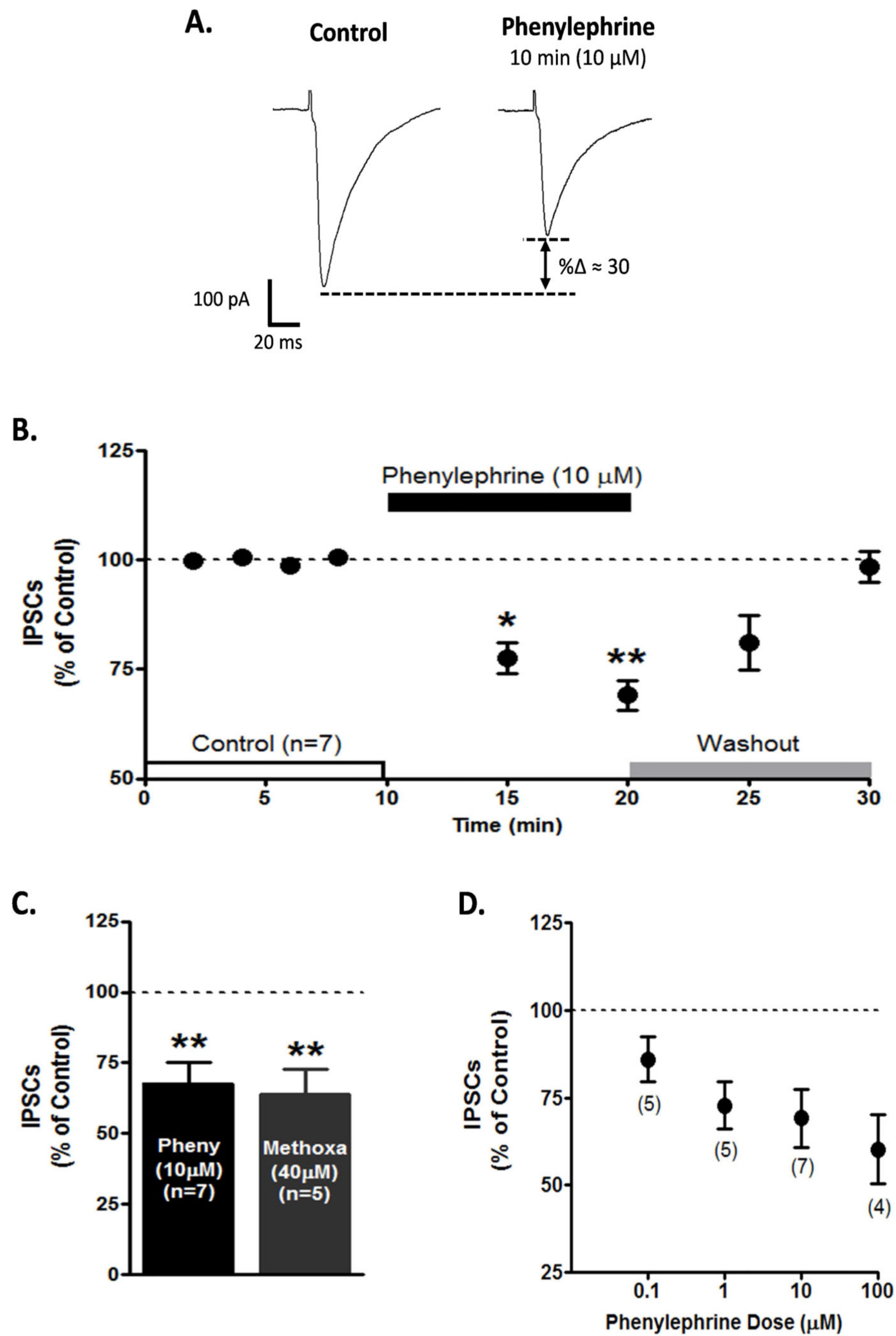

Figure 1. Bath application of phenylephrine reduced $GABA_A$ IPSCs amplitude in putative VTA DA neurons

A. Representative recordings from the same cell, showing that phenylephrine's superfusion (10 μm) induced a significant reduction in $GABA_A$ IPSCs amplitude in a putative VTA DA cell voltage clamped at −70 mV. **B.** Summary time course of the effect of phenylephrine bath application on $GABA_A$ IPSCs amplitude recorded from 7 putative VTA DA neurons at 8 min of control (2 min intervals), 5 and 10 min phenylephrine (10 μM) and 5 and 10 min washout. A 5 and 10 min phenylephrine application caused amplitude reduction of $GABA_A$

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IPSCs. There was a rapid return to control levels upon phenylephrine removal. **C.** Bar graph showing that, on average, phenylephrine (n=7) and methoxamine (n=5) application resulted in a ~30% decrease in $GABA_A$ IPSCs amplitude. **D.** Dose-response curve of phenylephrine's effect on $GABA_A$ IPSCs. Phenylephrine-induced decrease was dose-dependent over the concentration range of 0.1 –100 μM. *$p < 0.05$, ** $p < 0.01$; One-way ANOVA, Newman-Keuls post hoc.

