

Regulation of rat mesencephalic GABAergic neurones through muscarinic receptors

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Central dopamine neurones are involved in regulating cognitive and motor processes. Most of these neurones are located in the ventral mesencephalon where they receive abundant intrinsic and extrinsic GABAergic input. Cholinergic neurones, originating from mesopontine nuclei, project profusely in the mesencephalon where they preferentially synapse onto local GABAergic neurones. The physiological role of this cholinergic innervation of GABAergic neurones remains to be determined, but these observations raise the hypothesis that ACh may regulate dopamine neurones indirectly through GABAergic interneurones. Using a mesencephalic primary culture model, we studied the impact of cholinergic agonists on mesencephalic GABAergic neurones. ACh increased the frequency of spontaneous IPSCs ($151 \pm 49\%$). Selective activation of muscarinic receptors increased the firing rate of isolated GABAergic neurones by $67 \pm 13\%$. The enhancement in firing rate was Ca^{2+} dependent since inclusion of BAPTA in the pipette blocked it, actually revealing a decrease in firing rate accompanied by membrane hyperpolarization. This inhibitory action was prevented by tertiapin, a blocker of GIRK-type K^+ channels. In addition to its excitatory somatodendritic effect, activation of muscarinic receptors also acted presynaptically, inhibiting the amplitude of unitary GABAergic synaptic currents. Both the enhancement in spontaneous IPSC frequency and presynaptic inhibition were abolished by 4-DAMP (100 nM), a preferential M3 muscarinic receptor antagonist. The presence of M3-like receptors on mesencephalic GABAergic neurones was confirmed by immunocytochemistry. Taken together, these results demonstrate that mesencephalic GABAergic neurones can be regulated directly through muscarinic receptors. Our findings provide new data that should be helpful in better understanding the influence of local GABAergic neurones during cholinergic activation of mesencephalic circuits.

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The central dopaminergic system regulates major physiological functions such as motivation, mood, cognition and motor behaviour. It is also implicated in the pathophysiology of schizophrenia, drug dependence and Parkinson's disease (Grace, 1991; Kalivas, 1993; Nestler & Aghajanian, 1997). The fine tuning of the firing rate of dopamine neurones is important in the regulation of dopamine (DA) release in projection areas, namely the nucleus accumbens, dorsal striatum and prefrontal cortex (Suaud-Chagny *et al.* 1992). Most dopaminergic cell bodies are localized in the substantia nigra (SN) and the ventral tegmental area (VTA), two nuclei located in the ventral part of the mesencephalon (Dahlstrom & Fuxe, 1964). These structures receive monoaminergic, cholinergic, glutamatergic as well as GABAergic afferents

(Walaas & Fonnum, 1980; Clarke *et al.* 1987; Grenhoff *et al.* 1993). The GABAergic input to dopamine neurones arises from striatal projection neurones but also from GABAergic neurones that are intrinsic to the ventral mesencephalon. The VTA and SN contain about 75–85% dopamine neurones and 15–25% GABAergic neurones (Bayer & Pickel, 1991; Johnson & North, 1992b). Although many of these are concentrated in the reticulata part of the SN, others are dispersed amongst the dopamine neurones and are difficult to identify. GABAergic interneurones are in a privileged position to regulate the activity of dopamine neurones. In addition, other evidence suggests that dopamine neurones are under tonic regulation by GABAergic afferents (Wolf *et al.* 1978; Westerink *et al.* 1996). The physiological importance of such local

GABAergic input to dopamine neurones is well illustrated by the excitatory action of opioids on dopamine neurones. This excitation depends on inhibition of the firing rate of GABAergic interneurons due to the activation of μ -type receptors that are expressed selectively on these neurones in the VTA/SN region (Johnson & North, 1992a). The regulation of VTA GABAergic neurones by μ -opioid receptors has also been observed in a primary culture system (Bergevin *et al.* 2002), under conditions where direct effects of pharmacological agents on GABAergic neurones are more easily detected.

