M5 Muscarinic Receptors Are Required for Prolonged Accumbal Dopamine Release after Electrical Stimulation of the Pons in Mice

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Midbrain dopamine neurons are activated directly by cholinergic agonists or by stimulation of the cholinergic neurons in the laterodorsal tegmental nucleus (LDT) of the pons in rats. In urethane-anesthetized mice, electrical stimulation of the LDT resulted in a rapid, stimulus-time-locked increase in dopamine release in the nucleus accumbens (NAc), followed several minutes later by a prolonged increase in dopamine release. In mutant mice with truncated M5 receptors, the prolonged phase of dopamine release was absent, but the initial, rapid phase of dopamine release was fully observed. We conclude that M5 muscarinic receptors on midbrain dopamine neurons mediate a prolonged facilitation of dopamine release in the NAc. These results imply that M5 muscarinic receptors play an important role in motivational behaviors driven by dopamine activity in the accumbens.

Key words: laterodorsal tegmental nucleus; scopolamine; acetylcholine; nucleus accumbens; reward; gene-targeted mice

The rewarding and stimulating effects of drugs of abuse, such as amphetamine and cocaine, result from increased release of dopamine from terminals in the basal forebrain (Blaha and Phillips, 1996). The rewarding effects of nicotine and hypothalamic brain stimulation occur primarily via cholinergic activation of dopamine-containing neurons in the midbrain ventral tegmental area (VTA) that project to limbic targets such as the nucleus accumbens (NAc) (Corrigall et al., 1994; Yeomans and Baptista, 1997). Acetylcholine-containing neurons in the laterodorsal tegmental nucleus (LDT) of the pons are a principal source of this excitatory cholinergic input to VTA dopaminergic neurons (Clements and Grant, 1990; Oakman et al., 1995; Blaha et al., 1996a).

Recently, we characterized the role of the LDT in NAc dopamine activity using repetitive chronoamperometry with dopamine-selective electrodes to measure dopamine efflux in the NAc of urethane-anesthetized rats. Physiologically relevant electrical stimulation of the LDT evoked a three-component pattern of change in dopamine efflux in the NAc (Forster and Blaha, 2000). The first, the rapid excitatory component, was time-locked to the stimulus and dependent on activation of ionotropic glutamate receptors and nicotinic acetylcholine receptors (AChRs) located in the VTA. The second, the inhibitory component, was dependent on activation of muscarinic (M2-like) autoreceptors located in the LDT. The third, the prolonged excitatory component, was dependent on activation of muscarinic AChRs (mAChRs) located in the VTA.

M5 mRNA is the only mAChR subtype marker to be localized definitively to the cell bodies of dopaminergic neurons in the VTA (Vilaro et al., 1990; Weiner et al., 1990; Reever et al., 1997). M5 mRNA loss in the VTA is associated with 6-hydroxydopamine-induced dopaminergic cell death, implying that these receptors are produced by dopamine cells in the midbrain (Vilaro et al., 1990; Reever et al., 1997). Little is known about the role of the M5 mAChR subtype in brain function, because of the lack of selective M5 receptor agonists or antagonists. However, Yeomans et al. (2000) used antisense oligonucleotide for the M5 mAChR to show that these receptors in the VTA are important for brain-stimulation reward in rats. This implies that these VTA mAChRs mediate dopamine-related reward, because the activation of dopamine neurons in the VTA is considered an important component of brain-stimulation reward (Blaha and Phillips, 1990).

M5 mutant mice are characterized by a deletion in the third intracellular loop of the M5 mAChR (Takeuchi et al., 2001). These mice show higher spontaneous water intake, preferences for fluid over food in food-deprived conditions, and decreased salivation in response to pilocarpine compared with wild-type controls, implicating the M5 mAChR in peripheral secretory processes. These mice also provide an excellent opportunity to study the role of the M5 mAChR in brain functioning. In this study, we aim to identify the importance of the M5 mAChR subtype in mediating accumbal dopamine activity by measuring

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the pattern of dopamine efflux in the NAc elicited by LDT electrical stimulation in wild-type and M5 mutant mice.

