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Roles of M1 muscarinic acetylcholine receptor subtype in regulation of basal ganglia function and implications for treatment of Parkinson's disease

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***Abbreviations*:** ACh, acetylcholine; BQCA, benzylquinolone carboxylic acid; CCh, carbachol; PD, Parkinson's Disease; *i.p.*, intraperitoneal; *s.c.*, subcutaneous; mAChR, muscarinic ACh receptor; MSNs, medium spiny neurons; STN, subthalamic nucleus; SNr, substantia nigra par reticulata; M1 receptor, muscarinic ACh receptor subtype 1; PAM, positive allosteric modulator;

ABSTRACT

Antagonists of the muscarinic acetylcholine receptors (mAChRs), were among the first treatments for Parkinson's disease (PD). However, the clinical utility of mAChR antagonists is limited by adverse effects associated with blockade of multiple mAChR subtypes. Understanding of the roles of specific mAChR subtypes in regulating basal ganglia and motor function could lead to development of novel agents that have antiparkinsonian activity with fewer adverse effects. Using novel, highly selective M1 antagonist, VU0255035, and M1 positive allosteric modulator (PAM), BQCA, we investigated the roles of M1 receptors in cholinergic excitation and regulation of synaptic transmission in striatal medium spiny neurons (MSNs) and neurons in the subthalamic nucleus (STN) and substantia nigra par reticulata (SNr). Electrophysiological studies demonstrate that M1 activation has excitatory effects on MSNs but plays little or no role in mAChR-mediated increases in firing frequency or regulation of synaptic transmission in STN and SNr neurons. Based on this profile, M1-selective antagonists may have weak antiparkinsonian activity but would not have the full efficacy observed in non-selective mAChR antagonists. Consistent with this, the M1-selective antagonist VU0255035 partially reversed reserpine-induced akinesia and decreased haloperidol-induced catalepsy in rats, but did not have the full efficacy observed with the nonselective mAChR antagonist scopolamine. These results suggest that the M1 receptor participates in the overall regulation of basal ganglia function and antiparkinsonian effects of mAChR antagonists but that other mAChR subtype(s) also play important roles at multiple levels of the basal ganglia motor circuit.

INTRODUCTION

Parkinson's disease (PD) is a common movement disorder with primary motor symptoms including resting tremor, rigidity and bradykinesia (Davie, 2008). The pathophysiological hallmark of this disorder is a loss of dopamine neurons in the subtantia nigra pars compacta. Traditional therapies are based on dopamine replacement strategies and include Levodopa (L-DOPA) and dopamine receptor agonists (Chen and Swope, 2007;Davie, 2008). These drugs are initially effective in virtually all PD patients but eventually fail in most patients due to the emergence of motor complications such as motor fluctuations and dykinesias. Thus, there is a need to develop understanding roles of other neurotransmitter systems in regulating function of the basal ganglia motor circuit in hope of developing novel strategies for treatment of PD.

Cholinergic systems provide one of the most important neuromodulators of basal ganglia function (Lester, et al., 2010). Interestingly, muscarinic acetylcholine receptor (mAChR) antagonists were among the first available treatments for PD and are still used for treatment of this disorder (Chen and Swope, 2007;Katzenschlager, et al., 2003). Unfortunately, clinical utility of mAChR antagonists is limited by the central and peripheral adverse effects some of which are likely mediated by blockade of mAChR subtypes that are not involved in regulation of basal ganglia motor function. In addition, it is not entirely clear what regions of the basal ganglia motor circuit are involved in the antiparkinsonian actions of muscarinic antagonists. The primary effects of ACh in regulating basal ganglia and motor function are often thought to be mediated by its actions in the striatum, where ACh is released from tonically active cholinergic interneurons that project to neighboring neurons within the striatum, including MSNs (Graybiel, 1990;Pisani, et al., 2007). However, ACh also plays important roles in regulating other structures in the basal ganglia. For instance, the subthalamic nucleus (STN) and the output nuclei

of the basal ganglia, substantia nigra reticulata (SNr) and internal globus pallidus, receive cholinergic innervation from the pedunculopontine tegmental nucleus (PPN) (Bevan and Bolam, 1995;Lavoie and Parent, 1994a;Lavoie and Parent, 1994b). Electrophysiological studies reveal an increase in burst firing in STN and SNr neurons in parkinsonian animals and patients with PD (Delong and Wichmann, 2007). If the PPN cholinergic projection contributes to increases in activity of STN and/or SNr neurons, this could exacerbate parkinsonian motor disability and thus could also provide an important site of action for mAChR antagonists. Indeed, it has been shown that microinjection of mAChR antagonists into the STN has antiparkinsonian effects in a rodent model of PD (Hernandez-Lopez, et al., 1996). Therefore, it is important to develop a detailed understanding of the physiological roles of individual mAChR subtypes that mediate cholinergic regulation of basal ganglia function, which could provide the basis for the development of improved anticholinergic therapies for PD.

The mAChRs are class A G protein-coupled receptors (GPCRs) and include five subtypes, termed M1 – M5. Of the five mAChR subtypes, M1 receptors are the most abundant mAChR subtypes expressed in the brain including the striatum and proposed to play important roles in a variety of brain functions, including motor control, as well as attention, memory, and sleep-wake cycle regulation (Felder, et al., 2000). Unfortunately, it has been difficult to develop a detailed understanding of the physiological roles of each mAChR subtype because of the lack of pharmacological tools that are highly selective for individual subtypes. Recently, we developed and characterized a panel of novel compounds that are highly selective for M1 and M4 mAChR subtypes, including selective M1 antagonist VU0255035 and M1 positive allosteric modulator (PAM) BQCA (Sheffler, et al., 2009;Shirey, et al., 2009). In the present studies, we took the advantage of these selective M1 ligands to determine the roles of M1 in modulation of

membrane excitability of striatal MSNs and neurons in the STN and SNr. In addition, we assessed the involvement of M1 in cholinergic modulation of synaptic transmission in STN and SNr with the goal of focusing on cholinergic modulation in the indirect pathway, particularly the GABAergic transmission in STN neurons and glutamatergic transmission in SNr neurons. The body of literature suggests that modulation of transmission through the indirect pathway can have antiparkinsonian activity (for review, see (Johnson, et al., 2009) and could therefore contribute to the antiparkinsonian effect of mAChR antagonists. Finally, we tested the hypothesis that selective M1 antagonists have antiparkinsonian activity in rodent models of PD.

METHODS

Animals. All animals used in present studies were group housed with food and water available *ad libitum*. Animals were kept under a 12 h light/dark cycle with lights on from 6 AM to 6 PM and were tested during the light phase unless stated otherwise. All experimental procedures were approved by the Vanderbilt University Animal Care and Use committee and followed the guidelines set forth by the *Guide for the Care and Use of Laboratory Animals*.