Cholinergic receptors control GABA release in several structures of the CNS (Baba *et al.* 1998; Guo & Chiappinelli, 2000; Xu *et al.* 2001) including the VTA (Grillner *et al.* 2000; Erhardt *et al.* 2002; Mansvelder *et al.* 2002). The majority (65%) of cholinergic projections from mesopontine nuclei (laterodorsal tegmentum (LDT) and pediculopontine tegmentum (PPT)) impinge on DA transporter-negative neurones (presumed GABAergic neurones) (Garzon *et al.* 1999). This preferential innervation suggests the hypothesis that acetylcholine (ACh) may exert its action on dopamine neurones at least in part by affecting local GABAergic neurones. ACh can act through nicotinic ionotropic receptors or through muscarinic G-protein coupled receptors. Previous studies using single-cell RT-PCR have shown the expression of various nicotinic receptor subunits in GABAergic (α_3 , α_4 , β_2 and β_3 subunits) and dopamine (α_3 to α_7 and β_2 to β_4 subunits) neurones of the VTA (Charpentier *et al.* 1998; Klink *et al.* 2001). Of the five cloned muscarinic receptors, only M3 and M5 mRNA have been detected in the ventral midbrain (Vilaro *et al.* 1990; Weiner *et al.* 1990). M2, M3, M4 and M5 immunolabelling or binding has also been reported (Levey, 1993; Zubieta & Frey, 1993). The specific cellular localization of muscarinic receptors in the mesencephalon has not yet been explored (Vilaro *et al.* 1990; Zubieta & Frey, 1993). The activation of cholinergic receptors by the injection of cholinergic agonists in the VTA of living rats leads to an increase in the firing rate of dopamine neurones and to an increase in the concentration of DA in projection areas (Imperato *et al.* 1986; Gronier & Rasmussen, 1998; Blaha & Winn, 1993; Gronier *et al.* 2000). Similarly, electrical stimulation of the PPT or LDT induces a significant increase in striatal DA concentration by a mechanism that requires the M5 muscarinic receptor (Forster *et al.* 2002). The net enhancement in DA release in such *in vivo* experiments is complex and involves three successive phases: a rapid but brief enhancement, a short-lasting decrease and a long-duration elevation. Finally, using extracellular stimulation in a midbrain slice preparation, Grillner *et al.*

(2000) demonstrated that muscarine diminishes evoked inhibitory postsynaptic currents (IPSCs) through M3-like muscarinic receptors. These later experiments were done in brain slices, where it is particularly difficult to distinguish between GABAergic synapses originating from intrinsic neurones and GABAergic synapses originating from striatal GABAergic neurones. In summary, although the net effect of muscarinic activation in the VTA/SN is excitatory and may depend in part on some direct cholinergic projections onto dopamine neurones, new data are required to understand the influence of local GABAergic neurones in this regulation.

To test the hypothesis of a direct regulation of VTA/SN GABAergic neurones by cholinergic receptors, we took advantage of a VTA/SN primary culture model. Our results show that cholinergic drugs increase the firing rate of GABAergic interneurons in a Ca^{2+} -dependent manner and decrease the amplitude of unitary IPSCs, at least in part through a M3-like muscarinic receptor.

Methods

Cell culture

Primary cultures were prepared as previously described (Congar *et al.* 2002) with protocols derived from Cardozo (1993) and Sulzer *et al.* (1998). Two types of cultures were used. In standard cultures, neurones were plated onto coverslips uniformly covered with astrocytes. In microdot cultures, neurones were plated on small islands of astrocytes previously plated onto microdroplets of substrate. This permitted the establishment of single neurone cultures and electrophysiological recordings of GABA-mediated autaptic currents (mediated by synapses established by a neurone onto its own dendrites) (Bekkers & Stevens, 1991; Michel & Trudeau, 2000; Bergevin *et al.* 2002). This allowed unambiguous identification of GABAergic neurones in cultures that otherwise also contained dopamine neurones.

Prior to plating, coverslips used for standard cultures were coated with collagen/poly-L-lysine. Coverslips used for microdot cultures were coated successively with poly-L-ornithine, agarose (0.05%) and microdroplets of collagen. A monolayer of mesencephalic astrocytes was then applied for both culture types. To prepare astrocyte cultures, neonatal (P0 to P2) Sprague–Dawley rats were cryoanaesthetized by placing them on crushed ice for 2 min. Brains were quickly removed from the skull and placed in ice-cold dissociation solution. A 2 mm³ block of ventral midbrain tissue was rapidly isolated from a 1 mm slice cut at the level of the midbrain flexure.

The tissue was then digested with papain (Worthington Biochemical Corp., Lakewood, NJ, USA) for 60 min at 37°C before being gently triturated using glass pipettes of decreasing diameters until a single-cell suspension was obtained. Cells were plated and grown in culture flasks and maintained in Basal Medium Eagle with Earl's Salts (Invitrogen, Burlington, ON, Canada) supplemented with penicillin–streptomycin, GlutaMAX-1 (Invitrogen), 10% fetal calf serum (Invitrogen) and Mito+ serum extender (VWR Canlab, Montréal, QC, Canada). After the first 48 h, culture flasks were vigorously washed with cold medium to remove most neurones, leaving only tightly adherent astrocytes. After reaching confluence, astrocytes were gently trypsinized, washed, collected, counted and plated at a concentration of 100 000 (standard cultures) or 60 000 (microdot cultures) living cells per millilitre. Twenty-four hours after plating, astrocytes covered most of the available surface and further division was inhibited with 5-fluoro-2-deoxyuridine (FUDR).