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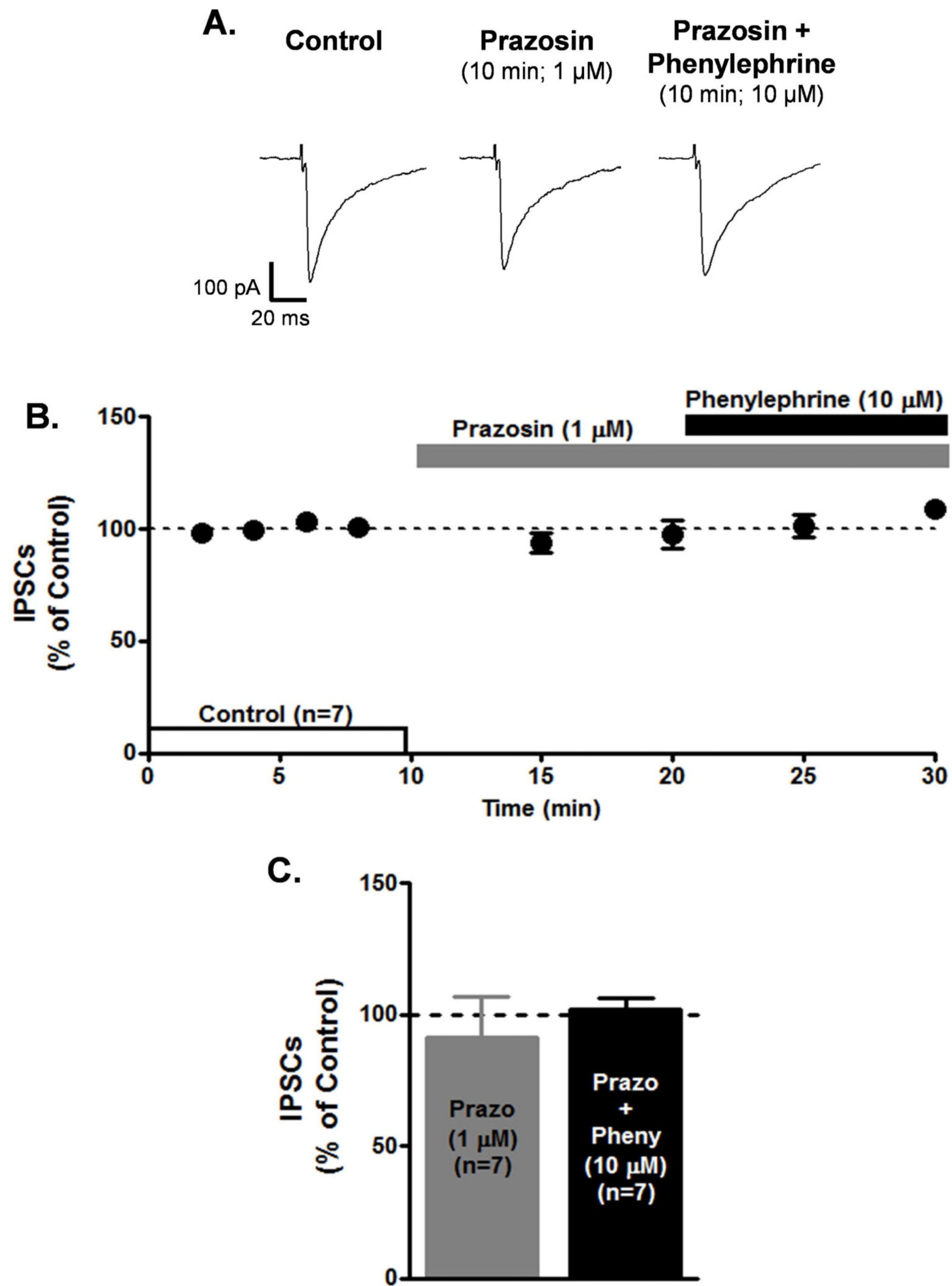

Figure 2. α1-AR antagonist, prazosin, blocks phenylephrine's effect on GABA$_A$ IPSCs

A. Representative recordings from a neuron showing that the α1-AR antagonist prazosin (1 μM) completely abolishes the phenylephrine-induced decrease in GABA$_A$ IPSCs amplitude. **B.** Prazosin superfusion leaves GABA$_A$ IPSCs amplitude unaltered. Phenylephrine (10 μM) and prazosin (1 μM) co-superfusion prevents phenylephrine-induced decrease of GABA$_A$ IPSCs amplitude. **C.** Summary illustrating the time course of prazosin actions. Note that prazosin alone has no effect on GABA$_A$ IPSCs amplitude. Each point represents the mean ± SEM of n=7.