In vitro Electrophysiology. Coronal brain slices (290-300 µm) containing the striatum were obtained from C57Bl/6Hsd mice [postnatal day 21 (P21) - P27; Harlan, Indianapolis, IN]. Sagittal brain slices (290 - 300 µm) containing the STN and SNr were obtained from or Sprague-Dawley rats (P16 - P22; Charles River, Wilmington, MA). Animals were anesthetized with isoflorane and brains were rapidly removed from skulls and submerged in ice-cold modified artificial cerebrospinal fluid (ACSF) oxygenated with 95% O_2/5% CO_2. The modified ACSF was composed of (in mM) 220 glucose, 2.5 KCl, 8 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 D-glucose. Brain slices containing the striatum or STN and SNr were cut using a Vibratome 3000 (Vibratome, St. Louis, MO). Slices were incubated in oxygenated ACSF at 32°C for 30 min and then maintained at room temperature (20-22°C) afterward until transferred to a recording chamber. The recording chamber was continuously perfused with oxygenated ACSF containing (mM) 126 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 1.3 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 D-glucose.

Whole cell or cell-attached recordings were made from visually identified striatal MSNs, STN neurons or SNr neurons under an Olympus BX50WI upright microscope (Olympus, Lake Success, NY). A low-power objective (4 X) was used to identify brain region and a 40 X water

immersion objective coupled with Hoffman optics was used to visualize the individual neurons of interest. Whole cell current- or voltage-clamp signal was amplified using an Axon Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Patch pipettes were prepared from borosilicate glass (Sutter Instruments, Novato, CA) using a Narishige puller (model PP-830; Narishige International USA, East Meadow, NY). The electrode resistance was 3–5 MΩ when filled with the following intracellular solution (in mM): 120 K-$MeSO_4$, 1 $MgCl_2$, 0.1 $CaCl_2$, 10 HEPES, 1 EGTA, 12 phosphocreatine, 0.4 GTP and 2 ATP. The pH of the pipette solution was adjusted to 7.3 with 1 M KOH, and osmolarity was adjusted to 290-295. Striatal MSNs, subthalamic neurons and SNr GABAergic projection neurons were identified based on previously established electrophysiological characteristics (Beurrier, et al., 1999;Richards, et al., 1997;Wilson, 2004). The change of excitability of MSN was assessed in current clamp mode by monitoring the change of membrane potential and change in the number of spike discharges in response to near rheobase depolarization current injection. The change of excitability of STN neurons and SNr neurons was determined by monitoring the change in frequency of spontaneous firing. IPSCs in STN neurons or EPSCs in SNr neurons were evoked every 10 seconds using a concentric bipolar tungsten electrode (Frederick Haer Company, Bowdoinham, ME) placed in the internal capsule rostral to the STN or the SNr. IPSCs were recorded at a holding potential of -55 mV in the presence of ionotropic glutamate receptor antagonists AP-5 (50uM, Tocris Bioscience, Ellisville, MO) and DNQX (20 uM, Tocris Bioscience, Ellisville, MO). EPSCs were recorded at a holding potential of −60 mV in the presence of $GABA_A$ receptor antagonist (-)-bicuculine methobromide (20 μM, Tocris Bioscience, Ellisville, MO). To determine the role of M1 in MSNs, STN and SNr neurons, we used selective M1 antagonist VU0255035 and M1 PAM BQCA. Use of these tools required us to establish the CRC for CCh for each response so

that we could use the appropriate CCh concentrations for each experiment. Thus, to assess the effects of these selective M1 ligands on CCh-induced responses, we first determined the dose-response relationship of CCh responses in MSNs, STN, and SNr neurons. This allowed us to use concentrations of CCh that provide EC20 and EC80 responses. We then used an approximate EC80 concentration of CCh for studies of the effect of the M1 antagonist VU0255035 and an EC20 CCh concentration to measure potentiation by BQCA. All drugs were bath applied. The electrical signal was low-pass-filtered at 3 kHz, digitized at 20 kHz, and acquired using a Clampex9.2/Digidata 1332 system (Molecular Devices, Sunnyvale, CA). ClampFit (Molecular Devices, Sunnyvale, CA), Origin (OriginLab, Northampton, MA), and MiniAnalysis (Synaptosoft, Decatur, GA) were used for data analysis.

Behavioral studies. Male Sprague-Dawley rats (250-300g, approximately 9-10 weeks old) were used in the behavioral studies. For reserpine-induced akinesia, rats were injected with reserpine (3 mg/kg, s.c., dissolved in 1% acetic acid) and returned to their home cages for 2 hr followed by random assignment to treatment groups and administration of a dose of either VU0255035 (0.3, 1 or 3 mg/kg, i.p.), scopolamine (0.1, 0.3 or 1mg/kg, s.c.) or vehicle. Animals were then placed in a locomotor activity chamber for 30 min. Thirty minutes after administration of the test compound or vehicle (2.5 hr post reserpine-injection), motor activity was recorded for an additional 30 min in the locomotor activity chamber equipped with 16 x 16 infrared beams (Hamilton-Kinder, Poway, CA).

For haloperidol-induced catalepsy, rats were injected with haloperidol (0.75 mg/kg, i.p., dissolved in 0.2% lactic acid) and monitored for catalepsy 2 hr later. Two hours after haloperidol injection, rats were randomly assigned to treatment groups and then given a single administration of either VU0255035 (0.3, 1 or 3 mg/kg, i.p.), scopolamine (0.3, 1 or 3mg/kg, s.c.) or vehicle.

Thirty minutes later (2.5 hr post haloperidol injection), catalepsy was assessed using a horizontal bar placed 6 cm from the testing surface. The forepaws of each rat were placed gently on the bar with the body positioned at an angle of ~45° to the testing surface. The latency for the rat to remove one or both forepaws from the bar was measured manually using a stop watch with cut-off time of 60 sec. Any rat that remained on the bar between 45-60 seconds was considered to be cataleptic.

Statistics. Group data were presented as mean ± SEM. The data from electrophysiological studies were compared using the t-test, and data from behavioral studies were compared using Dunnett's test. $p < 0.05$ was considered to be statistically significant.