MATERIALS AND METHODS

Animals. Seven wild-type male mice (strain CD1 × 129SvJ) were obtained from the Adelaide Resources Center (South Australia, Australia). Eight male mice (CD1 × 129SvJ) were obtained from the University of Toronto (Toronto, Ontario, Canada); these mice were homozygote mutants for the M5 mAChR gene, characterized by a deletion in the third intracellular loop of the M5 mAChR (Takeuchi et al., 2001). All mice were matched for size and age, ranging between 35 and 46 gm and between 5 and 6 months of age. Both wild-type and mutant mice were acclimatized for 1 month after transportation before being used in the following experiment.

Surgery. Mice were anesthetized with urethane (1.5 gm/kg, i.p.; ICN Biochemicals Inc., Sydney, New South Wales, Australia) and supplemented 30 min later (0.5 gm/kg, i.p.). Each mouse was mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) within a mouse head-holder adaptor (Stoelting, Kiel, WI), ensuring the skull was flat. Body temperature was maintained at 36 ± 0.5°C with a temperature-regulated heating pad (TC-831; CWE Inc., New York, NY). A single, concentric, bipolar stimulating electrode (SNE-100; Rhodes Medical Co., Woodland Hills, CA) was implanted into the left LDT of each mouse [coordinates: anteroposterior (AP), −1.72 mm from lambda; mediolateral (ML), +0.25 mm; and dorsoventral (DV), −3.5 mm from dura; adjusted from Franklin and Paxinos (1997) to allow for strain differences]. A single stearate-modified graphite-paste electrochemical recording electrode (Blaha and Jung, 1991), which permitted *in vivo* measurement of changes in dopamine oxidation current without interference from other oxidizable compounds in brain extracellular fluid (Blaha and Phillips, 1996; Blaha et al., 1996b), was implanted into the left NAc of each mouse [coordinates: AP, +1.5 mm from bregma; ML, +1.5 mm; and DV, −4.8 mm from dura; adjusted from Franklin and Paxinos (1997)]. An Ag–AgCl reference and stainless-steel auxiliary electrode combination were placed in contact with contralateral cortical tissue 3 mm posterior from bregma.

Electrochemical recordings. After implantation of all electrodes, the operating characteristics of the electrochemical recording electrodes were evaluated *in vivo* before each experiment. This consisted of several voltametric sweeps recorded within the NAc (triangular wave potentials applied from −0.15 to +0.45 V vs Ag–AgCl; ramp rate 0.1 V/sec) (Blaha and Jung, 1991). After confirmation of the viability of the recording electrode for dopamine, repetitive chronoamperometric measurements of oxidation current using an electrometer (Echempro; GMA Technologies, Inc., Vancouver, Canada) were made by applying a potential pulse from −0.15 to +0.3 V vs Ag–AgCl to the recording electrode for 1 sec at 30 sec intervals and monitoring the oxidation current at the end of each 1 sec pulse (Blaha and Phillips, 1996). After at least 30 min of baseline chronoamperometric recordings, LDT stimulation was applied and changes in dopamine oxidation currents were monitored for a 60 min period.

Electrical stimulation. A series of cathodal monophasic current (400 μA) pulses (0.5 msec duration) were delivered to the concentric, bipolar stimulating electrode implanted in the LDT via an optical isolator and programmable pulse generator (Iso-Flex/Master-8; A.M.P.I., Jerusalem, Israel). Each electrical stimulation of the LDT consisted of a 1 sec, 35 Hz train of pulses (1 sec intertrain interval) applied over a 60 sec period (total number of pulses, 1050). These parameters were designed to mimic spontaneous firing patterns of the LDT in awake, naturally aroused animals (Steriade et al., 1990) and have been shown (at 800 μA) to elicit a three-component phasic pattern of change in dopamine efflux in the NAc of urethane-anesthetized rats (Forster and Blaha, 2000).