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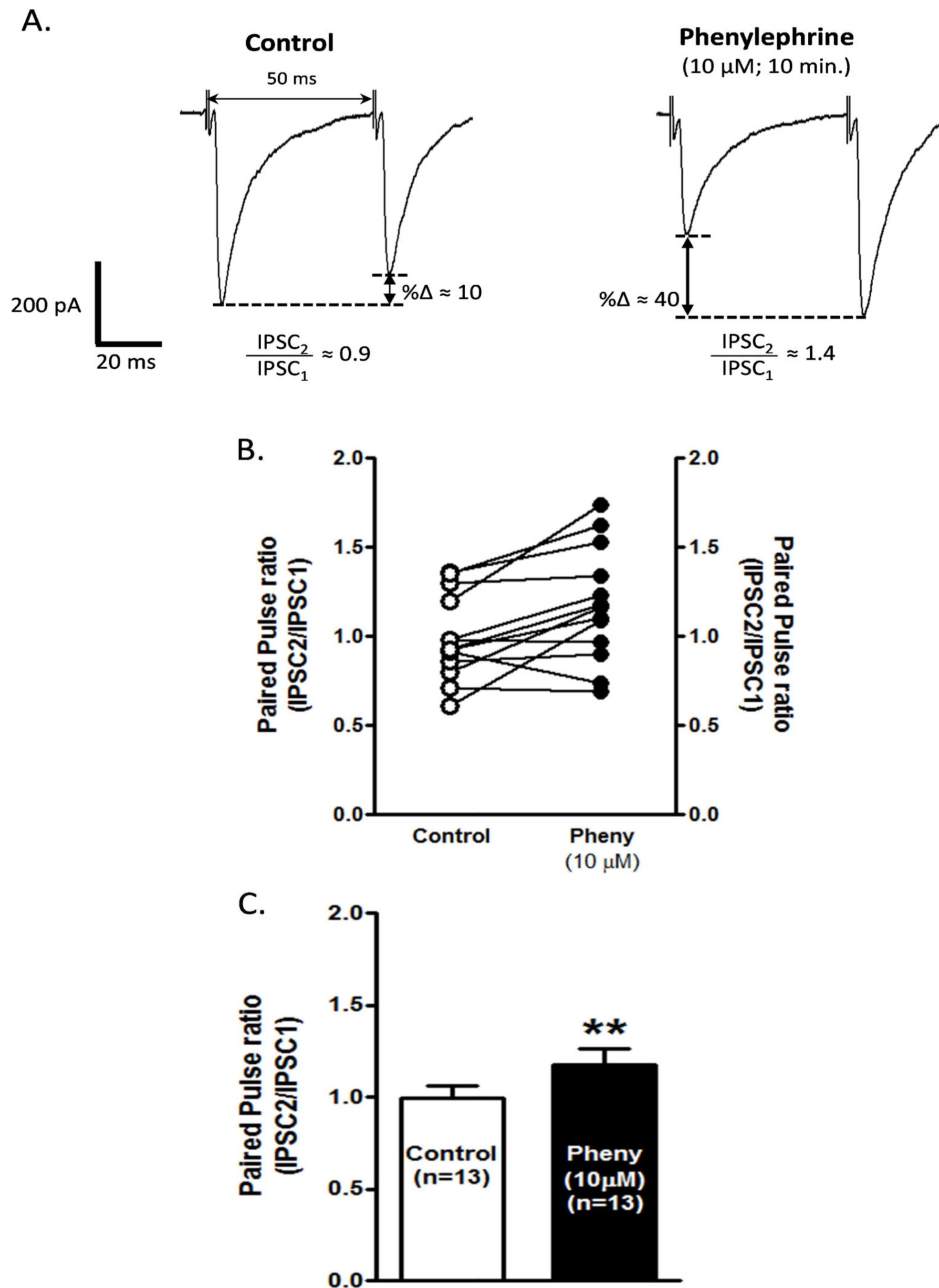

Figure 3. Phenylephrine increases paired-pulse ratio in putative VTA DA neurons

A. Representative recordings from a neuron illustrating that phenylephrine superfusion (10 μM, 10 min application), induces a significant increase in paired pulse ratio (PPR = IPSC2/IPSC1) in a putative VTA DA cell voltage clamp at a-70 mV. Time interval between consecutive EPSCs is 50 ms. **B.** Bar graph showing that phenylephrine-induced increase in PPR is statistically significant (n=13) **C.** Graph summary of the changes in PPR after 10 min phenylephrine (10 μM) bath application. **p < 0.01, paired t-test.