RESULTS

Activation of M1 mAChR has excitatory effects in striatal MSNs. Striatal MSNs were identified based on their electrophysiological characteristics described previously, including a hyperpolarized resting membrane potential, inward rectification and delayed action potential discharges in response to near rheobase current injection (Fig.1A). Using novel compounds that are highly selective for M1 subtype, we studied the involvement of M1 mAChRs in modulation of membrane excitability of MSNs by monitoring the changes in membrane potential and the number of spike discharges in response to a near threshold depolarization current pulse in current clamp condition. The amplitude of the depolarization current pulse was adjusted such that only 1-3 spikes were elicited prior to any pharmacological manipulation. Bath application of muscarinc agonist carbachol (CCh) increased excitability of MSNs in a concentration-dependent manner. As illustrated in Fig. 1B, C and F, 0.5 uM and 5 uM depolarized the membrane potential (ΔVm) by 1.27 ± 0.42mV (n = 6) and 5.13 ± 0.95 mV (n = 9), respectively, and increased the number of spike discharge in response to the depolarization current pulse (change in # of spikes/pulse) by 2.17 ± 0.60 (n = 6) and 13.0 ± 1.3 (n = 9), respectively. The mAChR antagonist VU0255035, which has been shown to be highly selective for M1 relative to the M2-M5 subtypes (Sheffler, et al., 2009), blocked the CCh-induced excitation of MSNs. As shown in Fig. 1D and F, in the presence of 5 uM VU0255035, CCh (5 uM) only slightly depolarized the membrane potential (ΔVm = 1.70 ± 0.38 mV, n = 7, compared to application of 5uM CCh alone with ΔVm = 5.13 ± 0.95 mV, n = 9, $p < 0.01$) and marginally increased the number of spikes/pulse (3.43 ± 0.72, n = 7, compared to 13.0 ± 1.3 with 5 uM CCh alone, n = 9, $p < 0.0001$). Furthermore, the novel selective M1 PAM BQCA (Shirey, et al., 2009) potentiated submaximal concentration of CCh induced excitation in MSNs. As shown in Fig. 1E and F, in

the presence of 3uM BQCA, CCh (0.5 uM) caused a robust depolarization of membrane potential ($\Delta Vm = 4.86 \pm 1.38$ mV, n = 8, compared to 1.27 ± 0.42 mV with 0.5 uM CCh alone, n = 6, $p < 0.05$) and a marked increase in the number of spikes/pulse (11.5 ± 1.90, n = 8, compared to 2.17 ± 0.60 with 0.5 uM CCh alone, n = 6, $p < 0.005$). The results suggest that muscarinc excitation of MSNs is mediated by M1 mAChR subtype. It is worth noting that BQCA alone also caused slight excitation in MSNs (Fig. 1E and F), the number of spikes/pulse increased by 1.75 ± 0.53 ($p < 0.05$, n = 8) although the change in Vm, 1.21 ± 0.54 mV, did not reach statistically significant level ($p = 0.06$, n = 8), which suggested that BQCA might potentiate endogenous ambient acetylcholine action on M1 receptors in MSNs.

M1 mAChRs are not involved in cholinergic excitation in STN and SNr neurons. Both glutamatergic STN neurons and GABAergic SNr neurons displayed spontaneous rhythmic firing. We used cell-attached recordings to access the effect of CCh on the firing frequency of STN and SNr neurons and determined the effect of M1 antagonist VU0255035 on the CCh-induced responses. As shown in Fig. 2A-D, bath application of CCh increased spontaneous firing of STN and SNr neurons in a concentration dependent manner. CCh at concentrations of 0.3uM, 1 uM and 10 uM increased the firing rate of STN neurons to $137.8 \pm 7.8\%$ (n = 7), $163.0 \pm 7.6\%$ (n = 8) and $219.0 \pm 7.0\%$ (n = 7), respectively (Fig. 2A and C). For SNr neurons, CCh at concentrations of 3 uM, 10 uM and 30 uM increased the firing rate to $124.1 \pm 2.1\%$ (n = 3), $153.7 \pm 14.0\%$ (n = 5) and 216.3 ± 23.5 % (n = 4), respectively (Fig. 2B and D). Selective M1 antagonist VU0255035 failed to block the excitation induced by submaximal concentration of CCh in STN and SNr neurons (Fig. 2E-H). In the presence of 5 uM VU0255035, 1 uM CCh increased the firing rate to $178.6 \pm 17.6\%$ of the control value in STN neurons (n = 6), compared with $163.0 \pm 7.6\%$ in the absence of VU0255035 (n = 8, $p > 0.1$, Fig. 2E and G). For SNr

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neurons, 10 uM CCh increased the firing rate to 151.1 ± 8.2% of the control value when 5 uM VU0255035 was co-applied (n = 5), compared with 153.7± 9.8% in the absence of VU0255035 (n = 5, p > 0.5; Fig. 2F and H). These data suggest that M1 activation does not play a major role in cholinergic modulation of neuronal excitability in these two nuclei. We noted that higher concentrations of CCh were needed in SNr neurons than STN neurons and MSNs to induce increased firing rates. This probably reflects a combination of different mAChR subtypes that mediate CCh responses in SNr neurons, STN neurons and MSNs, and/or differences in receptor reserve in these neuronal populations.

M1 mAChRs are partially involved in cholinergic inhibition of GABAergic synaptic transmission in STN neurons but not involved in cholinergic inhibition of excitatory transmission in SNr neurons. Whole-cell voltage clamp recordings were used to determine the involvement of M1 mAChR subtype in cholinergic modulation of GABAergic transmission in STN neurons and glutamatergic transmission in SNr neurons. Using CCh, M1 antagonist VU0255035 and M1 PAM BQCA, we found that mAChR activation inhibits IPSCs in STN neurons and this cholinergic inhibition is mediated in part by M1 receptors. As illustrated in Fig. 3, application of CCh at 0.3 uM, 1 uM and 10 uM reduced IPSC amplitude by 13.3 ± 6.1 % (n = 5), 42.6 ± 2.6 % (n = 6), and 53.9 ± 3.5 % (n = 7), respectively. M1 antagonist VU0255035 partially blocked the CCh-induced inhibition of IPSCs (Fig. 3). In the presence of 5 uM VU0255035, CCh (1 uM) reduced IPSC amplitude by 35.2 ± 1.8% (n = 7), compared to 42.6 ± 2.6 % with 1 uM CCh alone (n = 6, p < 0.05). To confirm the M1 involvement, we determined whether the effect of CCh could be potentiated by M1 PAM BQCA. As shown in Fig. 3, in the presence of 10 uM BQCA, 0.3 uM CCh inhibited IPSC amplitude by 36.3 ± 3.8 % (n = 6), significantly greater than the effect of 0.3 uM CCh alone (13.3 ± 6.1 %, n = 5, p < 0.05). Taken together, the results suggest

that M1 plays a role in cholinergic inhibition of GABAergic synaptic transmission in STN. However, based on the failure of saturating concentrations of VU0255035 to fully block the response to a relatively low concentration of CCh, it is likely that other mAChR subtypes are also involved.