To prepare neurones, neonatal (P0 to P2) Sprague–Dawley rats were cryoanaesthetized and a mesencephalic slice was prepared, as described before. The ventral mesencephalic part of the slice was rapidly isolated. As for the astrocyte preparation, the tissue was digested with papain for 30 min at 37°C, before being gently triturated. The dissociated cells were then collected by centrifugation, counted and diluted at a density titrated to optimize neuronal viability in standard culture conditions (240 000 living cells per millilitre) and to maximize the number of single neurones in microdot cultures (100 000 living cells per millilitre). Further astrocytic division was inhibited by a second addition of FUDR 24 h after neurones were plated. Neurone culture were maintained in Neurobasal A/BME (2:1) medium, penicillin–streptomycin, GlutaMAX-1, 10% fetal calf serum (Hyclone Laboratories, Logan, UT, USA) and B27 serum extender (Invitrogen). Kynurenate (0.5 mM) was added to the culture medium 7 days after neurones were plated in order to prevent excitotoxicity. Cell cultures were incubated at 37°C in a 5% CO₂ atmosphere. Experiments were performed on neurones between 10 and 30 days after plating. All procedures were approved by the animal ethics committee of the Université de Montréal.

Electrophysiology

All electrophysiological procedures were performed at room temperature using a WARNER PC-505 or PC-501 patch-clamp amplifier (Warner Instrument Corp, Hamden, CT, USA). Signals were filtered at 2 kHz, digitized at 5 kHz, recorded and analysed with pCLAMP

8 software (Axon Instruments, Union City, CA, USA). Patch pipettes were prepared with borosilicate glass and had a resistance of 5 ± 1 M Ω . Cells were perfused with an extracellular solution containing (mM): NaCl 140, KCl 5, MgCl₂ 2, CaCl₂ 2, *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulphonic acid) (Hepes) 10, glucose 10, sucrose 6 (pH 7.35; 295 mosmol l⁻¹). Cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μ M) was added to the extracellular bathing solution while recording spontaneous inhibitory postsynaptic currents (sIPSCs) in order to block glutamatergic (AMPA/kainate) fast excitatory postsynaptic currents. Tetrodotoxin (TTX; 0.5 μ M) (Alomone labs, Jerusalem, Israel) was also added to the bathing solution while recording miniature inhibitory postsynaptic currents (mIPSCs). Spontaneous IPSCs and mIPSCs were recorded using a caesium methylsulphate intrapipette solution containing (mM): CsMeSO₄ 120, CsCl 20, NaCl 5, EGTA 1, ATP (Mg salt) 4, GTP (Tris salt) 0.5, Hepes 10 (pH 7.35; 295 mosmol l⁻¹). Autaptic inhibitory postsynaptic currents (autaptic IPSCs) and action potentials were recorded with a potassium methylsulphate intrapipette solution containing (mM): KMeSO₄ 120, KCl 20, NaCl 5, phosphocreatine (Tris salt) 10, ATP (Mg salt) 4, GTP (Tris salt) 0.5, Hepes 10, EGTA 0.1 (pH 7.35; 295 mosmol l⁻¹). To evoke autaptic IPSCs, isolated neurones were stimulated every 15 s with a brief (1 ms) voltage command pulse to +20 mV from a holding potential of –50 to –40 mV. To measure outward whole-cell voltage-dependent K⁺ currents, identified GABAergic neurones were stepped in 10 mV increments from –70 mV to +50 mV. Experiments were performed in the presence of TTX (0.5 μ M) to block voltage-dependent Na⁺ currents. Evoked postsynaptic currents and voltage-dependent K⁺ currents were recorded and analysed with pCLAMP 8 (Axon instruments). All other electrophysiological events (IPSCs and action potentials) were analysed with Mini Analysis Software (version 5) by Synaptosoft Inc. (Leonia, NJ, USA). For mIPSCs analysis, a threshold was fixed at 6 ± 1 pA. For sIPSCs analysis, we chose an arbitrary threshold of 25 pA that permitted exclusion of most mIPSCs. This procedure prevented the influence of mIPSCs on the quantification of sIPSC amplitude or frequency. SR 95531 (Gabazine) (5 μ M), a potent and reversible GABA_A receptor antagonist, was used to confirm the GABAergic nature of synaptic responses in most electrophysiological experiments.