Systemic scopolamine injections. After observing at least two comparable chronoamperometric responses to LDT stimulation (each monitored for a 60 min period), mice were injected with the nonselective mAChR antagonist scopolamine hydrobromide (5 mg/kg, i.p.; Sigma, Sydney, New South Wales, Australia). In urethane-anesthetized rats, this dose blocks mAChR-mediated dopamine efflux in the NAc elicited by LDT stimulation (Forster and Blaha, 2000). The LDT was then stimulated 30 min after injection, because the antagonist effects of systemic scopolamine peak within 40–90 min after injection (Chapman et al., 1997), and stimulated every 60 min thereafter until the LDT-stimulated pattern of NAc dopamine efflux was comparable with the prescopolamine response.

Data analysis. Prestimulation baseline chronoamperometric currents were normalized to zero current values, with stimulated changes in the baseline oxidation current signal presented as absolute changes (increases as positive and decreases as negative) in dopamine oxidation current. The maximal LDT-elicited dopamine oxidation current change for each of the three components of the triphasic response was obtained for each animal before and after scopolamine injection for both wild-type and M5 mutant mice. The mean duration (in minutes) and peak current (in nanoamperes) for each of the three components of the LDT-elicited triphasic response were compared before and after scopolamine (two-tailed paired *t* tests) and between wild-type and M5 mutant mice responses (two-tailed unpaired *t* tests), with the exception of the duration of the third component, which was absent in M5 mutant and scopolamine-treated mice.

To estimate the kinetics of the rise and the decay of the third component, the contributions of the first and second components were removed from the data by subtracting the LDT-elicited mean changes in dopamine oxidation current recorded from M5 mutant mice (see Fig. 3*A*) from those recorded from wild-type mice (see Fig. 2*A*) in the absence of scopolamine. The resultant temporal profile was then divided into two functions (rise and decay) at the asymptote of the curve (23 min after stimulation). Each function was plotted on a semilogarithmic scale, and a linear regression analysis was performed to assess whether each function described a linear process. The half-life (in minutes) for each curve function was then measured at 50% of the maximum dopamine oxidation current.

Histology. On completion of data collection, an iron deposit was made in the LDT stimulation site by passing DC (100 μA for 5 sec) through the stimulating electrode. Mice were then killed with a 0.25 ml intracardial infusion of urethane (0.345 gm/ml). Brains were removed, immersed overnight in 10% buffered formalin containing 0.1% potassium ferricyanide, and then stored in 30% sucrose/10% formalin solution until sectioning. After fixation, 30 μm coronal sections were cut at −30°C, with a Prussian blue spot (resulting from a redox reaction of ferricyanide) marking the stimulation site. The placements of the electrochemical recording electrodes and stimulating electrodes were determined under a light microscope and recorded on representative coronal diagrams (Franklin and Paxinos, 1997).

RESULTS

Stereotaxic placements of electrodes

The locations of electrochemical recording and electrical stimulating electrode tips for all mice are shown in Figure 1. The placement of the active surfaces of the recording electrodes was confined within the core of the NAc (Fig. 1*A*) in the range of 0.98–1.18 mm anterior to bregma and 4.20–4.75 mm ventral to dura for wild-type mice and 0.98–1.34 mm anterior to bregma and 4.25–4.85 mm ventral to dura for M5 mutant mice. Stimulating electrodes were accurately positioned within the dorsal aspect of the posterior LDT (Fig. 1*B*) in the range of 5.20–5.52 mm posterior to bregma and 3.25–3.60 mm ventral to dura for wild-type mice and 5.40–5.52 mm posterior to bregma and 3.40–3.65 mm ventral to dura for M5 mutant mice. The placements of both stimulation and recording electrodes in the present experiment were similar in relation to the NAc and LDT to those used previously in rats (Forster and Blaha, 2000).