Muscarinic receptor activation also inhibited excitatory synaptic transmission in SNr neurons. CCh at 10 uM and 100 uM reduced EPSC amplitude by 36.4 ± 4.7 % (n = 6) and 67.9 ± 2.8 % (n = 4), respectively (Fig. 4). To determine if M1 mAChRs are involved in CCh-induced reduction of EPSCs, we assessed the effect of M1 antagonist VU0355025 on the CCh action. Application of 5 uM VU0355025 failed to block 10 uM CCh-induced inhibition of EPSCs (41.5 ± 3.4%, n = 5, compared with 36.4 ± 4.7 % in the absence of VU0355025, n = 6, $p > 0.1$). This result suggests that M1 mAChRs are not involved in mAChR mediated inhibition of excitatory transmission in the SNr.

Effect of VU0355025 on rodent models of PD. To determine if M1 selective antagonist VU0255035 has any antiparkinsonian efficacy, we tested VU0255035 in two preclinical models of PD that are commonly used to test drugs for their ability to reverse parkinsonian motor deficits, reserpine-induced akinesia and haloperidol-induced catalepsy in rats, in comparison with the effects of the nonselective mAChR antagonist scopolamine. We found that VU0225035 partially reversed akinesia induced by reserpine (3mg/kg, s.c.) as evidenced by in a dose-dependent increase in locomotor activity with 3 mg/kg being most effective (Fig. 5A, n = 8 per group, * $p < 0.05$, compared to vehicle group, Dunnett's test). In addition, VU0255035 reduced haloperidol (0.75 mg/kg, i.p.)-induced catalepsy, as indicated by a decrease in the latency to withdrawal of the forepaws when placed on a horizontal grid (Fig. 5C). The cutoff for termination of the experiment was 60 sec. As shown in Fig. 5C, the withdrawal latency

significantly reduced for 0.3 mg/kg and 1 mg/kg dosing groups, compared to the vehicle group ($n = 8$ per group, * $p < 0.05$, Dunnett's test). In comparison, we tested the nonselective mAChR antagonist scopolamine in these two animal models and found that scopolamine had more robust effects in reversing reserpine (3 mg/kg, s.c.)-induced akinesia as well as haloperidol (0.75 mg/kg, i.p.)-induced catalepsy (Fig. 5B and D). These results suggest that additional mAChR subtype(s) other than M1 are also participated in antiparkinsonian actions of muscarinic antagonists.

DISCUSSION

Hypercholinergic tone in the basal ganglia has long been proposed to be associated with motor deficits of PD. Most previous studies have focused on cholinergic modulation of striatal function as the primary site of action of cholinergic modulation of motor function (Barbeau, 1962;Pisani, et al., 2007). However, previous animal and human studies provide strong evidence that changes in activity in the indirect pathway of the basal ganglia motor circuit can contribute to motor dysfunction in Parkinson's patients. For instance, STN neurons are hyperactive in Parkinson's patients and display increased incidence of burst activity (Delong and Wichmann, 2007). The glutamatergic STN neurons send a primary excitatory projection to GABAergic neurons in the SNr, and this contributes to over excitation of SNr GABAergic neurons in PD patients (Delong and Wichmann, 2007). Interestingly, inhibition of activity of either STN or SNr neurons using surgical lesions or deep brain stimulation can reduce parkinsonian motor disability (Bergman, et al., 1990;Delong and Wichmann, 2007;Starr, et al., 1998).

The actions of mAChR activation at each level of the basal ganglia assessed in the present studies could contribute to parkinsonian motor impairments. Likewise, the antiparkinsonian efficacy of mAChR antagonists may involve actions at the level of the striatum as well as the STN and SNr. Muscarinic agonists increase activity of STN neurons (Feger, et al., 1979;Flores, et al., 1996) and microinjection of mAChR antagonists in the STN alleviates motor deficits in an animal model of PD (Hernandez-Lopez, et al., 1996). Similarly, mAChRs are present on SNr neurons (Cross and Waddington, 1980), which receive cholinergic innervations from the PPN (Lavoie and Parent, 1994). Activity of SNr neurons increases following electrophoresis of ACh in vivo (Collingridge and Davies, 1981), and microinjection of muscarinic agonists in the SNr induces parkionsonian-like motor deficits that are blocked by the

muscarinic antagonist scopolamine (De Montis, et al., 1979;Turski, et al., 1984). These previous studies are consistent with the present findings that CCh has excitatory effects and reduces inhibitory transmission in STN and SNr neurons and suggest that, in addition to the striatum, the STN and the SNr could also be target for the actions of anticholinergic drugs in reducing motor symptom of PD.

The present studies employed highly selective M1 ligands, M1 antagonist VU0255035 and M1 PAM BQCA. The selectivity of these two compounds has been rigorously evaluated in our previous studies (Shirley et al. 2009; Sheffler et al. 2009). For instance, BQCA has been shown to lack effect on responses to activation in cell lines expressing M2 – M5, lack effect in cellular backgrounds lacking M1 expression and in broad profiling for activity at other GPCRs and other CNS targets, and lack effect in M1 knockout (KO) mice (Shirley et al. 2009). For VU0255035, a similar series of studies have been performed to rigorously evaluate activity at all mAChR subtypes as well as multiple other potential targets. We found that VU0255035 has 75-fold or greater selectivity in antagonizing CCh-induced responses in cells expressing M1 relative to those expressing M2, M3, M4 or M5 (Sheffler et al. 2009). We also found that CCh has no effect on M1-mediated responses in M1 KO mice that are blocked by VU0255035, including evaluation of responses to VU0255035 in hippocampal and cortical neurons and the present studies the muscarinic excitation of MSNs that have been shown to be absent in M1 KO mice (Shirley et al. 2009; Sheffler et al. 2009; Shen et al. 2005).

The present finding that the M1 mAChR subtype is responsible for excitatory effects of mAChR activation on striatal MSNs but not neurons in the STN and SNr, suggests that inhibition of multiple mAChR subtypes, including M1, is likely important for the overall effects of mAChR antagonists on basal ganglia function and motor activity. Importantly, while M1 is not involved

in direct excitatory effects on STN and SNr neurons, our studies suggest that M1 does participate in the cholinergic depression of IPSCs in STN neurons (Shen and Johnson, 2000). The primary source of GABAergic inhibitory inputs to STN neurons are from the external segment of globus pallidus (van der Kooy, et al., 1981). In parkinsonism, GABAergic pallidosubthalamic transmission is reduced (Delong and Wichmann, 2007), which would contribute, at least in part, to the hyperactivity of STN neurons. Thus, M1 activation and disinhibition of STN could further increases the activity of STN neurons and exacerbate the parkinsonian motor deficits.

