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Differential effects of methylphenidate and cocaine on GABA transmission in sensory thalamic nuclei

Belén Goitia[1,2], **Mariana Raineri**[1,2], **Laura E. González**[1,&], **José L. Rozas**[1], **Edgar Garcia-Rill**[3], **Verónica Bisagno**[2,#], and **Francisco J. Urbano**[1,#,*]

[1] Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE- CONICET-UBA), Intendente Guiraldes 2670, pabellón 2, piso 2, Ciudad Universitaria, (C1428BGA)-Buenos Aires, Argentina.

[2] Instituto de Investigaciones Farmacológicas (ININFA- CONICET-UBA-), Junín 956, piso 5, C1113-Buenos Aires, Argentina.

[3] Center for Translational Neuroscience, University of Arkansas for Medical Sciences, 4301 West Markham St., Little Rock, AR 72205, USA. Phone: (501)-686-5166. Fax: (501)-526-7928.

Abstract

Methylphenidate (MPH) is widely used to treat children and adolescents diagnosed with attention deficit/hyperactivity disorder. Although MPH shares mechanistic similarities to cocaine, its effects on GABAergic transmission in sensory thalamic nuclei are unknown. Our aim was to compare cocaine and MPH effects on GABAergic projections between thalamic reticular and ventrobasal (VB) nuclei.

Mice (P18-30) were subjected to *binge*-like cocaine and MPH *acute* and *sub-chronic* administrations. Cocaine and MPH enhanced hyperlocomotion, though *sub-chronic* cocaine-mediated effects were stronger than MPH effects. Cocaine and MPH *sub-chronic* administration altered paired-pulse and spontaneous GABAergic input differently. The effects of cocaine on evoked paired-pulse GABA-A mediated currents changed from depression to facilitation with the duration of the protocols used, while MPH induced a constant increase throughout administration protocols. Thalamic reticular nucleus GAD67 and VB $Ca_V3.1$ protein levels were measured using Western blot in order to better understand their link to increased GABA release. Both proteins were increased by *sub-chronic* administration of cocaine.

These results suggest that cocaine and MPH produced distinct presynaptic alterations on GABAergic transmission. MPH showed effects on GABAergic transmission that seems less disruptive than cocaine. Unique effects of cocaine on postsynaptic VB calcium currents might explain deleterious cocaine effects on sensory thalamic nuclei. These results might help to understand the impact of MPH repetitive administration on sensory thalamic nuclei.

Keywords

GABAergic transmission; Thalamic reticular nucleus; Methylphenidate; Cocaine; Ventrobasal thalamic nucleus; T-type calcium channels

[*] ***Corresponding author:***fjurbano@fbmc.fcen.uba.ar Phone: (+54)-11-4576-3368. Fax: (+54)-11-4576-3321. Phone: (+54)-11-4961-6784. Fax: (+54)-11-4963-8593..
[#] *authors contributed equally.*
[&]Present address: Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET, (C1428ADN) Buenos Aires, Argentina.

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Introduction

Chronic abuse of cocaine is associated with major neuro-psychiatric conditions (Devlin and Henry 2008). Acute *binge-like* administration of cocaine was able to alter the intrinsic properties of thalamocortical neurons and spontaneous GABAergic transmission, resulting in enhancements of EEG low frequency activity in mice (Urbano *et al.*2009). Systemic administration of T-type calcium channel blockers *in vivo* prevented hyperlocomotion and GABAergic neurotransmission enhancement onto Ventrobasal (VB) neurons after acute *binge*-like cocaine administration (Bisagno *et al.*2010), suggesting a key role for T-type channels in cocaine effects on specific thalamic GABAergic networks.

The thalamic reticular nucleus (TRN) is a thin layer of GABAergic neurons that project to sensory thalamic nuclei (Spreafico *et al.* 1991), and its cells are interconnected by GABAergic terminals (Sun *et al.* 2012) and gap junctions (Landisman *et al.* 2002). In rodents, there is a lack of GABAergic interneurons in the VB nucleus, and the inhibition necessary for proper sensory perception is provided by GABAergic TRN afferents (De Biasi *et al.* 1997). TRN neurons have intrinsic properties that allow them to generate action potentials and membrane potential oscillations at a wide range of frequencies (reviewed by Steriade 2005). TRN neurons express $Ca_V3.2$ and $Ca_V3.3$ T-type calcium channel subunits (Talley *et al.* 1999), although recent studies have also confirmed the presence of $Ca_V3.1$ subunits (Kovács *et al.* 2010). TRN rhythmicity is modulated by monoamines and GABA (Pinault and Deschênes 1992; Shammah-Lagnado *et al.* 1996; Rutter *et al.* 1998; Rodríguez *et al.* 2011).

Methylphenidate (MPH), another psychostimulant that has some abuse liability (Chait 1994), is widely used to treat children and adolescents diagnosed with Attention deficit/ hyperactivity disorder (ADHD) (Biederman *et al.* 1999). In humans, MPH has reinforcing effects (associated with increased extracellular dopamine levels by blocking the dopamine transporter, DAT) after intravenous administration (Volkow *et al.* 1999a). Lowered predisposition to drug abuse during adulthood has been described after early exposure to MPH in humans (Biederman *et al.* 1999) and in animal models (Carlezon *et al.* 2003). However, other authors have suggested otherwise (Brandon *et al.* 2001, Volkow and Insel 2003). Differences in pharmacodynamics between cocaine and MPH in humans have been associated with the lack of cross-sensitization of preadolescent MPH use (Guerriero *et al.* 2006). MPH administration normalized EEG low frequency activity (Clarke *et al.* 2003), suggesting direct involvement of the TRN in the etiology of ADHD. Single MPH administration has been shown to block GABAergic transmission from the TRN through the activation of D4 receptors both *in vitro* (Florán *et al.* 2004) and *in vivo* (Erlij *et al.* 2012). Nevertheless, the effects of repetitive MPH administration on GABAergic transmission between sensory thalamic nuclei remain unknown.

Cocaine has been shown to inhibit monoamine transporters (DAT, SERT and NET), elevating synaptic levels of dopamine (Wise and Bozarth 1987; Ritz *et al.* 1987; Howes *et al.* 2000), norepinephrine and serotonin (Glowinski and Axelrod 1966; Ross and Renyi 1969; Pan *et al.* 1994; Howes *et al.* 2000). MPH mainly inhibits DAT and NET, but not SERT, inducing rapid increases in extracellular dopamine levels in the basal ganglia (Kuczenski and Segal 1997). MPH affinity for DAT *in vivo* is comparable to that of cocaine (Volkow *et al.* 1999b), but whole-brain dopamine kinetics mediated by MPH are slower than those of cocaine (Volkow *et al.* 1999a; Volkow and Swanson 2003). In adult ADHD patients, MPH increased dopamine in the ventral striatum while reducing their symptoms (Volkow *et al.* 2012).

The aim of this work was to compare the effects in mice of *binge*-like cocaine and MPH *acute* (1-DAY) and *sub-chronic* (3-DAY) administration on locomotor activity and GABAergic transmission from the TRN onto VB neurons. Our results showed that both cocaine and MPH enhanced hyperlocomotion, though cocaine-mediated effects were stronger than MPH after *sub-chronic* administration. Both cocaine and MPH changed paired-pulse evoked and spontaneous GABAergic transmission from TRN. While cocaine drastically increased paired-pulse ratios only 24 hours after 3-DAY, MPH enhanced them from 1-DAY up to 3-DAY administrations. Cocaine induced a greater spontaneous GABA minis frequency compared to MPH after 1-DAY, but not for the 3-DAY administrations. The effects of cocaine on thalamic GABAergic transmission and postsynaptic calcium currents we observed could underlie drastic alterations in the protein expression of GAD and/or postsynaptic T-type channels. Western blot analysis revealed an increase in $Ca_V3.1$ and GAD67 levels after sub-chronic administration of cocaine.