LDT-evoked dopamine efflux in the NAc

Electrical stimulation of the LDT in urethane-anesthetized wild-type mice elicited a three-component pattern of change in dopamine oxidation current (dopamine efflux) in the NAc (Fig. 2*A*). These responses were similar in form to those recorded from urethane-anesthetized rats (Forster and Blaha, 2000). The mean (±SEM) peak magnitude and the duration of change in dopamine oxidation currents (in nanoamperes) for each component, averaged across all wild-type mice, are shown in Table 1. Specifically, stimulation of the LDT evoked an initial, stimulus-time-locked increase in the dopamine signal above baseline levels (first component). At ~3 min after stimulation, the dopamine signal decreased to below prestimulation baseline levels (second com-

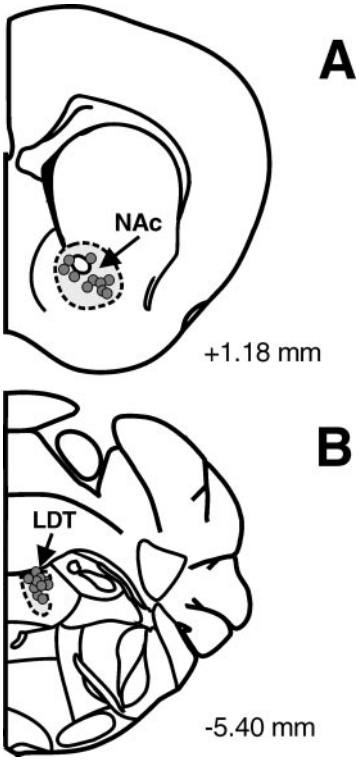

Figure 1. Representative coronal sections of the mouse brain showing the composite placements of electrochemical electrodes in the NAc (*A*) ($n = 15$) and stimulating electrodes in the LDT (*B*) ($n = 15$). *Filled circles* represent recording electrode and stimulating electrode tips. Sections were adapted from the atlas of Franklin and Paxinos (1997) and *numbers* correspond to millimeters from bregma.

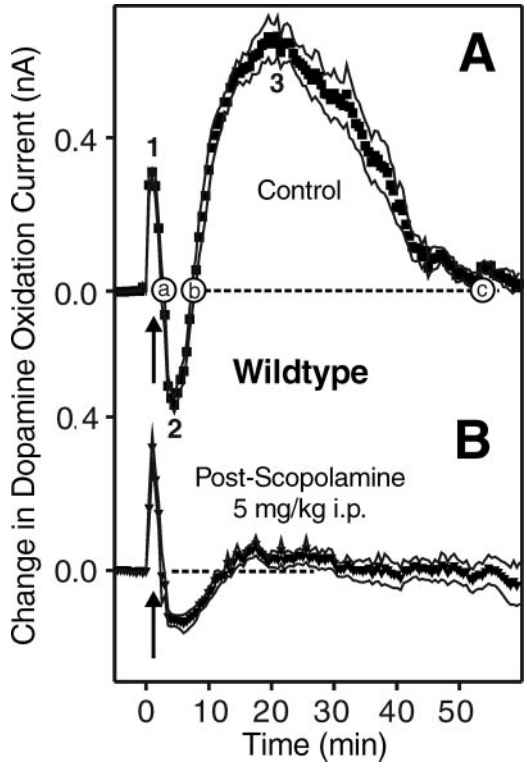

Figure 2. The effect of electrical stimulation applied to the LDT on dopamine oxidation currents recorded in the NAc before (*A*) and after (*B*) systemic injection of scopolamine (5 mg/kg, i.p.) in wild-type mice. *Numbers* in *A* refer to the peak response for each of the three components of the triphasic response; *lowercase letters* denote the duration of each component, with time of stimulus onset (zero) to *point a*, *point a* to *b*, and *point b* to *c* indicating the duration of components 1, 2, and 3, respectively. *Arrows* indicate the time of stimulus termination. *Points* represent the mean changes in the chronoamperometric responses ($n = 7$); *solid lines* represent the SEM; *dotted lines* correspond to prestimulation baseline levels.

ponent). At ~8 min after stimulation, this suppression in the dopamine signal was followed by a facilitatory third component that returned to baseline 42 min after stimulation (mean duration of the third component, 35.64 ± 2.66 min). This third component was twice as large in magnitude (Table 1) and >14 times longer than the profile of the first component.