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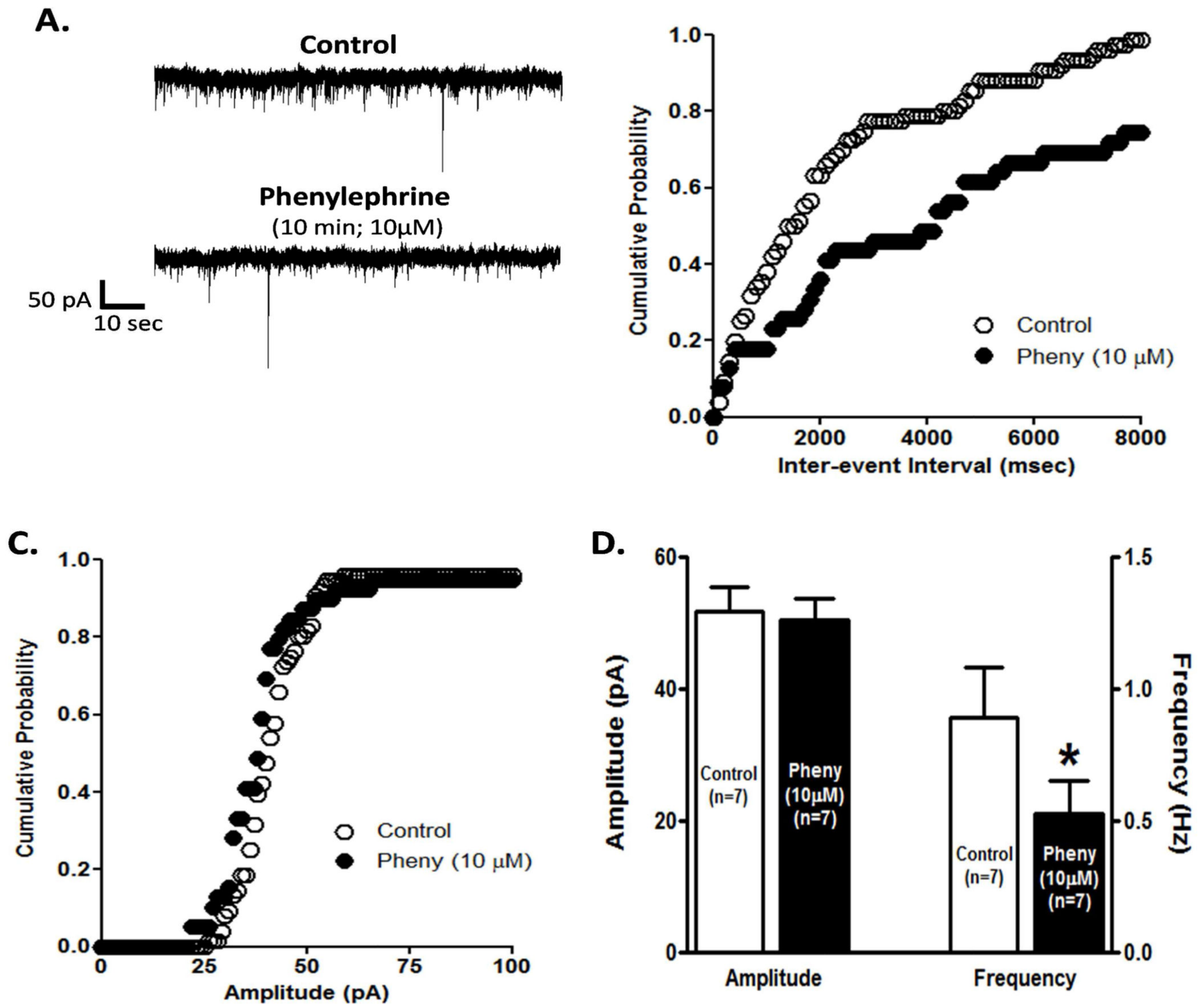

Figure 4. Phenylephrine decreases the frequency but not the amplitude of sIPSCs

A. Representative recordings from a cell illustrating that phenylephrine's application (10 μM) decreases sIPSC frequency but not the amplitude. The cell was voltage clamped at −70 mV during the recordings. **B.** Phenylephrine's superfusion (10 μM, 10 min application) results in a shift to the right of the inter-event interval cumulative distribution (K-S, p<0.05) implying a decrease in sIPSCs frequency. The plot was constructed from the cell used in part A. **C.** Phenylephrine does not shift the sIPSCs amplitude cumulative distribution. The plot was constructed from the cell used in A. **D.** Summary graph showing that phenylephrine decreased the mean frequency without affecting the mean amplitude of sIPSCs (n=7). *p < 0.05, paired *t*-test.

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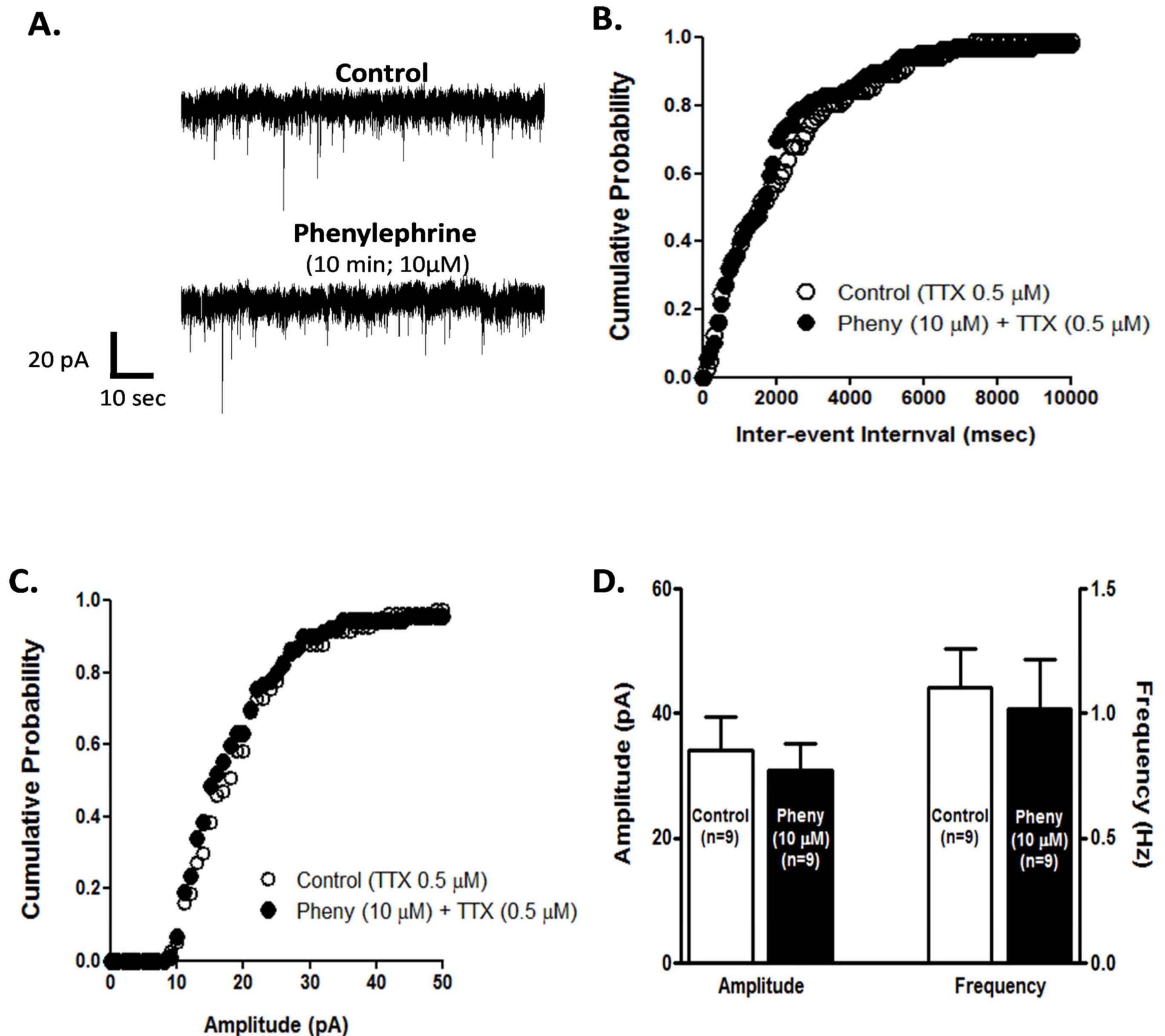