Interestingly, some of the muscarinic antagonists used in treatment of PD have been purported to have an "M1-like" preferential pharmacological profile leading some investigators to postulate that selective M1 blockade would be an effective strategy for treatment of PD (Giachetti, et al., 1986;Tien and Wallace, 1985). However, these clinically-available M1-preferring drugs are not sufficiently selective to ascribe their effects to M1 and our findings that M1 is involved in some but not all actions of mAChR activation in the basal ganglia suggests that blockade may not have the same efficacy as can be achieved with non-selective mAChR antagonists. Consistent with this, we found that the selective M1 antagonist VU0255035 has a modest effect in reducing parkinsonian motor disability in rodent models but does not achieve the efficacy that can be seen with the non-selective mAChR antagonist scopolamine. Importantly, the dose of VU0255035 used in these studies was previously shown to achieve high brain levels and maximal inhibition of M1 activation in the CNS (Sheffler et al., 2009). These results raise a need for developing novel highly selective ligands for the mAChR subtype(s) to allow development of a complete understanding of the roles of specific subtypes in regulating different aspects of basal ganglia function.

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While these studies do not suggest that M1 antagonists could provide sufficient efficacy to be used as stand-alone therapy for PD, it is possible that the modest efficacy achieved could be useful to augment other therapeutic approaches in PD patients. Also, it is possible that M1 antagonists could prove useful in some other basal ganglia disorders that have been shown to be effectively treated by non-selective mAChR antagonists, such as dystonia. It will be important to explore this possibility in future studies.

One concern associated with the use of M1 antagonists as therapeutic agents is possible adverse effects on cognitive functions, as nonselective mAChR antagonists have long been known to cause severe side effects such as impairment of learning and memory (Drachman and Leavitt, 1974). However, we recently reported that VU0255035 does not have any effects on acquisition of contextual fear conditioning in rats, a model of hippocampal-dependent learning, at doses that block pilocarpine-induced seizures (Sheffler et al, 2009). In contrast, the nonselective mAChR antagonist scopolamine causes a dose-dependent disruption in the acquisition of this conditioning response (Sheffler et al, 2009). These results are consistent with the previous reports using M1 mAChR KO mice, which demonstrate that mice lacking M1 mAChRs show no impairments in certain forms of hippocampus-dependent learning (Anagnostaras, et al., 2003;Miyakawa, et al., 2001), suggesting that M1 receptors play subtle roles in learning and memory. Taken together, these results suggest that selective M1 antagonists could be used as potential therapeutic agents in treatment of brain disorders such as PD and other movement disorders with limited side effects on cognitive functions.

In previous studies, the mechanisms underlying the overall excitatory effects of mAChR activation on MSN neurons have been evaluated in detail. For instance, mAChR activation inhibit various K^+ currents including KCNQ channel currents in MSNs and this effect was lost in

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M1 KO mice (Shen, et al., 2005). Another type of K^+ channels that regulate MSN excitability and are also modulated by mAChR signaling is Kir2 K^+ channels (Galarraga, et al., 1999;Shen, et al., 2007). Unlike KCNQ channels, which are uniformly inhibited by mAChR activation in MSNs reside on both direct and indirect pathways (Shen et al., 2005), Kir2 currents are differentially inhibited by muscarinic activation in MSNs depending on their projection targets; specifically, it preferentially reduces Kir2 channel currents in striatopallidal MSNs but has little effect in striatonigra MSNs (Shen, et al., 2005). In the present studies, we did not note large variation or bimodal distribution of CCh-induced responses and BQCA potentiation of CCh responses across MSNs. This suggests that the VU0255035 and BQCA sensitive, CCh-induced excitation in MSNs is likely to be primarily mediated by inhibition of KCNQ channels. However, future studies will be needed to determine whether M1 is responsible for modulation of other specific ion channels in these cells and it is likely that effects of M1 receptor activation on KCNQ channels along with Kir2 channels and possibly other ion channels contribute to the cholinergic modulation of overall excitability of MSNs.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Xiang, Thompson, Jones and Conn

Conducted experiments: Xiang and Thompson

Contributed new reagents or analytic tools: Lindsley and Conn

Performed data analysis: Xiang, and Thompson

Wrote or contributed to the writing of the manuscript: Xiang, Thompson, Jones and Conn

REFERENCES

Anagnostaras SG, Murphy GG, Hamilton SE, Mitchell SL, Rahnama NP, Nathanson NM and Silva AJ (2003) Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice. *Nat Neurosci* **6**:51-58.

Barbeau A (1962) The pathogenesis of Parkinson's disease: a new hypothesis. *Can Med Assoc J* **87**:802-807.

Bergman H, Wichmann T and Delong MR (1990) Reversal of experimental parkinsonism by lesions of the subthalamic nucleus. *Science* **249**:1436-1438.

Beurrier C, Bioulac B, Congar P and Hammond C (1999) Subthalamic nucleus neurons switch from single-spike activity to burst- firing mode. *Journal of Neuroscience* **19**:599-609.

Bevan MD and Bolam JP (1995) Cholinergic, GABAergic, and glutamate-enriched inputs from the mesopontine tegmentum to the subthalamic nucleus in the rat. *J Neurosci* **15**:7105-7120.

Chen JJ and Swope DM (2007) Pharmacotherapy for Parkinson's disease. *Pharmacotherapy* **27**:161S-173S.

Collingridge GL and Davies J (1981) The influence of striatal stimulation and putative neurotransmitters on identified neurones in the rat substantia nigra. *Brain Res* **212**:345-359.

Cross AJ and Waddington JL (1980) [3H] Quinuclidinyl benzylate and [3H] GABA receptor binding in rat substantia nigra after 6-hydroxy-dopamine lesions. *Neurosci Lett* **17**:271-275.

Davie CA (2008) A review of Parkinson's disease. *Br Med Bull* **86**:109-127.

De Montis GM, Olianas MC, Serra G, Tagliamonte A and Scheel-Kruger J (1979) Evidence that a nigral gabaergic--cholinergic balance controls posture. *Eur J Pharmacol* **53**:181-190.

Delong MR and Wichmann T (2007) Circuits and circuit disorders of the basal ganglia. *Arch Neurol* **64**:20-24.

Drachman DA and Leavitt J (1974) Human memory and the cholinergic system. A relationship to aging? *Arch Neurol* **30**:113-121.

Feger J, Hammond C and Rouzaire-Dubois B (1979) Pharmacological properties of acetylcholine-induced excitation of subthalamic nucleus neurones. *British Journal of Pharmacology* **65**:511-515.