In contrast, LDT stimulation in M5 mutant mice elicited only a two-component pattern of dopamine efflux in the NAc. The third, prolonged component was clearly absent (Fig. 3*A*). The magnitude and duration of the first excitatory component elicited from the M5 mutant mice did not differ significantly ($p = 0.44$ and $p = 0.57$, respectively) from the response recorded from wild-type mice (Table 1). The second inhibitory component elicited from M5 mutant mice reached a peak magnitude that was comparable ($p = 0.27$) with that obtained in the wild-type mice; however, this component was significantly longer ($p < 0.01$) in duration compared with that recorded from wild-type mice.

Effects of systemic mAChR blockade on LDT-evoked dopamine efflux in the NAc

Systemic administration of the mAChR antagonist scopolamine (5 mg/kg) to wild-type mice 30 min before LDT stimulation diminished the LDT-evoked second and third components in the NAc dopamine signal but did not alter the first component (magnitude, $p = 0.80$; duration, $p = 0.29$) (Fig. 2*B*). Specifically, the magnitude of the second component was significantly ($p < 0.01$) attenuated, whereas the duration of this component increased significantly ($p < 0.01$) compared with prescopolamine stimulated responses (Table 1). Pretreatment with scopolamine completely abolished the third component of LDT-elicited dopamine efflux in the NAc of wild-type mice ($p < 0.01$) (Fig. 2*B*, Table 1).

Systemic administration of scopolamine (5 mg/kg) to M5 mutant mice 30 min before LDT stimulation diminished the LDT-evoked second component in the NAc dopamine signal, similar to wild-type mice (Fig. 3*B*). Again, this was evidenced by a significant ($p < 0.01$) attenuation of the magnitude and increase in the duration of the second component compared with prescopolamine stimulated responses with no significant change in the magnitude ($p = 0.20$) and duration ($p = 0.50$) of the first component (Table 1). Scopolamine pretreatment in M5 mutant mice had no effect on baseline levels of the dopamine signal after the second component.

Kinetics of the third component

To measure the contribution of the M5 mAChR to LDT-elicited dopamine efflux, we subtracted the results obtained from the mutant mice from those of the wild-type mice. This subtraction revealed that the contribution of the M5 receptor was initiated 5 min after stimulation. In turn, these data were plotted on semilogarithmic scales to estimate the rise and decay rates of the M5 contribution to the third phase. Both rise and decay components were found to be linear ($r^2 = 0.81$ and 0.96, respectively; $p < 0.05$) and yielded half-life estimates of 5.5 min for the rise to the

Table 1. Effects of LDT electrical stimulation on dopamine oxidation currents recorded from the nucleus accumbens in wild-type and M5 mutant mice before and after scopolamine administration (5 mg/kg, i.p.)

		Mean change in accumbal dopamine oxidation current from baseline levels for each LDT-elicited component				
		First (+)		Second (−)		Third (+)
Group (*n*)	Drug condition	Peak (nA)	Duration (min)	Peak (nA)	Duration (min)	Peak (nA)
Wild-type (7)	Control	0.310 ± 0.03	2.64 ± 0.48	−0.265 ± 0.07	4.50 ± 0.87	0.663 ± 0.16
Wild-type (7)	Scopolamine	0.322 ± 0.11	2.43 ± 0.45	−0.126 ± 0.04[a]	11.86 ± 4.27[b]	0.024 ± 0.03[a]
M5 mutant (8)	Control	0.282 ± 0.09	2.44 ± 0.86	−0.230 ± 0.04	19.19 ± 9.06[b]	−0.021 ± 0.05[c]
M5 mutant (8)	Scopolamine	0.338 ± 0.04	2.69 ± 0.46	−0.096 ± 0.03[a]	14.38 ± 8.98[b]	0.013 ± 0.03

[a]Significant decrease compared with relevant control group ($p < 0.01$).
[b]Significant increase compared with wild-type controls ($p < 0.01$).
[c]Significant decrease compared with wild-type controls ($p < 0.01$).