Figure 5. Phenylephrine had no effect on mIPSCs frequency or amplitude

A. Representative recordings from a neuron illustrating that in presence of TTX (0.5 µM) phenylephrine (10 µM) does not change the frequency or amplitude of mIPSCs. The neuron was voltage clamped at −70 mV during the recordings. **B.** Phenylephrine's superfusion (10 µM, 10 min. application) does not shift the inter-event interval cumulative distribution of mIPSCs. The plot was constructed from the cell used in A. **C.** Phenylephrine does not shift the amplitude cumulative distribution of mIPSCs. The plot was constructed from the cell used in A. **D.** Summary graph showing that phenylephrine's superfusion in the presence of TTX had no effect on frequency or amplitude mIPSCs (n=9).

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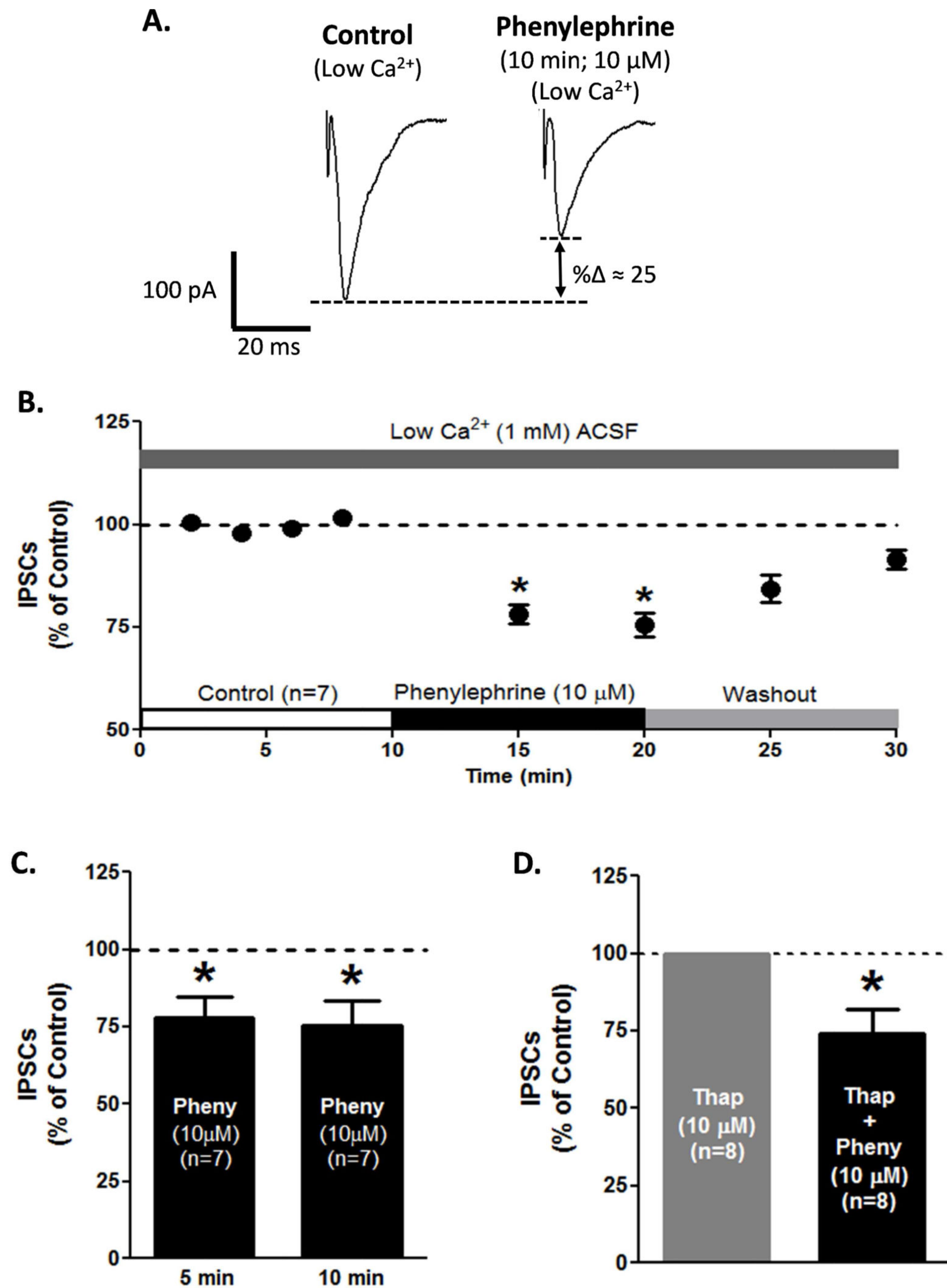