Our results suggest a considerable dysregulation of thalamic GABAergic transmission and postsynaptic calcium currents by cocaine, which might underlie its long-lasting neurotoxic effects. Also, MPH induced steady-state alterations of GABAergic transmission changes, which would result in long-lasting, potentially permanent changes in sensory thalamic processing.

Materials and Methods

Animals

18-30 days old male C57BL/6 mice from the Central Animal Facility at University of Buenos Aires were used. Principles of animal care were in accordance with CONICET (2003), and approved by its authorities using *OLAW//ARENA* directives (NIH, USA).

Drug administration

Cocaine and methylphenidate were administered *i.p.* using "*binge-like*" protocols (Spangler *et al.* 1993) for 1-DAY (3x15mg/kg, 1h apart) or for 3-DAY (*sub-chronic*; 3x15mg/kg, 1h apart, each day for 3 days) (Fig. 1A). Control animals received saline injections equally timed.

Thalamic slices

Thalamocortical slices were obtained as previously described (Bisagno *et al.* 2010), 1h after last "*binge-like*" administration, except when 24h after 3-DAY was used. Slices were allowed to recover at 35°C for at least 30 minutes in a psychostimulant-free ACSF (Bisagno *et al.* 2010).

Whole-cell patch-clamp recordings

Recordings were made at 20-24°C using patch clamp electrodes (Bisagno *et al.* 2010). Inhibitory postsynaptic miniatures currents (minis) were recorded in the presence of TTX (3μM) and analyzed using Mini Analysis (Synaptosoft, NJ, USA). Inter-event interval curves were fitted to a single exponential equation (SigmaPlot, Systat Softwares, CA, USA), and median mini intervals were compared across groups.

Inhibitory postsynaptic currents (IPSCs) were evoked in the presence of DL-AP5 (50μM) and CNQX (20μM) (40–200μs; 200–1000μA) using bipolar electrodes (FHC Inc, ME, USA) positioned at the boundary between the TRN and VB nuclei (Zhang *et al.* 1997). Bicuculline (50μM) was used to confirm that currents were mediated by GABA-A receptors (Fig. 1B). Paired pulses at 10Hz or 40Hz were used, and IPSCs were normalized to the amplitude of first IPSC. Voltage-dependent calcium currents were recorded with a ramp-like

protocol ($0.3mV*ms^{-1}$; [Bisagno *et al.* 2010]). Voltage ramps reduced the rundown of calcium currents, allowing us to accurately calculate low voltage activated (LVA, T-type) and high voltage activated (HVA, mediated by P/Q-type, ω-Agatoxin-IVA-sensitive calcium currents; see Urbano *et al.* 2009) peak currents ratios (Bisagno *et al.* 2010). Signals were recorded using a MultiClamp 700 amplifier commanded by pCLAMP 10.0 (Molecular Devices, CA, USA). Low concentrations of mibefradil (20μM) were used to block T-type currents (Fig. 1C), as previously described (Bisagno *et al.* 2010).

Behavioral studies

Mouse locomotor activity was recorded with an automated system (Ethovision XT7.0, Noldus, The Netherlands) as previously described (Bisagno *et al.* 2010). Total distance traveled (cm) was quantified for a total of 30 minutes prior to injections (baseline), and 45 minutes following the last injection of a *binge*.

Tissue homogenization and Western blot

TRN and VB were dissected from 350μm thick slices on an ice-cold stage, collected in plastic tubes and stored (-80°C). Samples were thawed and homogenized in RIPA buffer containing protease inhibitors at 4°C. After centrifugation (20 min at 21,500*g*), protein levels were determined with a BCA protein assay kit (Thermo Scientific, IL, USA), and 40μg from each sample were incubated with cracking buffer (Laemmli 1970) for 10 minutes at 100°C. Samples were run on a 10% polyacrylamide resolving gel and proteins were transferred to a nitrocellulose membrane (Sigma-Aldrich, MO, USA). The blot was probed with specific primary antibodies, including rabbit anti-GAD67/65 (1:10,000, Chemicon, MA, USA), rabbit anti-actin (1:100, Sigma-Aldrich, MO, USA), and rabbit anti-$Ca_V3.1$ (1:200, Chemicon, MA, USA). Secondary antibody was anti-rabbit conjugated to HRP (1:1000, Dako, Denmark). Blots were developed with a chemiluminescent HRP substrate (Immobilon Western, EMD Millipore Co., MA, USA), and chemiluminescence was visualized with a CCD camera (LAS-1000, Fujifilm, Japan). Signal intensity was quantified using ImageJ 1.43m software (http://imagej.nih.gov/ij/index.html, NIH, USA). Bands corresponding to GAD67 and $Ca_V3.1$ were normalized to actin, and all samples were normalized to the saline group's mean.

Statistical analysis

InfoStat software (Univ. Nacional de Córdoba, Argentina) was used for statistical comparisons. Statistics were performed using Student's t-test or one-way ANOVA (unless otherwise stated) and Tukey-Kramer or LSD Fisher multiple comparisons post hoc tests when applicable. Differences were considered significant if $p<0.05$. Whenever the data did not comply with assumptions of the parametric tests, non-parametric Wilcoxon-Mann-Whitney or Kruskal Wallis tests were performed followed by paired comparisons. Data presented as mean ± standard error of the mean.

Materials

Cocaine-HCl was purchased from Sigma-Aldrich (USA), and methylphenidate-HCl (Mallinckrodt Inc., USA) was a generous donation from Osmotica Pharmaceuticals S.A. (Buenos Aires, Argentina). During electrophysiological recordings the following drugs were used: DL-AP5, CNQX, TTX, bicuculline and mibefradil (all from Sigma-Aldrich, MO, USA).

Results

Repetitive methylphenidate (MPH) binge administration induced milder changes in hyperlocomotion than cocaine

We initially compared the effects of cocaine and MPH on locomotor activation after 1-DAY acute *binge* administration (Fig. 2A). Cocaine and MPH induced higher hyperlocomotion compared to saline but did not differ from each other (Fig. 2A; Kruskal-Wallis ANOVA, H=11.08, $p<0.05$; cocaine *vs*. saline: $p<0.001$; MPH *vs*. saline: $p<0.05$; cocaine *vs*. MPH: $p>0.05$). After 3-DAY binge administrations both cocaine and MPH induced hyperlocomotion (Fig. 2B; ANOVA, $F_{(2,25)}=17.88$, $p<0.01$; Tukey-Kramer post hoc test). Higher hyperlocomotion was observed after 3-DAY cocaine *binge* administration compared to the responses mediated by a 1-DAY binge. However, MPH-administered mice showed similar hyperlocomotion after 1-DAY and 3-DAY administration. No cocaine or MPH-mediated effects on hyperlocomotion were observed 24h after the last injection (Fig. 2C, ANOVA, $p>0.05$).