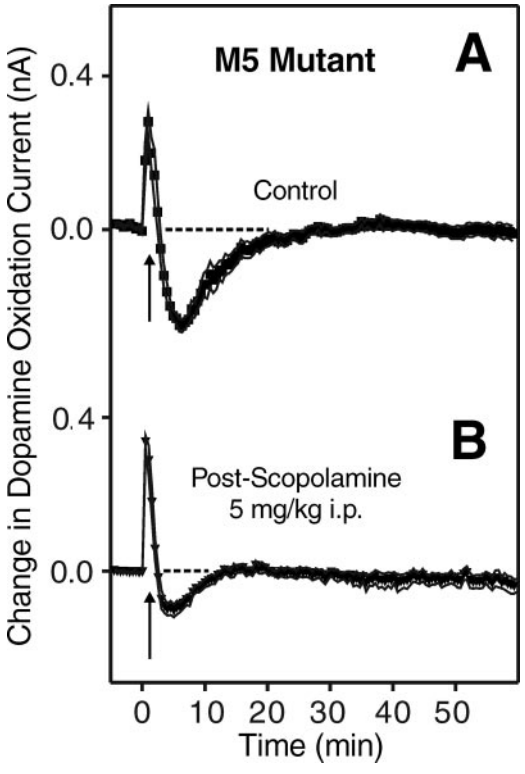

Figure 3. The effect of electrical stimulation applied to the LDT on dopamine oxidation currents recorded in the NAc before (*A*) and after (*B*) systemic injection of scopolamine (5 mg/kg, i.p.) in M5 mutant mice. *Arrows* indicate the time of stimulus termination. *Points* represent the mean changes in the chronoamperometric responses ($n = 8$); *solid lines* represent the SEM; *dotted lines* correspond to prestimulation baseline levels.

peak from initiation and 16.5 min for the decay to baseline for the third component.

DISCUSSION

The present results are the first to indicate that M5 mAChRs are important for mediating cholinergic excitation of dopamine release in the NAc. Electrical stimulation of the LDT evoked a three-component phasic pattern of change in dopamine efflux in the NAc of wild-type mice. The prolonged facilitatory third component of dopamine efflux evoked by LDT stimulation was found to be dependent on the integrity of M5 mAChRs, because it was completely absent in M5 mAChR-truncated mice.

We have shown previously that electrical stimulation of the LDT in rats evokes a similar three-component pattern of change in NAc dopamine efflux (Forster and Blaha, 2000). Furthermore, the third, prolonged facilitatory component of dopamine efflux in rats was blocked by intra-VTA infusion of the nonselective mAChR antagonist scopolamine in a manner comparable with that observed presently in wild-type mice treated systemically with scopolamine. These data suggest that M5 mAChRs localized in the VTA (Vilaro et al., 1990; Weiner et al., 1990) mediate the prolonged activation of dopamine efflux elicited by LDT stimulation.

M5 mAChR kinetics

We have shown that LDT stimulation facilitates NAc dopamine efflux in two distinct phases (Fig. 2*A*, Table 1). The first phase, activated immediately with LDT stimulation and lasting <3 min, is mediated by ionotropic glutamatergic and nicotinic cholinergic receptors in the VTA (Forster and Blaha, 2000). The second, activated 8 min after LDT stimulation and lasting 35 min on average, is mediated entirely by a slow-activating metabotropic M5 mAChR. M5 mAChRs have been found previously to mediate prolonged salivation (15–60 min) in response to pilocarpine administration in mice (Takeuchi et al., 2001). Also, *N*-methylscopolamine binding to M5 mAChRs in cultured cells has been found to be the slowest of all mAChRs (association half-life of 6 min, dissociation half-life of 20.5 min) (Ferrari-Dileo et al., 1994). Despite obvious methodological differences, these time constants are strikingly similar to our own half-life rise (5.5 min) and decay (16.5 min) estimates of the M5 mAChR-mediated component. These results support our hypothesis that M5 mAChRs serve a unique role in mediating prolonged excitatory effects on midbrain dopamine neurons (Yeomans et al., 2001).