Immunocytochemistry

Cells were fixed in paraformaldehyde (4%), permeabilized with Triton X-100 (0.1%) and incubated in a blocking

solution containing bovine serum albumin (0.5%), Triton X-100 (0.1%) and goat serum (5%). Primary mouse monoclonal (1 : 100) or rabbit polyclonal (1 : 500) (Sigma) antibodies raised against GABA were used to identify GABAergic neurones. Anti-tyrosine-hydroxylase mouse monoclonal (1 : 1000) (Pelfrez Biological, AK, USA) or rabbit polyclonal (1 : 5000) (Sigma) antibodies were used to identify dopamine neurones. The presence of M3 and M5 muscarinic receptors was tested with subtype-specific rabbit polyclonal antibodies from Alomone Laboratories (1 : 300). Primary antibodies were detected using Alexa-488 or Alexa-546-labelled secondary antibodies (1 : 200) (Molecular Probes Inc., Eugene, OR, USA). Images of immunofluorescent labelling were acquired using a Hamamatsu Orca-II digital-cooled CCD camera using Esee and Isee software (Inovision Corporation, Raleigh, NC, USA).

Drugs

All chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada) except where otherwise indicated. The GIRK-type K⁺ channel toxin blocker tertiapin was obtained from Alomone Laboratories.

Data processing

A proportion of neurones were insensitive to cholinergic agonists under our experimental conditions. Because this was essentially all-or-none, analyses were restricted to responsive cells only. To determine objectively if a cell was responsive or not, all neurones were analysed to determine whether the measured parameter changed by more than 2 standard deviations away from control value in response to the application of the cholinergic agonist ACh or muscarine. A third of all recorded cells were thus discarded.

Data presented in the text are expressed as means $\pm$ s.e.m. Statistical comparisons were performed using Student's paired *t* test or a one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* pairwise comparisons, as appropriate. A probability value of $P < 0.05$ was considered indicative of a significant difference.

Results

Acetylcholine increases the frequency of spontaneous GABAergic postsynaptic currents (sIPSCs)

Because mesencephalic neurones receive abundant projections from cholinergic structures, we expected

that the activation of cholinergic receptors would affect the physiology of GABAergic VTA/SN neurones. We first recorded spontaneously generated IPSCs to evaluate global effects of the natural agonist, acetylcholine (ACh), on GABAergic neurones. Because GABAergic synapses impinge on essentially all neurones in these cultures, whole-cell voltage-clamp recordings were performed in randomly selected neurones in standard primary dissociated cultures. ACh (10 μ M) elicited a large increase of sIPSC frequency (Fig. 1A). The effect was rapid and lasted until the washout of ACh. The maximal increase was to $251 \pm 49\%$ ($n = 55$) of the control period (Fig. 1B). The average basal frequency of sIPSCs increased from 3.8 ± 0.5 Hz to 6.7 ± 0.6 Hz ($n = 55$; $F = 9.85$ (DF = 2), $P < 0.001$) in the presence of ACh. The washout was complete 5 min later with the sIPSC frequency returning to 3.7 ± 0.5 Hz (Fig. 1C). Note that the increase of sIPSCs frequency did not show any obvious desensitization during the application of ACh. On the other hand, the average amplitude of sIPSCs was not affected by ACh ($n = 55$; $F = 0.729$ (DF = 2), $P = 0.484$) (Fig. 1D).

Muscarine increases sIPSC frequency in a dose-dependent manner

We used muscarine to evaluate the contribution of muscarinic receptors. Muscarine (10 μ M) increased sIPSC frequency to $157.2 \pm 23\%$ ($n = 11$) of the control period, with the maximal effect occurring 1 min after the beginning of drug application (Fig. 2A). The effect of muscarine outlasted the duration of drug application by a few minutes (Fig. 2B). Although this was not specifically quantified, the sIPSC pattern was often more regular in the presence of muscarine. This change in pattern disappeared during drug washout. Like in experiments with ACh, muscarine did not significantly affect sIPSC amplitude (Fig. 2B). The dose-response curve (Fig. 2C) demonstrates that the average increase in sIPSC frequency in the presence of muscarine reached a peak at 1 μ M ($152.9 \pm 19.2\%$ of control) ($P < 0.01$) and that a steady-state was reached at 10 μ M ($152.4 \pm 20.6\%$ of control) ($P < 0.001$).

A component of the excitation induced by ACh was mediated by nicotinic receptors since GABAergic neurones could still be excited when ACh was applied in the presence of atropine (1 μ M), a potent and broad-spectrum muscarinic receptor antagonist (F. J. Michel and L.-E. Trudeau, unpublished results). Because recent work has examined the expression of nicotinic receptor subunits in mesencephalic GABAergic neurones (Charpentier *et al.* 1998; Klink *et al.* 2001), and considering the paucity of data on muscarinic receptor actions in the mesencephalon, in

further experiments, we chose to focus our attention on the regulation of mesencephalic GABAergic neurones by muscarinic receptors. A dose of $10\ \mu\text{M}$ muscarine was used to obtain maximal effects.