Cocaine showed higher frequencies of spontaneous GABAergic minis than MPH while only cocaine altered postsynaptic low voltage activated (LVA)/high voltage activated (HVA) calcium current ratios

GABAergic minis (mIPSCs) recorded from VB neurons after an acute cocaine *binge* manifested higher frequencies compared to MPH and saline treatments (Figure 3A,B; Kruskal-Wallis ANOVA, cocaine *vs*. MPH: H=6.85, $p<0.01$; cocaine *vs*. saline: H=15.75 $p<0.05$). Mini intervals were not significantly different when comparing saline and MPH-treated slices (Figure 3A,B; $p>0.05$). After 3-DAY *sub-chronic* protocols, cocaine and MPH treatments showed higher frequencies than saline (Fig. 3C,D; Kruskal-Wallis ANOVA, H=9.9, $p<0.01$), while no differences were observed across groups 24h after the last 3-DAY *sub-chronic* injection (Fig 3 E,F; $p>0.05$).

One MPH *binge* did not change postsynaptic calcium current LVA/HVA ratios in VB neurons when compared to saline while ratios of 1-DAY cocaine-treated animals were significantly higher than for either saline or MPH (Figure 3G; One-way ANOVA, $F_{(2,38)}=7.6$, Tukey-Kramer post hoc test; saline *vs*. MPH, $p>0.05$; cocaine *vs*. MPH: $p<0.05$; cocaine *vs*. saline: $p<0.05$). No changes in LVA/HVA ratios were observed between saline and 3-DAY (1h after) cocaine and MPH treatments (Fig. 3H; ANOVA, $p>0.05$). Lower ratios were observed 24h after 3-DAY cocaine *binge* administration (Fig. 3I, One-way ANOVA, $F_{(2,29)}=5.8$, $p<0.01$), related to higher HVA, P/Q-type mediated current density without changes in T-type current density (253%±18 increment 24h after 3-DAY *vs*. saline, n=10; Kruskal Wallis ANOVA; H=13.9, $p<0.01$). No changes in LVA/HVA ratios were observed after repetitive MPH treatments compared to saline (Fig. 3H, I; $p>0.05$).

Cocaine and MPH differentially affected paired-pulse evoked GABAergic transmission

Paired-pulse ratios (PPRs; 2ndstimulus-evoked amplitude/1st stimulus-evoked amplitude) are widely accepted as a parameter to characterize presynaptic-dependent alterations. We compared ratios using both 10Hz and 40Hz frequencies of stimulation across all treatments. Mean PPR values were lower than one indicating that there was synaptic depression between stimuli. After 1-DAY *binge* treatment, 10Hz ratios were not significantly different across treatments (Fig. 4A, Kruskal-Wallis ANOVA, $p>0.05$). However, compared to cocaine and saline, 40Hz PPRs from the MPH group were higher both in control conditions (Fig. 4A, Kruskal-Wallis ANOVA, $p<0.05$) and after bath application of mibefradil (20μM; 20-40 minutes; Fig. 4B, Kruskal-Wallis ANOVA, $p<0.05$), suggesting no significant involvement of T-type channels on paired-pulse GABA release after 1-DAY *binge* treatment. We continued characterizing the effects of cocaine and MPH on evoked

GABAergic transmission in mice after *sub-chronic* administration protocols. Again, MPH elicited higher PPRs than saline and cocaine at both frequencies tested (Fig. 4C; 10Hz: ANOVA $F_{(2,61)}$=3.79, p<0.05; Tukey-Kramer post hoc test, MPH *vs.* saline, cocaine p<0.05; 40Hz: ANOVA $F_{(2,48)}$=8.64, p<0.01; Tukey-Kramer post hoc test, MPH *vs.* saline, cocaine, p<0.05). Cocaine did not change PPRs 1h after *binge* compared to saline at either 10Hz or 40Hz (Fig. 4C; ANOVA, p>0.05), while PPRs were significantly higher than MPH and saline 24h after 3-DAY cocaine *binge* treatment for both 10Hz and 40Hz (Fig. 4D; 10Hz: ANOVA $F_{(2,42)}$=20.27 p<0.01; Tukey-Kramer post hoc test, MPH *vs.* cocaine, p<0.05, and saline vs. cocaine, p<0.01; 40Hz: ANOVA $F_{(2,34)}$=18.8 p<0.01; Tukey-Kramer post hoc test, MPH, *vs.* cocaine, p<0.05 and saline vs. cocaine, p<0.01). Importantly, 40Hz PPRs 24h after 3-DAY cocaine *binge* treatment (Fig. 4D) showed ratios surpassing the threshold of 1.0, indicating pure facilitation during GABA transmission at high frequency. In the presence of mibefradil (20 μM), 24h after 3-DAY cocaine *binge* treatment 10Hz and 40 Hz PPR values were significantly reduced to saline levels (Fig. 4D, dashed grey bars; Tukey-Kramer post hoc test, 24-h after 3-DAY before vs. after mibefradil, p<0.05; saline vs. 24-h after 3-DAY in the presence of mibefradil, p>0.05). MPH ratios 24h after 3-DAY administration protocols were higher than saline only at 10Hz (Tukey-Kramer post hoc test, saline vs. MPH, p<0.05). PPRs after MPH administration were not significantly different comparing 1h (Fig. 4C) and 24h after 3-DAY *binge* (Fig. 4D).

In conclusion, MPH treatments increased PPRs compared to saline throughout all administration protocols used, being reversible 24h after 3-DAY treatment at 40Hz stimulation. However, only cocaine induced a rebound in PPR values 24h after 3-DAY after either 10Hz or 40Hz stimulation (Fig. 4E). Mibefradil reduced higher PPR values observed 24h after 3-DAY cocaine treatment.

Cocaine increased thalamic $Ca_V3.1$ protein levels

Cocaine effects on GABAergic PPRs and on LVA/HVA current ratios might be due to transient changes in TRN synaptic GAD67 or VB $Ca_V3.1$ protein levels. Fig. 5A shows GAD67 protein levels (measured by Western Blot) in the TRN 1h and 24h after 3-DAY *binge* protocol. No statistically significant differences were observed between cocaine- and saline-treated mice (p>0.05). On the other hand, $Ca_V3.1$ protein levels in VB nucleus were significantly higher 24h after 3-DAY *binge* protocols compared to saline (Fig. 5B; Student's t-test, t=4.0, p=0.002).

Discussion

The results presented here show distinct alterations by *sub-chronic binge-like* administrations of either cocaine or MPH on hyperlocomotion, pre-synaptic modulation of GABAergic transmission and postsynaptic calcium currents from sensory thalamic nuclei. Our results suggest that MPH-mediated effects were longer lasting than cocaine, while cocaine effects were more robust and changed significantly with the duration of the administration protocols.

Cocaine and MPH differentially affected hyperlocomotion

Cocaine- and MPH-mediated rapid enhancement in locomotion in rodents has been correlated with their ability to increase extracellular dopamine and norepinephrine levels in nucleus accumbens and caudate-putamen (Segal and Kuczenski 1992; 1999; 2001). Unlike cocaine, MPH fails to increase extracellular serotonin levels (Kuczenski and Segal 1997; Segal and Kuczenski 1999) due to its weak binding affinity for SERT (Pan *et al.* 1994; Gatley *et al.* 1996). MPH (*i.p.*) has been extensively used in mice at the same concentration range as cocaine (Kuczenski and Segal 2001; Drerup *et al.* 2010; Thanos *et al.* 2010). It is

agreed that MPH and cocaine might share neuronal pathways to exert their effects (Volkow *et al*. 1999b; Argento *et al*. 2012). Here, *binge-like* MPH administration, similar to non-prescribed, repetitive MPH self-administration described in adolescents (Morton and Stockton, 2000), was compared to *binge-like* cocaine, showing no change in hyperlocomotion between days of treatment, though enhancing hyperlocomotion above saline levels (Drerup *et al*. 2010). MPH-induced hyperlocomotion was insensitive to the T-type calcium channel blocker 2-octanol (0.07mg/kg, *i.p.; p*>0.05; data not shown), unlike what has been previously reported by our group for cocaine at the exact same dose (Bisagno *et al*. 2010). It has also been described that MPH increased dopamine levels in the TRN, reducing hyperlocomotion through activation of D4 receptors (Erlij *et al*. 2012), thus suggesting a limiting process that might explain why MPH repetitive administration did not increase hyperlocomotion levels above acute-mediated levels.