Figure 6. The α1-AR mediated decrease in $GABA_A$ IPSCs amplitude is independent of extracellular calcium concentration

A. Representative recordings from a neuron showing that low calcium ACSF (1 mM) did not block the phenylephrine-induced decrease of $GABA_A$ IPSCs in VTA DA cells. **B.** Bar graph showing that application of phenylephrine on low Ca^{2+} ACSF, did not block phenylephrine-induced decrease of $GABA_A$ IPSCs (10 μM, 5 and 10 min). **C.** Summary time course of 7 neurons illustrating the population effects. **D.** Bar graph showing that application of phenylephrine (10 μM, 5 and 10 min) on thapsigargin pre-treated slices did

not block phenylephrine-induce decrease of $GABA_A$ IPSCs. *$p < 0.05$, One-way ANOVA, Newman-Keuls *post-hoc*.

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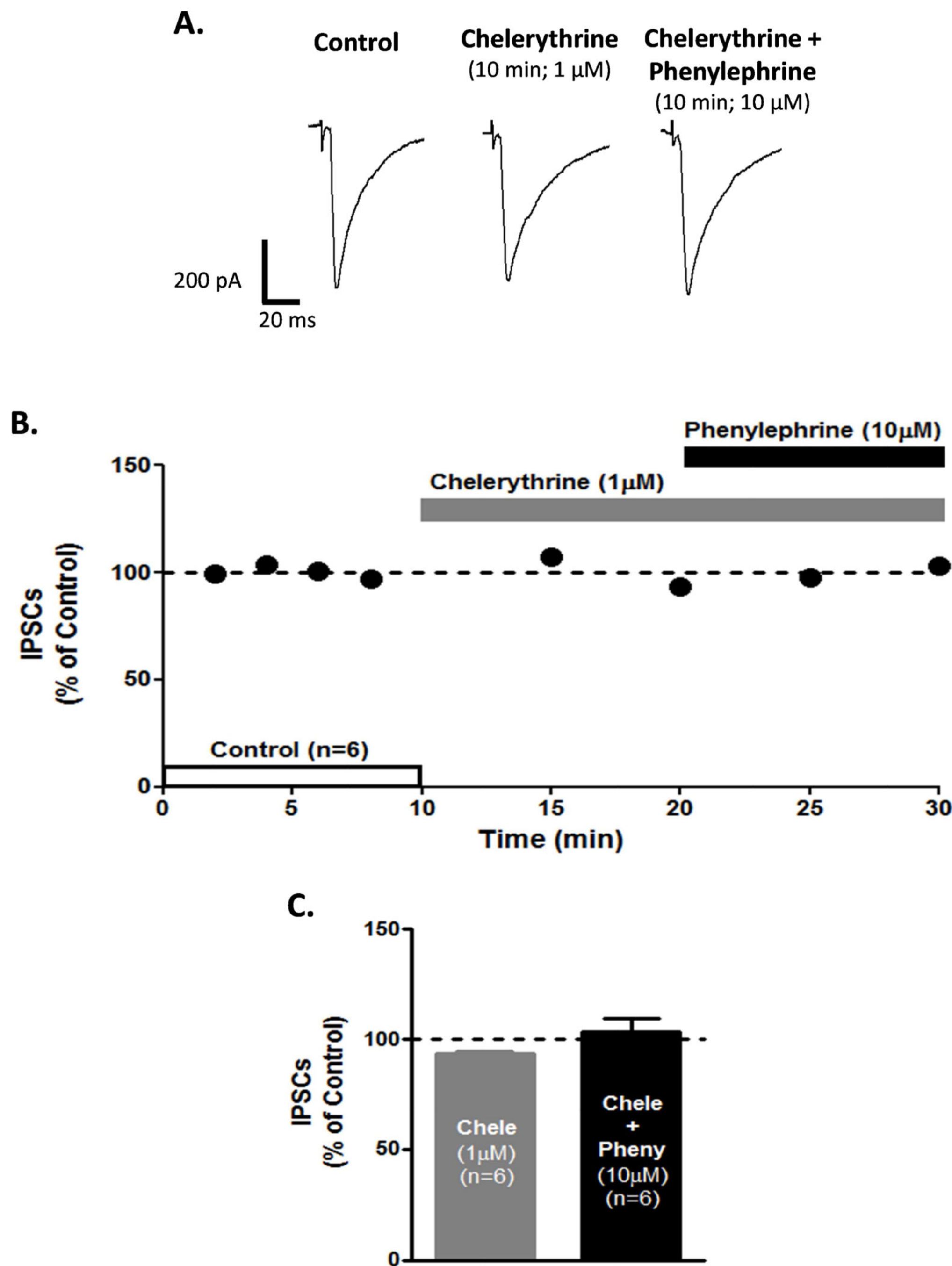