Muscarine regulates action potential firing rate in isolated GABAergic neurones

We took advantage of single-cell cultures to investigate more directly the regulation of GABAergic VTA/SN neurones by muscarinic receptors. In such single neurone cultures, individual GABAergic neurones establish autaptic synaptic connections (Bekkers & Stevens, 1991) and can thus be readily identified electrophysiologically

(Michel & Trudeau, 2000; Bergevin *et al.* 2002). This preparation facilitates the detection of direct drug effects. Single GABAergic neurones were patch-clamped and identified physiologically by the ability of SR95531 ($5\ \mu\text{M}$), a specific GABA_A receptor antagonist, to block autaptic currents evoked by single action potentials (see Fig. 5A). Under our experimental conditions, the reversal potential of GABAergic currents was approximately $-50\ \text{mV}$, thus providing an additional criterion to rapidly identify GABAergic synaptic events.

In these identified VTA/SN GABAergic neurones, current-clamp experiments demonstrated that muscarine increased action potential firing rate (Fig. 3A). At the

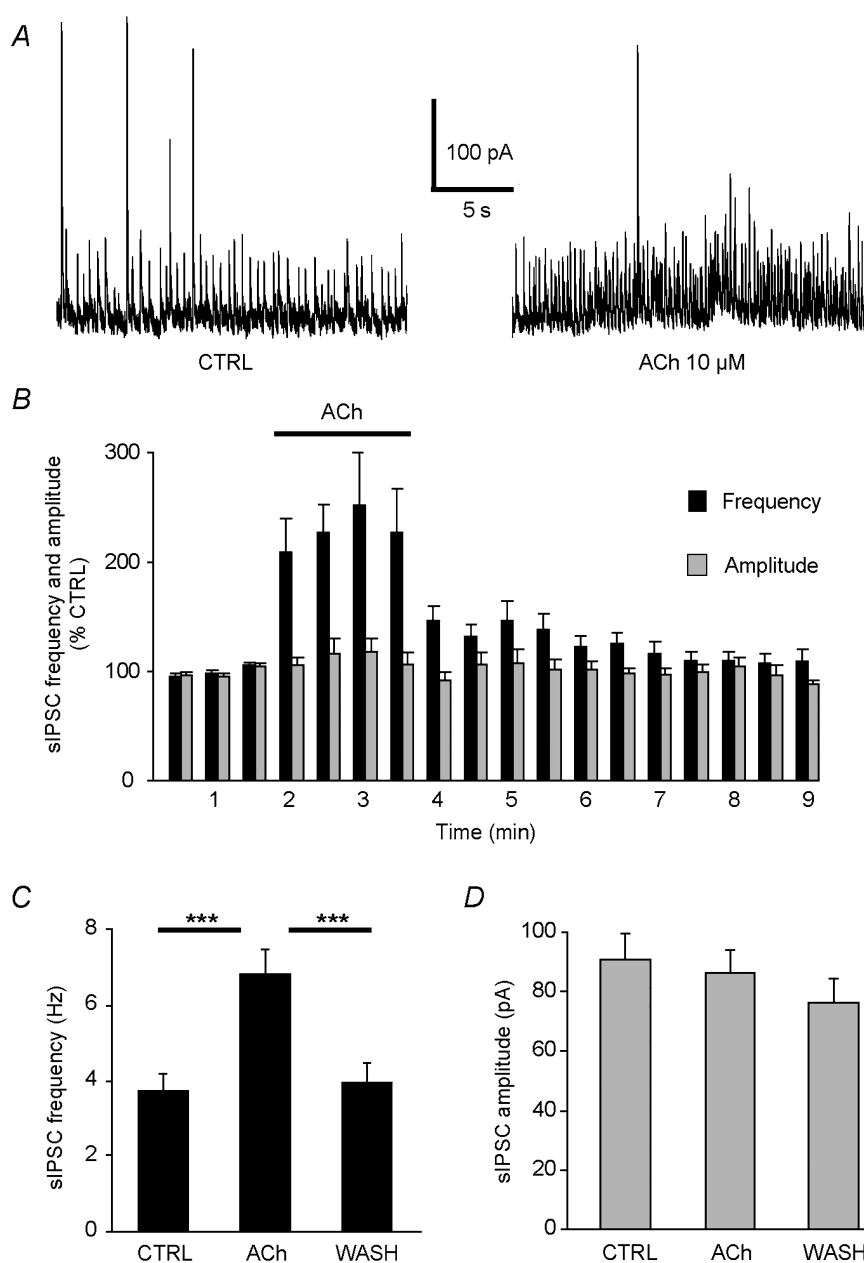


Figure 1. ACh enhances spontaneous IPSC frequency

A, whole-cell voltage-clamp recording from a cultured mesencephalic neurone that showed an increase in the frequency of sIPSCs in response to $10\ \mu\text{M}$ ACh. The neurone was voltage-clamped to $0\ \text{mV}$ in the presence of CNQX ($10\ \mu\text{M}$). GABAergic events appear as outward currents. B, bar graph illustrating the average time course of sIPSC frequency and amplitude. Data are presented in 30 s bins and were normalized relative to the control period. ACh ($10\ \mu\text{M}$) was applied for 2 min as shown by the black bar ($n = 55$). C, bar graph summarizing the effect of ACh on sIPSC frequency. The time period showing the maximal effect was chosen for each neurone. Data are represented in events per second (in Hz) ($n = 55$) (***) indicates $P < 0.001$. D, bar graph summarizing the average effect of ACh on sIPSC amplitude for the same time periods represented in C (in pA) ($n = 55$).

beginning of each recording, membrane voltage (V_m) was adjusted to stabilize the firing rate (average corrected V_m : -48.1 ± 0.9 mV). At this voltage, 14 of 29 cells were depolarized more than 2 standard deviations (see methods) above the resting potential in response to muscarine (5.4 ± 0.6 mV on average, $n = 14$, $F = 7.700$ (DF = 2), $P < 0.001$) (Fig. 3B), while five were hyperpolarized by more than 2 standard deviations. Finally, 10 of 29 neurones did not show any significant change in V_m . Pooled results including all 29 recorded cells show that muscarine tended to depolarize single GABAergic neurones, but the variability of responses was such that

the overall effect was not significant ($n = 29$, $F = 0.903$ (DF = 559), $P = 0.580$) (Fig. 3B). A late depolarizing phase was also often noted 5–10 min after washout of muscarine (Fig. 3B). This delayed effect was not accompanied by an increase in firing rate and was not further investigated. Finally, of the 17 cells that showed an increased firing rate in the presence of muscarine, 5 did not show any significant change in V_m . On average, for these 17 responsive neurones, basal firing rate averaged 1.41 ± 0.23 Hz. Muscarine induced an increase to $167.4 \pm 13.3\%$ of control (to 2.12 ± 0.30 Hz) ($n = 17$, $F = 2.923$ (DF = 2), $P < 0.001$). Washout was