Cocaine- and MPH-induced changes on GABAergic transmission from TRN: Monoamine synaptic levels vs. intrinsic properties

The effects of cocaine and MPH on GABA release were observed several hours after slicing, which suggests long-lasting effects from multiple basal ganglia/brainstem-TRN interactions (Contreras *et al*. 1993; Shammah-Lagnado *et al*. 1996). Nevertheless, PPR values from saline mice were not significantly different either across treatments or frequencies. Frequency-independence of PPR results presented here are in agreement with recent reports using similar PPR testing (Zhang *et al*. 2010).

It has been described that monoamine receptors can modulate GABA release from Globus Pallidus onto TRN. Indeed, activation of D4 dopaminergic receptors enhances 10Hz PPRs in Globus Pallidus afferents, without altering GABAergic transmission within TRN (Govindaiah *et al*. 2010). The fact that MPH is known to activate these receptors in the TRN (Erlij *et al*. 2012) and that cocaine can also increase monoamine levels in somatosensory thalamic nuclei (Rutter *et al*. 1998), suggest a more complex mechanism underlying the observed differences in PPRs. Changes in PPR during *sub-chronic* administrations of MPH affected both 10Hz and 40Hz stimulation, suggesting a modulation of TRN neurons at both frequencies as described *in vivo* (Pinault and Deschênes 1992). After such modulation, TRN neurons might need to recover from direct alteration (blocking/opening) of membrane ionic channels as well as GABAergic transmission after *sub-chronic* cocaine and MPH administrations (Shoji *et al*. 1998; Federici *et al*. 2005). Recent experiments made by our group have confirmed this hypothesis, showing that bath-applied MPH (10μM) did not have any effect on the firing frequency of TRN neurons, while cocaine (10μM) strongly reduced frequency of action potentials (data not shown; n=8). Thus, cocaine *sub-chronic* treatments might block TRN somatic activity (known to be required for the correct TRN-TRN inter-somatic excitatory activity; [Sun *et al*. 2012]), explaining the observed mean paired-pulse “rebound” 24h after 3-DAY cocaine administration. Milder MPH effects on intrinsic properties of TRN would explain the more modest, but sustained effects on PPR. GABA minis frequency increment by cocaine after one day (and up to three days) would be mediated by its direct effect on presynaptic TRN GABAergic terminals, regardless of action potential frequency at TRN somatic levels (i.e., consistently with our group's previous reports in the presence of TTX, [Urbano *et al*. 2009; Bisagno *et al*. 2010]).

Monoamine receptors have also been described to modulate intrinsic properties (e.g., T-type calcium channels) of sensory thalamic neurons. T-type calcium channels are involved in distal dendritic calcium transients in TRN neurons helping integrate dendritic GABAergic afferents (Crandall *et al*. 2010; Sun *et al*. 2012). It is accepted that only P/Q-type channels are located in both VB dendrites (Pedroarena and Llinás 1997) and TRN synaptic terminals in charge of GABA release (Iwasaki *et al*. 2000). Interestingly, serotonin, but not dopamine, has been reported to significantly affect T-type calcium currents (Berger and Takahashi

1990; Fraser and MacVicar 1991), suggesting a possible serotonin-based mechanism mediating cocaine-, but not MPH-induced changes in postsynaptic LVA/HVA currents ratios during *sub-chronic* administrations showed here. Different effects in T-type channels mediated by cocaine are consistent with unchanged MPH hyperlocomotion after 2-Octanol administration, but contrary to the absence of mibefradil effects on PPR after acute *binge* administration of cocaine.

Another possibility to explain the observed differences between MPH and cocaine *sub-chronic* administrations on GABA release PPR would be the existence of a MPH-mediated presynaptic inhibition of TRN GABA release (Federici *et al.* 2005), which would mediate the increment in mean ratio values observed in this work. On the contrary, cocaine would have a stronger blocking effect of intersomatic TRN GABAergic inhibition while simultaneously incrementing spontaneous minis (i.e., directly acting on presynaptic terminals), thus presenting probabilities of GABA release in the same range than saline treated terminals (i.e., an experimental condition characterized by smaller mean PPRs, [Zhang *et al.* 1997]). Accordingly, a rebound in PPR values 24h after the 3-DAY cocaine treatment can be seen as a compensatory mechanism. The involvement of T-type calcium channels on intercellular TRN GABAergic inhibition is consistent with the observed reduction of PPR values 24 h after 3-DAY administration using bath-application of mibefradil. A mibefradil-mediated reduction in low-threshold spikes mediated by T-type channels at TRN somatic level (Crandall *et al.* 2010; Sun *et al.* 2012) would reduce GABA release between TRN neurons. Thus, lower inhibition of TRN neurons would dis-inhibit GABA release onto VB neurons, ultimately inducing higher probabilities of GABA release (i.e., provoking depression such as PPR values). We further tested this hypothesis by comparing PPR values obtained 24h after 3-DAY *vs.* a 4-DAY treatment (animals sacrificed 1h after the last injection). The 4-DAY cocaine administration further reduced PPRs values, although they were significantly higher than saline PPRs (n>15, saline; n>23 cocaine; data not shown).

Therefore, our results support the hypothesis of a cocaine-mediated over-stimulation of GABA release by altering TRN inter-somatic inhibition. In addition, dissimilar effects of MPH and cocaine in sensory thalamic nuclei might have a correlation with changes in intracellular calcium concentrations in sensory thalamic neurons, as previously reported in cortical areas (Du *et al.* 2006). Further studies are needed in order to characterize intracellular, downstream events that might explain the observed differences between cocaine- and MPH-induced changes in thalamic GABAergic PPR values.

GAD67 and $Ca_V3.1$ protein levels

Transient, rebound-like cocaine-mediated effects on thalamic GABAergic transmission and postsynaptic calcium currents could underlie drastic alterations in the protein expression of GAD or postsynaptic $Ca_V3.1$ T-type channels. Western blot results presented here about cocaine-induced changes in GAD67 or $Ca_V3.1$ protein levels can be considered new, having no precedent study published to the best of our knowledge. Nevertheless, Western blot quantification has been used to report a GAD level increment after cocaine withdrawal in the hypothalamus (Ma *et al.* 2008). Here no significantly different levels of GAD67 (predominantly located at a TRN somatic level; [Esclapez *et al.* 1994]) were observed up to 24h after 3-DAY *binge* administration. However, one extra day of administration did increase GAD67 (data not shown), supporting the idea that cocaine might drastically reduce presynaptic GABA levels, leading to higher GAD protein synthesis.