Felder CC, Bymaster FP, Ward J and DeLapp N (2000) Therapeutic opportunities for muscarinic receptors in the central nervous system. *J Med Chem* **43**:4333-4353.

Flores G, Hernandez S, Rosales MG, Sierra A, Martines-Fong D, Flores-Hernandez J and Aceves J (1996) M3 muscarinic receptors mediate cholinergic excitation of the spontaneous activity of subthalamic neurons in the rat. *Neurosci Lett* **203**:203-206.

Galarraga E, Hernandez-Lopez S, Reyes A, Miranda I, Bermudez-Rattoni F, Vilchis C and Bargas J (1999) Cholinergic modulation of neostriatal output: a functional antagonism between different types of muscarinic receptors. *J Neurosci* **19**:3629-3638.

Giachetti A, Giraldo E, Ladinsky H and Montagna E (1986) Binding and functional profiles of the selective M1 muscarinic receptor antagonists trihexyphenidyl and dicyclomine. *Br J Pharmacol* **89**:83-90.

Graybiel AM (1990) Neurotransmitters and neuromodulators in the basal ganglia. *Trends Neurosci* **13**:244-254.

Hernandez-Lopez S, Flores G, Rosales MG, Sierra A, Martinez-Fong D and Aceves J (1996) Muscarinic antagonists microinjected into the subthalamic nucleus decrease muscular rigidity in reserpinized rats. *Neurosci Lett* **213**:157-160.

Johnson KA, Conn PJ and Niswender CM (2009) Glutamate receptors as therapeutic targets for Parkinson's disease. *CNS Neurol Disord Drug Targets* **8**:475-491.

Katzenschlager R, Sampaio C, Costa J and Lees A (2003) Anticholinergics for symptomatic management of Parkinson's disease. *Cochrane Database Syst Rev*CD003735.

Lavoie B and Parent A (1994a) Pedunculopontine nucleus in the squirrel monkey: cholinergic and glutamatergic projections to the substantia nigra. *J Comp Neurol* **344**:232-241.

Lavoie B and Parent A (1994b) Pedunculopontine nucleus in the squirrel monkey: projections to the basal ganglia as revealed by anterograde tract-tracing methods. *J Comp Neurol* **344**:210-231.

Lester DB, Rogers TD and Blaha CD (2010) Acetylcholine-dopamine interactions in the pathophysiology and treatment of CNS disorders. *CNS Neurosci Ther* **16**:137-162.

Miyakawa T, Yamada M, Duttaroy A and Wess J (2001) Hyperactivity and intact hippocampus-dependent learning in mice lacking the M1 muscarinic acetylcholine receptor. *J Neurosci* **21**:5239-5250.

Pisani A, Bernardi G, Ding J and Surmeier DJ (2007) Re-emergence of striatal cholinergic interneurons in movement disorders. *Trends Neurosci* **30**:545-553.

Richards CD, Shiroyama T and Kitai ST (1997) Electrophysiological and immunocytochemical characterization of GABA and dopamine neurons in the substantia nigra of the rat. *Neuroscience* **80**:545-557.

Sheffler DJ, Williams R, Bridges TM, Xiang Z, Kane AS, Byun NE, Jadhav S, Mock MM, Zheng F, Lewis LM, Jones CK, Niswender CM, Weaver CD, Lindsley CW and Conn PJ (2009) A Novel Selective Muscarinic Acetylcholine Receptor Subtype 1 (M1 mAChR) Antagonist Reduces Seizures Without Impairing Hippocampal-Dependent Learning. *Mol Pharmacol.*

Shen KZ and Johnson SW (2000) Presynaptic dopamine D2 and muscarine M3 receptors inhibit excitatory and inhibitory transmission to rat subthalamic neurones in vitro. *Journal of Physiology* **525**:331-341.

Shen W, Hamilton SE, Nathanson NM and Surmeier DJ (2005) Cholinergic suppression of KCNQ channel currents enhances excitability of striatal medium spiny neurons. *J Neurosci* **25**:7449-7458.

Shen W, Tian X, Day M, Ulrich S, Tkatch T, Nathanson NM and Surmeier DJ (2007) Cholinergic modulation of Kir2 channels selectively elevates dendritic excitability in striatopallidal neurons. *Nat Neurosci* **10**:1458-1466.

Shirey JK, Brady AE, Jones PJ, Davis AA, Bridges TM, Kennedy JP, Jadhav SB, Menon UN, Xiang Z, Watson ML, Christian EP, Doherty JJ, Quirk MC, Snyder DH, Lah JJ, Levey AI, Nicolle MM, Lindsley CW and Conn PJ (2009) A selective allosteric potentiator of the M1 muscarinic acetylcholine receptor increases activity of medial prefrontal cortical neurons and restores impairments in reversal learning. *J Neurosci* **29**:14271-14286.

Starr PA, Vitek JL and Bakay RA (1998) Deep brain stimulation for movement disorders. *Neurosurg Clin N Am* **9**:381-402.

Tien XY and Wallace LJ (1985) Trihexyphenidyl--further evidence for muscarinic receptor subclassification. *Biochem Pharmacol* **34**:588-590.

Turski L, Havemann U and Kuschinsky K (1984) Role of muscarinic cholinergic mechanisms in the substantia nigra pars reticulata in mediating muscular rigidity in rats. *Naunyn Schmiedebergs Arch Pharmacol* **327**:14-17.

van der Kooy D, Hattori T, Shannak K and Hornykiewicz O (1981) The pallido-subthalamic projection in rat: anatomical and biochemical studies. *Brain Res* **204**:253-268.

Wilson CJ (2004) Basal ganglia, in *The Synaptic Organization of the Brain* (Shepherd GM ed) pp 361-413, Oxford University Press, New York.

FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. VU0255035 blocks and BQCA potentiates the CCh induced excitation in MSNs. **A.** Electrophysiological properties of strital MSNs. Membrane potential responses to series of hyperpolarization and depolarization current steps in a typical striatal MSN recorded in current clamp condition (left), and voltage-current relationship of this MSN showing the inward rectification (right). **B-C.** Membrane potential responses to a depolarization current step in control and after application of different concentrations of CCh (C, 5 uM; D, 0.5 uM) in MSNs, showing CCh excites MSNs in a dose dependent manner. **D-E.** Membrane potential responses to a depolarization current step in control, during application of 5 uM VU0255035 and co-application of VU0255035 with 5 uM CCh in a MSN (D); and in control, during application of 3 uM BQCA and co-application of BQCA with 0.5uM CCh in another MSN (E). **F.** Bar graphs summarize the changes in membrane potential (left) and number of spikes per pulse in response to the depolarization current step (right) following application of different ligands. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, #$p < 0.0001$.