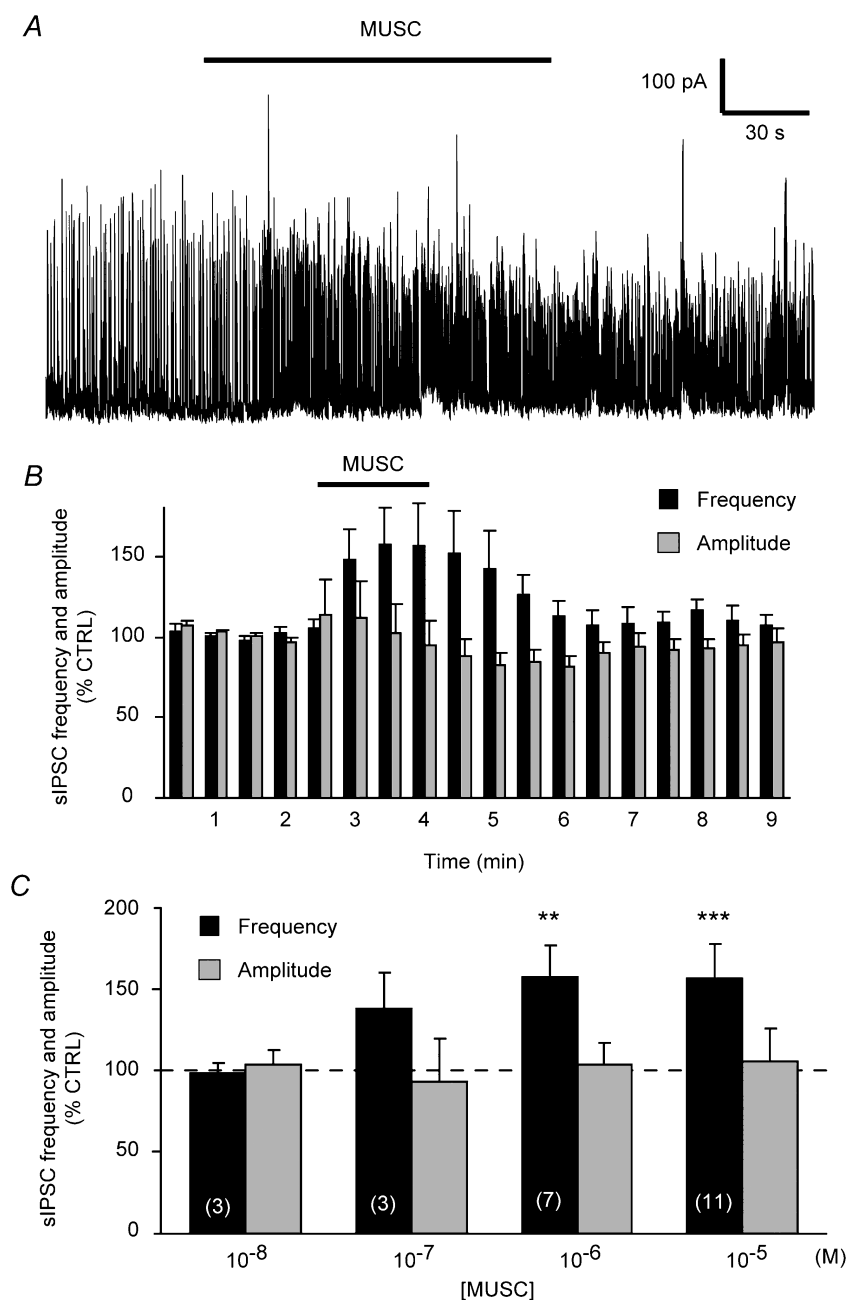


Figure 2. Enhancement of sIPSC frequency through muscarinic receptors

A, whole-cell voltage-clamp recording from a neurone showing an increase in sIPSC frequency in response to $10 \mu\text{M}$ muscarine (MUSC). The drug was applied for 2 min (black bar). The holding potential was 0 mV. B, bar graph illustrating the average time course of sIPSC frequency and amplitude in response to muscarine. Data were normalized and presented in 30 s bins ($n = 11$). C, bar graph representing the dose-dependent effect of muscarine on sIPSC