The role of T-type channels in sensory thalamic nuclei has been recently expanded, describing that both presynaptic TRN and postsynaptic VB neuronal types share $Ca_V3.1$ subunits containing T-type calcium channels (Kovács *et al.* 2010). In light of these new

reports, the observed changes in VB postsynaptic calcium currents ratios (LVA/HVA) throughout cocaine *sub-chronic binge*-like administrations may be suggesting the existence of a compensatory expression of T-type channels by VB and/or TRN terminals. There was an increment in $Ca_V3.1$ subunits expression 24h after a 3-DAY treatment, but not at any time tested right after cocaine was administered (including 4-DAY administration, data not shown). It is worth noticing that plasma cocaine levels were expected to be totally washed out after 24h.

Both cocaine-mediated magnitude and time-delayed effects in GAD and $Ca_V3.1$ protein levels are illustrative of the long lasting, deleterious effects that this stimulant can exert over sensory thalamocortical processing.

Functional implications of sustained cocaine TRN alterations for thalamocortical interactions

Results from this study suggest that cocaine and MPH are able to enhance synaptic GABAergic transmission at both low (10Hz) and high frequency (40Hz) stimulation of TRN axons as well as hyperlocomotion after *sub-chronic* administration protocols. This may result in the abnormal hyperpolarization of VB thalamocortical projecting neurons, leading to thalamocortical recurrent low frequency bursting activity of both TRN (Llinás and Geijo-Barrientos 1988; Huguenard and Prince 1992) and VB neurons (Jahnsen and Llinás 1984a,b; McCormick and Feeser 1990). Prolonged coherence between low-frequency burst-firing and high frequency thalamocortical activity during awake states has been suggested to disrupt sensory processing (McCormick and Feeser 1990), as well as induce alterations in nociception in mice (Liao *et al*. 2011), which are known to underlie multiple diseases known collectively as *thalamocortical dysrhythmia syndrome* (Llinás *et al*. 1999; Jeanmonod *et al*. 2003). T-type channel over-activation at the level of the TRN has been associated with pathophysiological behaviors including epilepsy (Steriade and Llinás 1988; Tsakiridou *et al*. 1995), a neurological disorder also associated with chronic administration of cocaine, but not MPH (Devlin and Henry 2008). An over activation of TRN-mediated GABAergic transmission would also alter sensory traffic through sensory relay nuclei in the thalamus, a mechanism thought to underlie major EEG abnormalities in ADHD patients (Rowe *et al*. 2005).

The results described here *using sub-chronic protocols*, confirm and expand our group's previous findings showing a dysregulation of thalamic GABAergic transmission and postsynaptic calcium current ratios as key mechanisms that might underlie the long-lasting deleterious effects of cocaine. MPH-induced changes in GABAergic transmission using repetitive administration protocols suggest that MPH might also alter sensory processing but in a less disruptive manner. Steady-state alterations by MPH are particularly important to understand the impact of MPH intake either as pharmacotherapy for ADHD patients or in non-prescribed stimulant abuse among healthy users. Future studies using longer, chronic protocols are still needed in order to determine whether the cocaine- and MPH-mediated effects described here might turn into permanent thalamic changes.

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Abbreviations

ADHD	attention deficit/hyperactivity disorder
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate
DAT	dopamine transporter
DL-AP5	DL-2-amino-5-phosphonovaleric acid
GAD	glutamic acid decarboxylase
HVA	high voltage activated
IPSC	inhibitory post synaptic current
LVA	low voltage activated
MPH	methylphenidate-HCl
NET	norepinephrine transporter
PPR	paired-pulse ratio
SERT	serotonin transporter
TRN	thalamic reticular nucleus
TTX	tetrodotoxin
VB	ventrobasal nucleus

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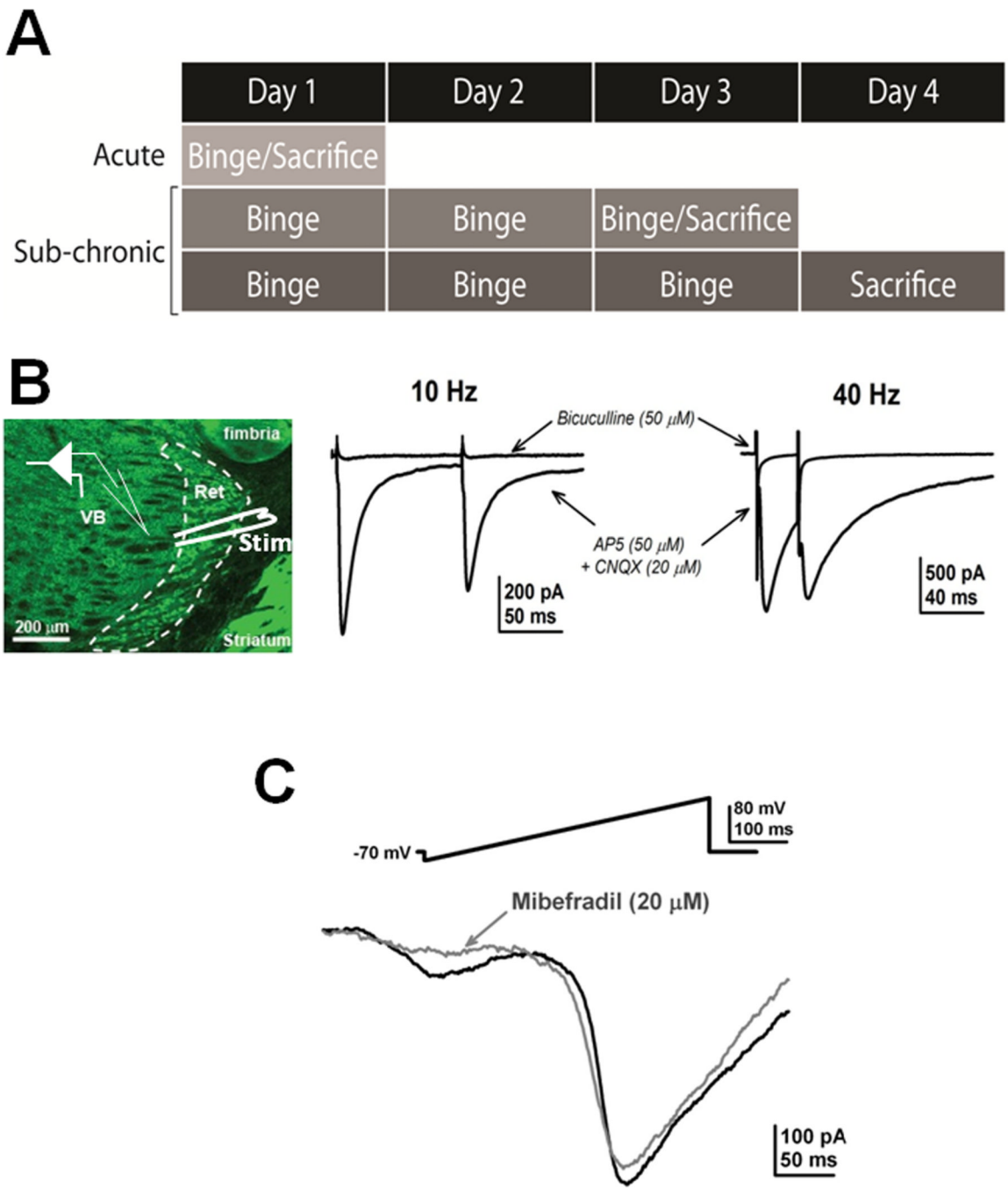

Figure 1. Cocaine and methylphenidate (MPH) administration protocols used. Representative evoked GABAergic synaptic and ramp-evoked calcium currents recorded from ventrobasal (VB) neurons

A, cocaine and MPH *binge* administration protocols used. **B, left**, GAD65/67-immunolabeled thalamocortical slice showing locations of reticular (Ret) and ventrobasal (VB) nuclei, as well as recording (VB) and stimulating electrodes (Stim.). **B, right**, representative evoked inhibitory postsynaptic currents (IPSCs) after 10Hz and 40Hz paired-pulse stimulation in the presence of 50μM AP5 and 20μM CNQX before and after Bicuculline bath application (50μM). Holding potential was -70mV. **C**, representative calcium currents generated by 500ms-long depolarizing ramps before (black line) and 20

minutes after 20μM mibefradil bath application (grey line). Mibefradil mainly reduced the low voltage activated T-type current component (arrow), while slightly affecting high voltage activated P/Q-type current amplitude (100 nM ω-Agatoxin-IVA-sensitive component; Urbano *et al.* 2009).