Figure 7. Involvement of the intracellular PKC signalling in the α1-AR mediated decrease in $GABA_A$ IPSCs amplitude

A. Representative recordings from a neuron showing that chelerythrine (1 μM), a selective PKC inhibitor, completely prevents the phenylephrine-induced decrease of $GABA_A$ IPSCs in VTA DA neurons. **B.** Summary time course illustrating that PKC selective inhibitor chelerythrine (1 μM) blocks the phenylephrine-induced decrease of $GABA_A$ IPSCs amplitude. Note that chelerythrine alone has no effect on $GABA_A$ IPSCs amplitude. Each point represents n=7 ± SEM. **C.** Bar graph showing that application of chelerythrine (Chele),

completely abolished phenylephrine's effect (10 μM; 10 min application) on $GABA_A$ IPSCs in VTA DA neurons.

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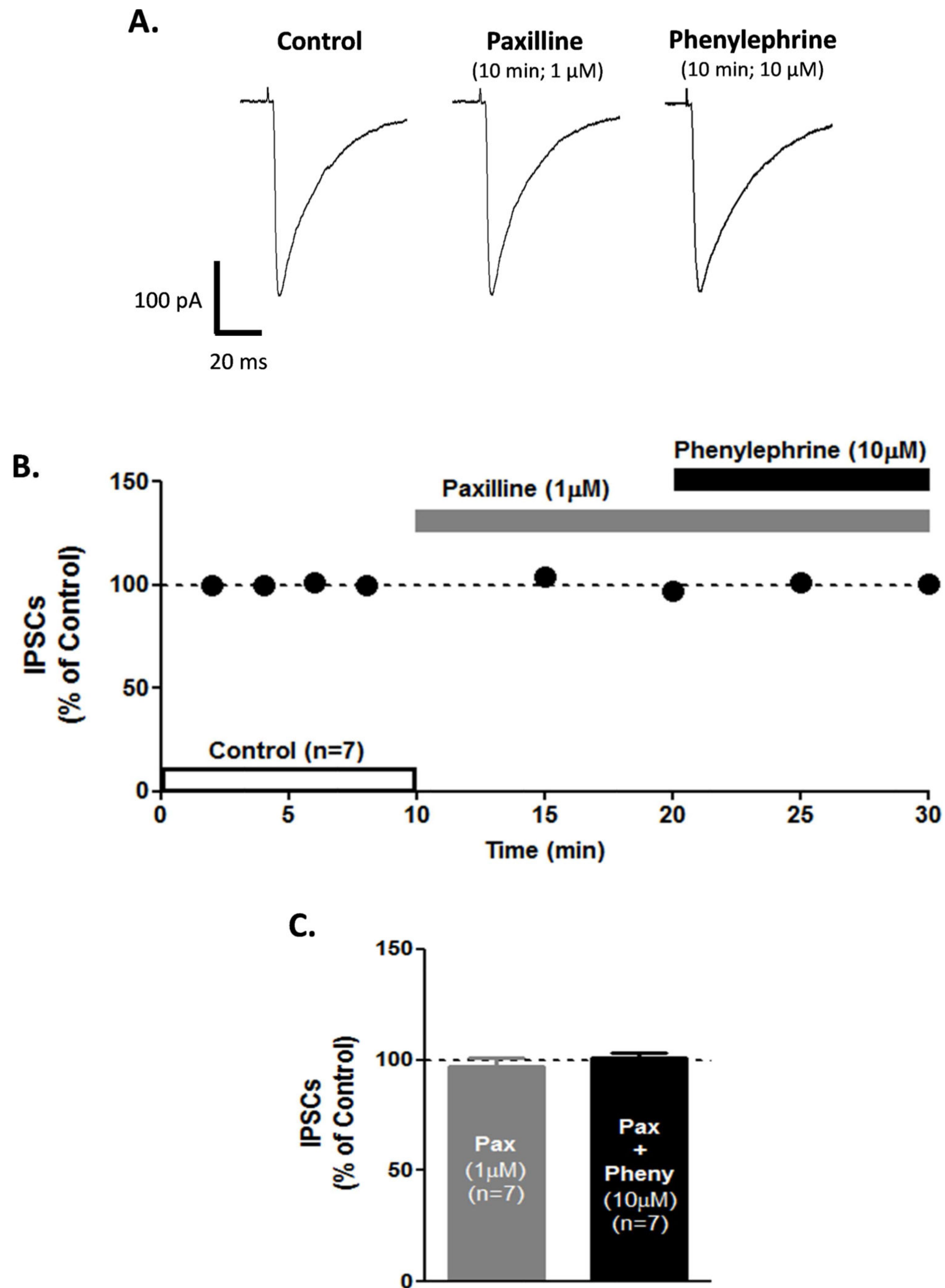