Figure 2. VU0255035 does not block the CCh-induced excitation in STN and SNr neurons. **A-B**. Sample traces (upper) and time courses of normalized spike frequency (lower) of cell-attached recordings from typical experiments, showing that CCh induces a concentration dependent increase in spike frequency of STN neurons (A) and SNr neurons (B). C-D. Bar graphs summarize the group data of CCh-induced increase in spike frequency of STN (C) and SNr neurons (D). **E-F.** Sample traces (upper) and time courses of normalized spike frequency (lower) of cell-attached recordings from representative cells, showing the effect of 1 uM or 10 uM CCh on spontaneous firing of STN neurons (E) or SNr neurons (F), respectively, in the absence (left) and presence of 5 uM VU0255035 (right). **G-H**. Summary of group data shows that VU0255035

(5uM) does not block CCh-induced increase in spike frequency of STN neurons (G) and SNr neurons (H). Low case letters in A, B, E and F indicate the time points where the sample traces are taken. Grouped data in C, D, G and H are normalized to the corresponding control values.

Figure 3. Cholinergic depression of inhibitory synaptic transmission in STN neurons is mediated in part by M1 mAChRs. **A**. Averaged IPSC traces obtained from typical experiments where the effects of the following ligands on IPSCs amplitude in STN neurons were examined, 0.3 uM CCh, 1uM CCh, 5 uM VU0225035, 5 uM VU0225035 with 1uM CCh, 10 uM BQCA, and 10 uM BQCA with 0.3 uM CCh. **B**. Bar graph summarizing partial blockade effect of VU0225035 and potentiation effect of BQCA on CCh-induced depression of IPSCs in STN neurons. * $p < 0.05$.

Figure 4. Cholinergic depression of excitatory synaptic transmission in SNr neurons is not mediated by M1 mAChRs. **A-B.** Averaged EPSC traces (upper) and time courses (lower) of the EPSC amplitude obtained from typical experiments, showing the effects of 10 uM and 100 uM CCh (A), and 5 uM VU0255035 with 10 uM CCh (B) on EPSC amplitude. **C.** Bar graph summarizes the group data showing that VU0255035 does not block the CCh-induced inhibition of EPSCs in SNr neurons.

Figure 5. Antiparkinsonian effects of VU0255035 in rodent models of PD, in comparison with the effect of scopolamine. **A, C**. Effects of VU0255035 at different concentrations on reserpine-induced akinesia (3mg/kg, s.c.) (A) and haloperidol (0.75 mg/kg, i.p.)-induced catalepsy (C) in rats (n = 8 per group), * $p < 0.05$, compared to the vehicle group. **B, D.** Effects of scopolamine at different concentrations on reserpine-induced (B) and haloperidol-induced motor deficits in rats (D) (n = 8 per group), * $p < 0.05$, compared to the vehicle group.

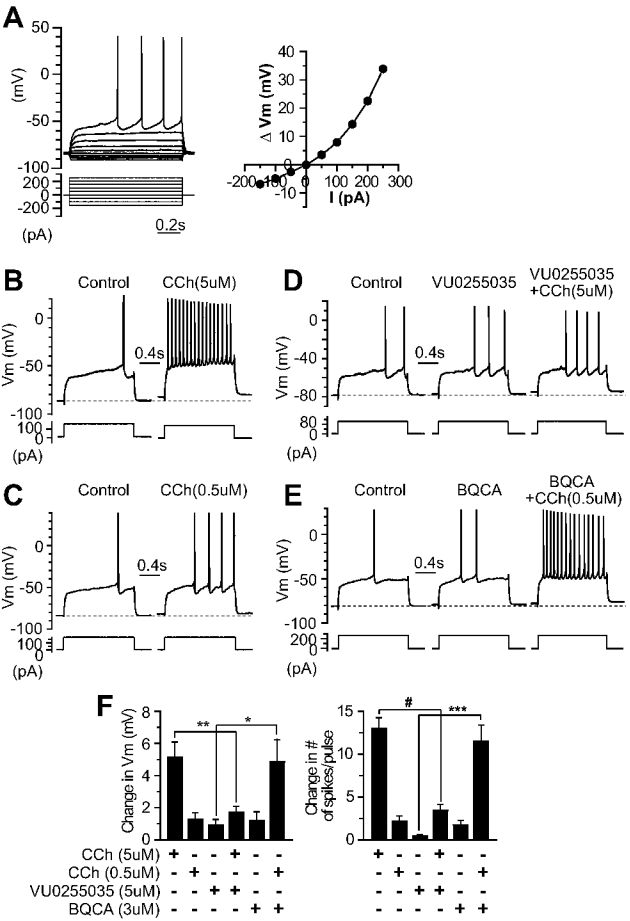
A
50
0
(mV)
-50
-100
200
0
-200
(pA)
0.2s
40
30
20
Δ Vm (mV)
10
0
-10
-200
-100
0
100
200
300
I (pA)
B
Control
CCh(5uM)
0.4s
Vm (mV)
0
-50
-100
100
0
(pA)
D
Control
VU0255035
VU0255035
+CCh(5uM)
0.4s
Vm (mV)
0
-40
-80
80
0
(pA)
C
Control
CCh(0.5uM)
0.4s
Vm (mV)
0
-50
-100
100
0
(pA)
E
Control
BQCA
BQCA
+CCh(0.5uM)
0.4s
Vm (mV)
0
-50
-100
200
0
(pA)
F
Change in Vm (mV)
8
6
4
2
0
**
*
Change in # of spikes/pulse
15
10
5
0
#
***
CCh (5uM) + - - + - -
CCh (0.5uM) - + - - - +
VU0255035 (5uM) - - + + - -
BQCA (3uM) - - - - + +