frequency and amplitude. The number of cells tested is indicated in parentheses for each dose.

** $P < 0.01$, *** $P < 0.001$.

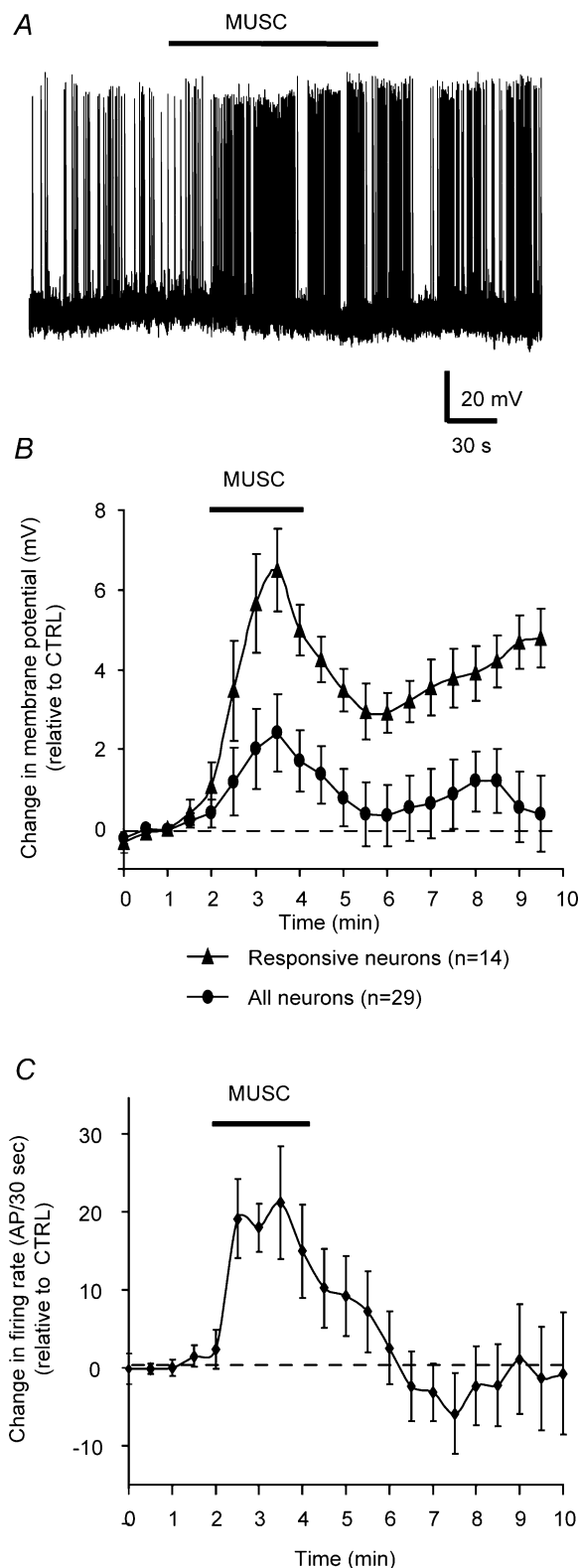


Figure 3. Muscarinic receptor activation increases firing rate
 A, current-clamp recording from an isolated mesencephalic GABAergic neurone showing an increase in firing rate in response to $10 \mu\text{M}$ muscarine (MUSC). Muscarine was applied for 2 min (black bar). B, graph representing the effect of muscarine on membrane potential. Results are expressed in 30 s bins and as a difference score (Δ) in