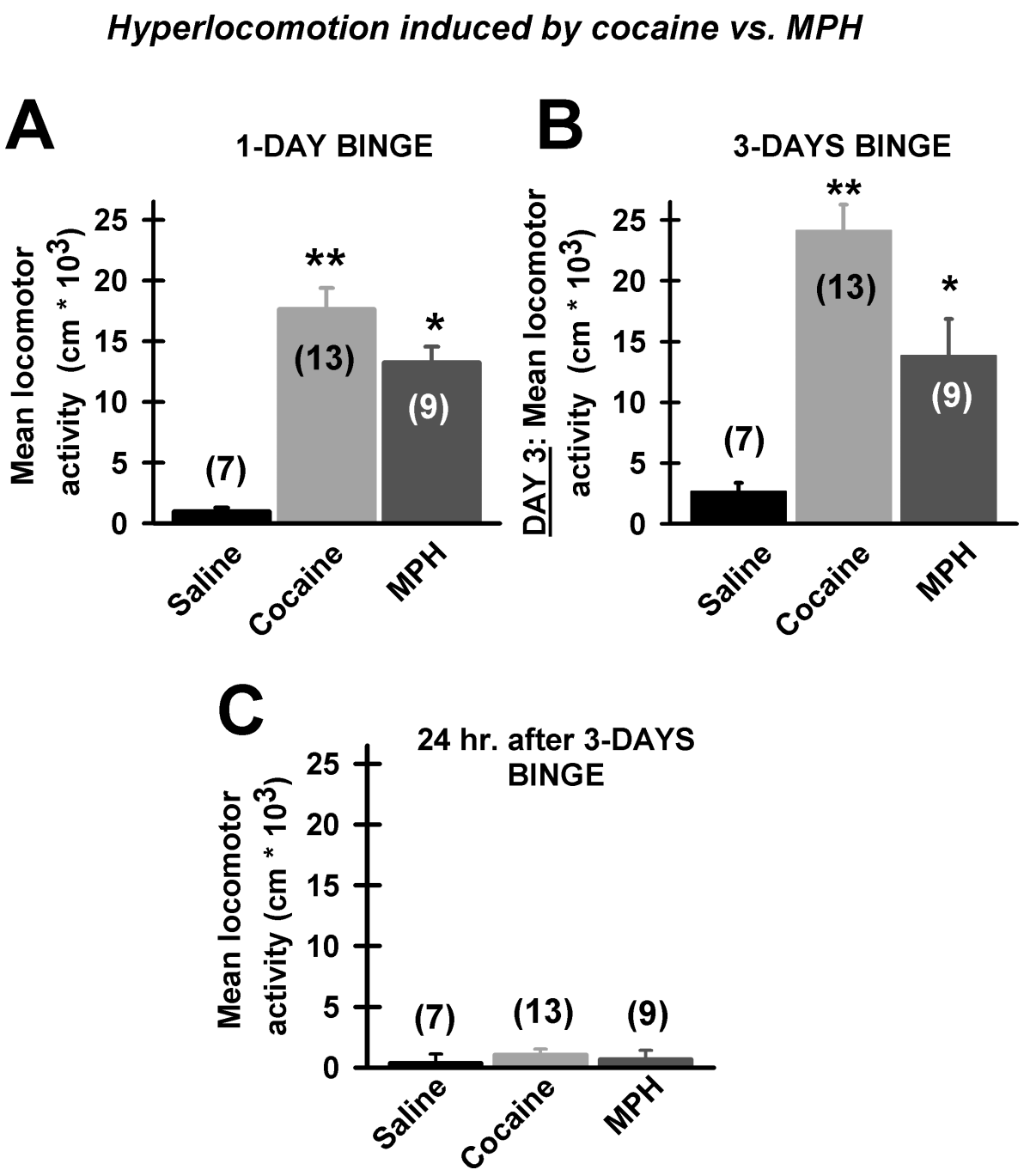

Figure 2. Repetitive methylphenidate (MPH) binge administrations induced milder changes in hyperlocomotion than cocaine

A, distance traveled (in cm $\times 10^3$) by mice *i.p.* injected with saline (black bar; 3 injections, 1h apart), 1-DAY cocaine (light grey bar), or MPH (dark grey bar) *binge*. ** $p<0.001$, cocaine *vs.* saline. * $p<0.05$, MPH *vs.* saline. **B**, distance traveled (in cm $\times 10^3$) by mice *i.p.* injected with saline (black bar), 3-DAY cocaine (light grey bar), or MPH (dark grey bar) *binge* 45 minutes after receiving the last injection. * $p<0.05$, MPH *vs.* saline. ** $p<0.01$, cocaine *vs.* saline. * $p<0.05$, cocaine *vs.* saline (LSD Fisher post hoc test). **C**, distance traveled (in cm $\times 10^3$) recorded 24h after the last injection for the same treatments (saline:

black bar; cocaine: light grey bar; MPH: dark grey bar). No statistical differences were found between treatments. Baseline values were recorded before injections on day one.