M5 mAChR interactions with other receptor types

The magnitude and duration of the excitatory first component of LDT-elicited dopamine efflux in the NAc were not affected either by scopolamine blockade of M5 mAChRs in wild-type mice or by truncation of the M5 mAChR (Table 1). This implies that there was no compensatory change in the function of ionotropic glutamatergic and nicotinic cholinergic receptors in the VTA, identified previously as mediating this rapid component (Forster and Blaha, 2000), despite transient or permanent inactivation of M5 receptors in this region.

In contrast to the first component, the duration of the inhibitory second component elicited by LDT stimulation was significantly prolonged (Table 1) after scopolamine or genetic inactivation of M5 mAChRs. Also, when the influence of the second component was subtracted from the third component recorded in

wild-type mice, the third component was seen to be initiated at 5 min rather than 8 min after stimulation. In rats, this inhibitory second component can be prevented by intra-LDT infusion of the M2 mAChR antagonist methoctramine (Forster and Blaha, 2000). Our present data suggest that activation of excitatory M5 mAChRs in the VTA competes in a progressive antagonistic manner with the inhibitory actions of M2-like autoreceptors in the LDT. Therefore, the inhibitory actions of M2-like mAChRs in the LDT appear to mask the initiation of the third-component increase in NAc dopamine efflux mediated by M5 mAChRs in the VTA. Together, the present findings emphasize the existence of an important regulatory balance between the actions of acetylcholine on M2-like autoreceptors in the LDT and the actions on M5 postsynaptic receptors in the VTA to modulate dopaminergic neurotransmission at terminal sites in the basal forebrain.

Functional significance

Behaviors dependent on dopamine activity in the NAc include initiation and maintenance of motivated behaviors, goal-directed locomotion, and mediation of natural and drug reinforcement (Willner and Scheel-Kruger, 1991; Bardo, 1998). Activation of ionotropic receptors on dopamine cells of the VTA by the LDT may play a role in initiation of NAc-driven incentive-related behaviors, whereas prolonged dopaminergic activity mediated by M5 mAChRs may serve to maintain these dopamine-dependent behaviors. This hypothesis is consistent with the recent finding that M5 mAChRs in the VTA are necessary for brain-stimulation reward (Yeomans et al., 2000).

The majority of studies examining the role of mesopontine nuclei in incentive-driven behaviors have focused on an anatomically adjacent cholinergic nucleus, the pedunculopontine tegmental nucleus (PPT). It is thought that the PPT is involved in making associations between environmental stimuli and rewards by mediating arousal or attentional mechanisms (Garcia-Rill, 1991; Inglis and Winn, 1995). In light of the present data, it is likely that the LDT serves a similar function in incentive behaviors via activation of M5 mAChRs in the VTA.

In addition to their potential significance in motivational behaviors, the role of the PPT in Parkinson's disease and the role of the PPT and LDT in schizophrenia are being actively explored (Zweig et al., 1989; Garcia-Rill et al., 1995; Yeomans, 1995; Pahapill and Lozano, 2000). Postmortem analyses of brain tissue from Parkinson's patients indicate a significant loss of cholinergic cells in the PPT, which is correlated with dopaminergic cell loss in the substantia nigra (Jellinger, 1988; Zweig et al., 1989). Conversely, postmortem analyses of certain populations of schizophrenic patients show increased cholinergic cell numbers in the pons (Karson et al., 1991; Garcia-Rill et al., 1995). Thus, midbrain M5 mAChR regulation of prolonged dopaminergic activity in the basal forebrain may represent a new target for pharmaceutical intervention in cases in which dopaminergic hypoexcitation or hyperexcitation is apparent. The observations that other receptor types in the VTA are not affected by transient or permanent inactivation of M5 mAChRs emphasize the feasibility of using agents selective to the M5 mAChRs to regulate midbrain dopaminergic cell activity *in vivo*.

Conclusion

Here we show for the first time that M5 mAChRs play a direct role in modulating basal forebrain dopaminergic transmission. Our results have demonstrated that these mAChRs, presumably in the VTA, are responsible for prolonged maintenance of dopaminergic activity in the NAc. Thus, M5 mAChRs may facilitate integration of motivational information from the pons to the basal forebrain, allowing appropriate incentive-related behaviors to occur.

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