Figure1

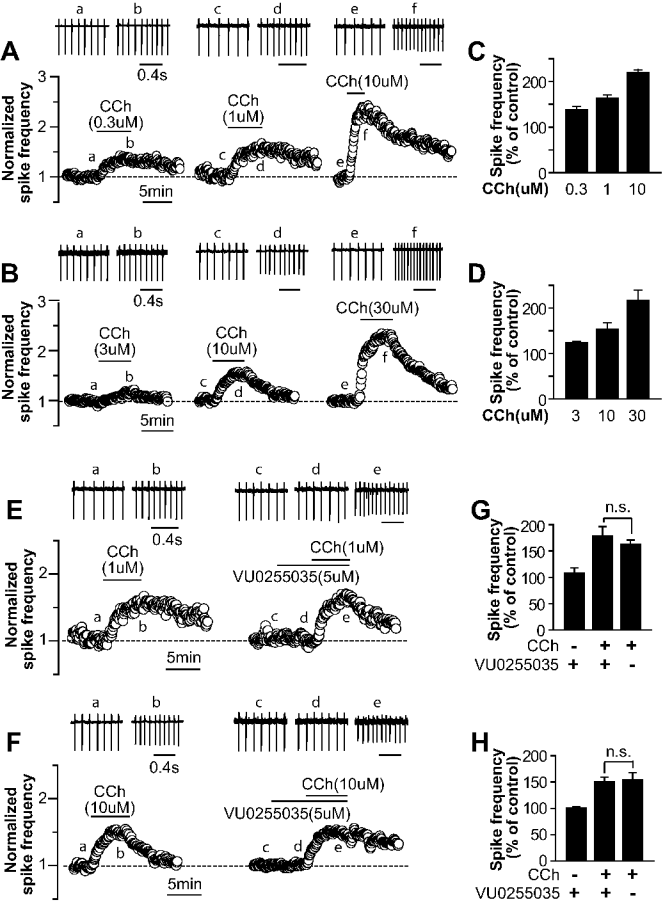
A
a
b
c
d
e
f
0.4s
CCh
(0.3uM)
CCh
(1uM)
CCh(10uM)
Normalized
spike frequency
5min
C
Spike frequency
(% of control)
CCh(uM) 0.3 1 10
B
CCh
(3uM)
CCh
(10uM)
CCh(30uM)
D
CCh(uM) 3 10 30
E
CCh
(1uM)
VU0255035(5uM)
G
n.s.
CCh - + +
VU0255035 + + -
F
CCh
(10uM)
H

Figure2

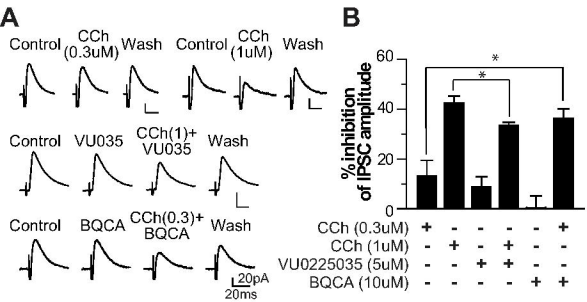
A
Control
CCh (0.3uM)
Wash
Control
CCh (1uM)
Wash
Control
VU035
CCh(1)+ VU035
Wash
Control
BQCA
CCh(0.3)+ BQCA
Wash
20pA
20ms
B
% inhibition of IPSC amplitude
60
40
20
0
*
*
CCh (0.3uM) + - - - - +
CCh (1uM) - + - + - -
VU0225035 (5uM) - - + + - -
BQCA (10uM) - - - - + +

Figure 3

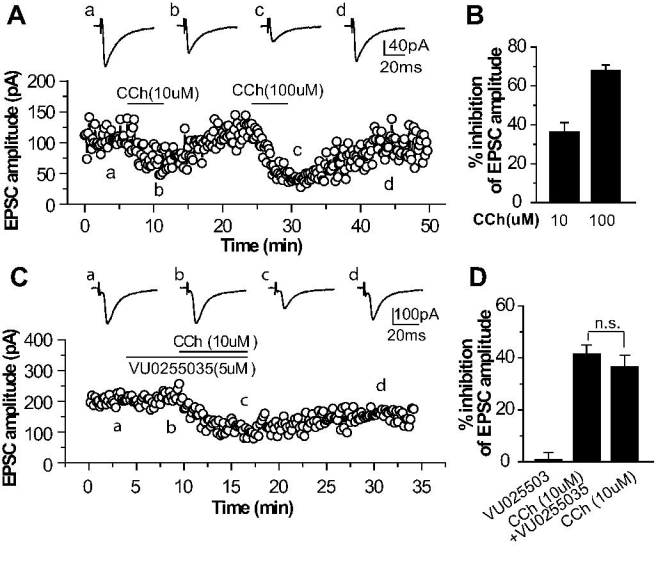
A
a
b
c
d
40pA
20ms
CCh(10uM)
CCh(100uM)
EPSC amplitude (pA)
Time (min)
B
% inhibition of EPSC amplitude
CCh(uM) 10 100
C
CCh (10uM)
VU0255035(5uM)
100pA
20ms
EPSC amplitude (pA)
Time (min)
D
% inhibition of EPSC amplitude
n.s.
VU025503
CCh (10uM) +VU0255035
CCh (10uM)

Figure 4

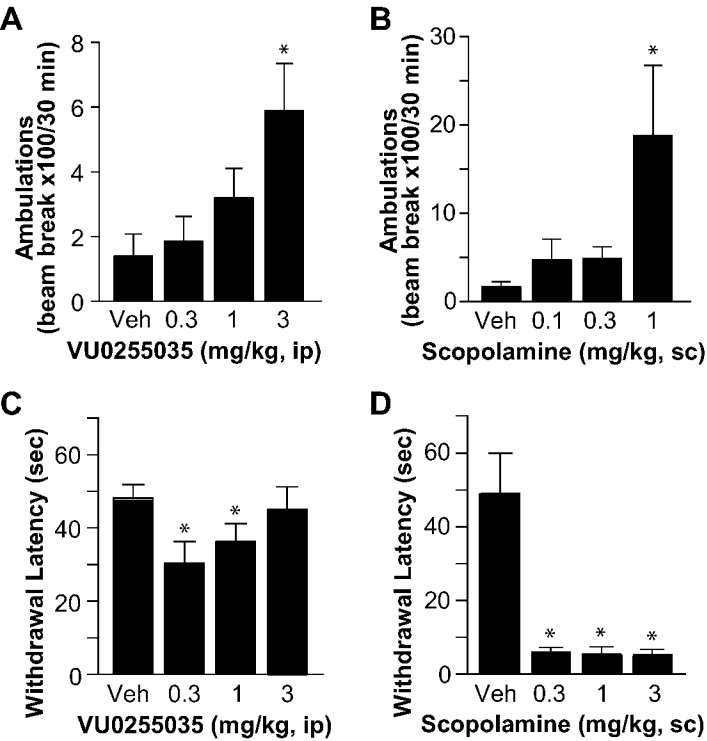
A
Ambulations (beam break x100/30 min)
8
6
4
2
0
*
Veh 0.3 1 3
VU0255035 (mg/kg, ip)
B
Ambulations (beam break x100/30 min)
30
20
10
0
*
Veh 0.1 0.3 1
Scopolamine (mg/kg, sc)
C
Withdrawal Latency (sec)
60
40
20
0
* *
Veh 0.3 1 3
VU0255035 (mg/kg, ip)
D
Withdrawal Latency (sec)
60
40
20
0
* * *
Veh 0.3 1 3
Scopolamine (mg/kg, sc)

Figure 5