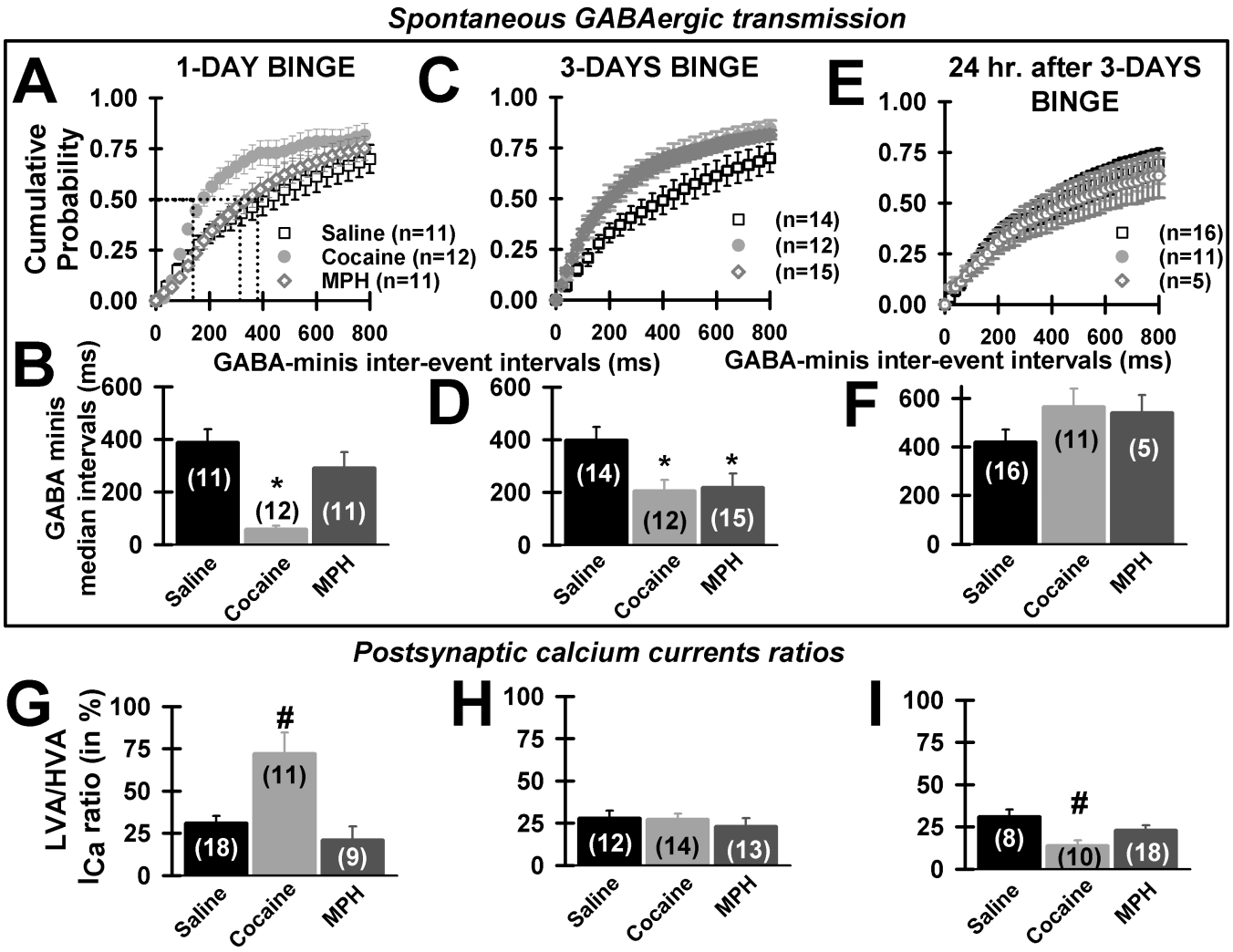

Figure 3. Cocaine showed higher frequencies of spontaneous GABAergic minis than MPH, and altered postsynaptic low voltage activated (LVA)/high voltage activated (HVA) calcium current ratios

A, cumulative probability plots of GABAergic mini inter-event intervals (mean ± standard error of the mean) recorded in VB neurons from mice injected with saline (black squares), 1-DAY cocaine *binge* (light grey circles), and 1-DAY MPH *binge* (dark grey diamonds). **B**, GABAergic mini median intervals from mice injected with saline (black bar), 1-DAY cocaine (light grey bar), or MPH (dark grey bar) *binge*. Median values (showed in 3A with dotted lines) were obtained after fitting individual cumulative probability plots to the function $y=y_0+a*\exp(-\text{Time (ms)}/\tau)$. * $p<0.01$, cocaine *vs*. saline, MPH. **C, D**, cumulative probability plots of GABAergic min inter-event intervals and median intervals recorded in VB neurons from mice treated with saline (black squares and bars), 3-DAY cocaine (light grey circles and bars), or MPH (dark grey diamonds and bars) *binge*, and sacrificed 1h after the last injection. Data presented and analyzed as in A and B. * $p<0.05$, saline *vs*. cocaine, MPH. **E, F**, cumulative probability plots of GABAergic mini inter-event intervals and median intervals recorded in VB neurons from mice treated with saline (black squares and bars), 3-DAY cocaine (light grey circles and bars), or MPH (dark grey diamonds and bars) *binge*, and sacrificed 24h after the last injection. Data presented and analyzed as in A and B. **G**, low voltage activated (LVA)/high voltage activated (HVA) calcium current ratios in VB neurons from mice treated with saline (black bar), 1-DAY cocaine (light grey bar), or MPH

(dark grey bar) *binge*. # $p<0.05$, cocaine *vs*. MPH, saline. **H**, LVA/HVA calcium current ratios for VB neurons from mice treated with saline (black bar), 3-DAY cocaine (light grey bar), or MPH (dark grey bar) *binge,* and sacrificed 1h after the last injection. **I**, LVA/HVA calcium current ratios for VB neurons from mice treated with saline (black bar), 3-DAY cocaine (light grey bar), or MPH (dark grey bar) *binge*, and sacrificed 24h after the last injection. # $p<0.01$, cocaine *vs*. saline, MPH (Tukey-Kramer post hoc test).