Figure 8. α1-AR-mediated decrease on $GABA_A$ IPSCs amplitude requires BK channel activation
A. Representative recording from VTA DA neuron showing that paxilline (1 μM, a selective BK channel inhibitor) prevents phenylephrine-induced decrease on $GABA_A$ IPSCs amplitude. **B.** Summary time course illustrating that paxilline superfusion blocks phenylephrine decrease of $GABA_A$ IPSCs amplitude. Note that paxilline alone has no effect on $GABA_A$ IPSCs amplitude. Each point represents n=7 ± SEM. **C.** Bar graph showing that paxilline application (Pax), completely abolished phenylephrine's effect (10 μM; 10 min application) on $GABA_A$ IPSCs in VTA DA neurons.

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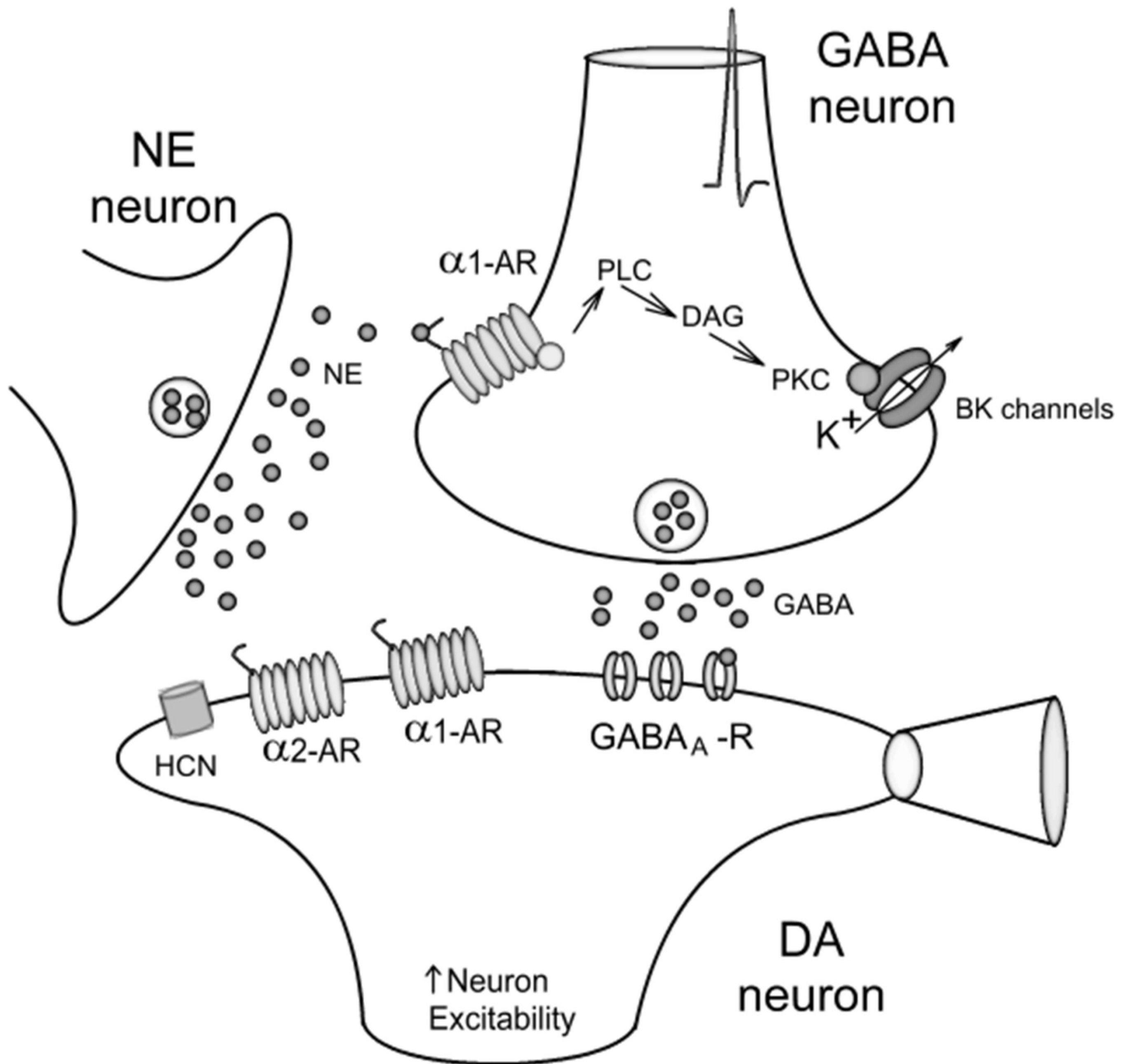

Figure 9. Schematic model to explain that α1-ARs presynaptic activation decrease GABA release onto VTA DA neurons by activation of PKC and BK channels

Stimulation of presynaptic α1-AR at inhibitory GABA terminals that project onto VTA DA neurons causes activation of PLC, via a Gq-mediated mechanism, resulting in DAG formation. The activated DAG stimulates a PKC phosphorylation, which may further increase BK channels activity hyperpolarizing the GABA terminal and therefore, decreasing GABA release. NE, norepinephrine; DA, dopamine; PLC, phospholipase C; DAG, diacylglycerol.