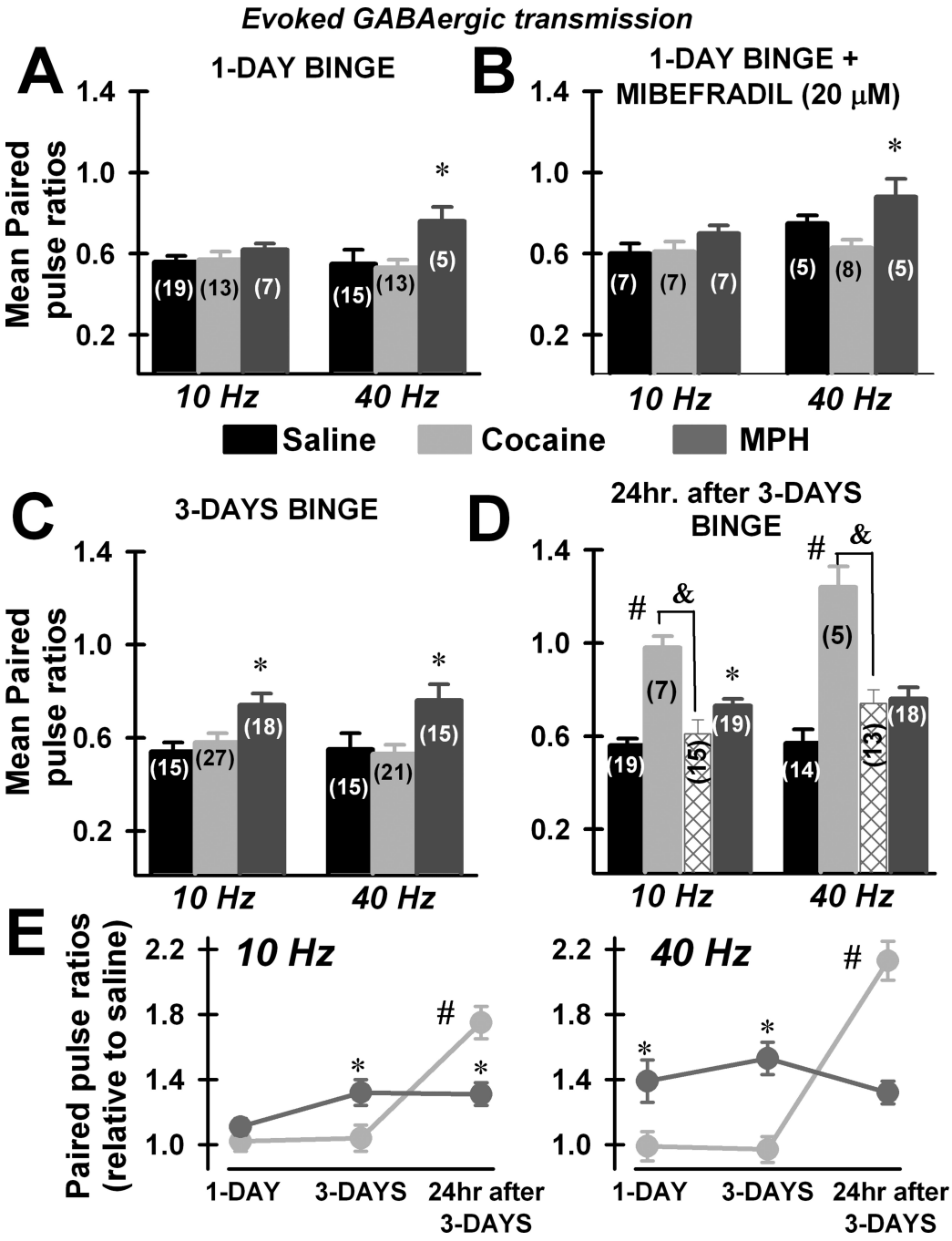

Figure 4. Cocaine and MPH differentially affected paired-pulse evoked GABAergic transmission

A, paired-pulse ratios (IPSCs 2nd stimulus/ IPSCs 1st stimulus) recorded from ventrobasal (VB) neurons after 10Hz and 40Hz paired-pulse stimulation of reticular thalamic nucleus (TRN) axons from mice treated with saline (black bars), 1-DAY cocaine (light grey bars), or MPH (dark grey bars) *binge*. **B**, paired-pulse ratios recorded from VB neurons after 10Hz and 40Hz paired-pulse stimulation of TRN axons in the presence of mibefradil (20 μM), from mice treated as in A (saline: black bars; cocaine: light grey bars; MPH: dark grey bars). * $p<0.05$, MPH *vs*. saline, cocaine. **C**, paired-pulse ratios recorded from VB neurons after 10Hz and 40Hz paired-pulse stimulation of TRN axons from mice treated with saline (black

bars), 3-DAY cocaine (light grey bars), or MPH (dark grey bars) *binge*, and sacrificed 1h after the last injection. * $p<0.05$, MPH *vs*. saline, cocaine. **D**, paired-pulse ratios recorded from VB neurons after 10Hz and 40Hz paired-pulse stimulation of TRN axons from mice treated with saline (black bars), 3-DAY cocaine (light grey bars), or 3-DAY cocaine in the presence of mibefradil (20 μM, dashed grey bars) or MPH (dark grey bars) *binge*, and sacrificed 24h after the last injection. * $p<0.05$, MPH *vs*. saline, cocaine. $^{\#}$ $p<0.01$, cocaine *vs*. saline, MPH. $^{\&}$ $p<0.05$, cocaine *vs*. cocaine+mibefradil. **E**, fold change of MPH and cocaine mean paired-pulse ratios (normalized to mean saline ratios) at 10Hz and 40Hz stimulation. Similar statistical significance was found when comparing relative ratios among treatments.

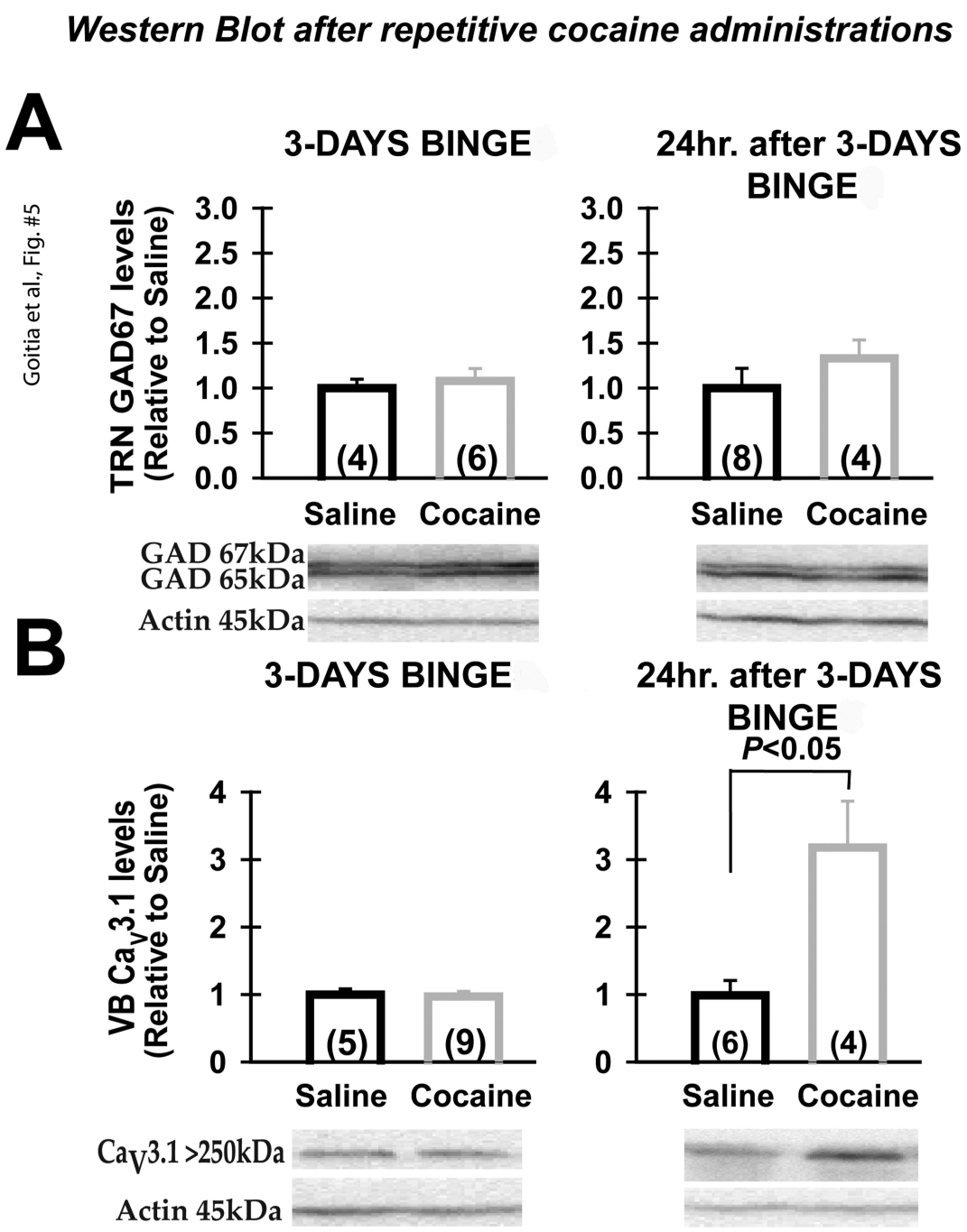

Figure 5. Repetitive administration of cocaine changed $Ca_V3.1$, but not GAD67 protein levels in ventrobasal (VB) and reticular (TRN) thalamic nuclei, respectively

A, GAD67 protein levels (normalized to actin and to the average value of the saline group) measured by Western blot in the TRN 1h and 24h after the last injection of a 3-DAY *binge* protocol. **B**, $Ca_V3.1$ protein levels (normalized to actin and to the average value of the saline group) measured by Western blot in the VB 1h and 24h after the last injection of a 3-DAY *binge* protocol. Statistical differences between cocaine and saline treated mice were found when animals were sacrificed 24h after the last injection.