almost complete after 5 min (1.32 ± 0.23 Hz) (Fig. 3C). Additional experiments were performed to determine whether overall changes in membrane resistance (R_m) could be detected. Recordings were performed under current-clamp mode under conditions where V_m was stabilized at -60 mV to prevent spontaneous firing. In the presence of muscarine, no significant change in R_m was detected ($96.9 \pm 3.2\%$ of control) ($n = 8$) (data not shown).

A rise in internal Ca^{2+} concentration is necessary for the effect of muscarine

Previous work has reported the expression of M3 and M5 muscarinic receptors in rodent mesencephalon (Weiner *et al.* 1990). Considering that a major signal transduction pathway for these receptors involves activation of phospholipase C via $G_{q/11}$ protein and intracellular Ca^{2+} elevations, we hypothesized that some of the physiological effects of muscarinic receptor activation in mesencephalic GABA neurones could be Ca^{2+} dependent. To investigate the possible interrelation between firing rate increases and intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), we added BAPTA (10 mM) in the intrapipette solution to buffer $[\text{Ca}^{2+}]_i$ and prevent any intracellular increase in the concentration of free Ca^{2+} . To allow diffusion of BAPTA, we waited for 5–10 min after rupture of the membrane patch to begin the recording. During the first few minutes of recording (before disappearance of synaptic currents due to dialysis of BAPTA in synaptic terminals), we checked the reversal potential of postsynaptic currents in isolated neurones and determined their phenotype. In GABAergic neurones dialysed with BAPTA, the effect of muscarine on action potential firing frequency was not only inhibited but was reversed (Fig. 4). By itself, intrapipette BAPTA induced a shift of the firing threshold. It was necessary to polarize neurones to more positive values to reach stable firing rates (-40.2 ± 2.4 mV ($n = 11$) versus 48.1 ± 0.9 mV without BAPTA) (Student's unpaired t test: $P < 0.001$). After such an adjustment of V_m , the basal firing frequency was equivalent to control (1.25 ± 0.40 Hz versus 1.41 ± 0.23 Hz for control). Muscarine failed to increase firing rate in all 11 recorded GABAergic

relation to the control period. ●, pooled data from all neurones tested ($n = 29$). ▲, data from neurones identified as responsive to muscarine ($n = 14$). C, graph representing the average effect of muscarine on action potential firing rate. Results are expressed in 30 s bins and as a difference score (Δ) in relation to the control period to minimize the effect of the variability in basal firing frequency across individual cells ($n = 17$).

neurons. In 8 out of 11 neurones, muscarine actually decreased firing rate to $54 \pm 11\%$ of control level (0.43 ± 0.09 Hz; $n = 8$, $F = 2.923$ (DF = 2), $P < 0.001$) (Fig. 4A, B and C). Washout was almost complete after 5 min (1.28 ± 0.53 Hz). Overall, across all recorded BAPTA-loaded neurones, V_m significantly decreased in response to muscarine from -40.2 ± 2.4 mV to -47.1 ± 3.6 mV ($n = 11$, $F = 3.434$ (DF = 2), $P < 0.001$) (Fig. 4D). Taken together, these results identify a major influence of $[Ca^{2+}]_i$ in the physiological effects of muscarine on GABAergic neurones.

We hypothesized that the membrane hyperpolarization and inhibition of firing rate caused by muscarine in the presence of intracellular BAPTA results from

activation of some G-protein-gated inward rectifying K^+ (GIRK) channels. To test this hypothesis, we used the selective GIRK channel blocker tertiapin (Jin & Lu, 1998, 1999), a toxin known to block ACh-regulated GIRKs (K_{ACh}) (Kitamura *et al.* 2000). Pre-application of tertiapin (30 nM) caused no major effect by itself but completely prevented both membrane hyperpolarization and firing rate inhibition caused by muscarine in BAPTA-loaded GABAergic neurones (Fig. 4C and D). Furthermore, in experiments performed without BAPTA in the patch pipette, tertiapin increased the proportion of GABAergic neurones showing a detectable depolarization of membrane potential in response to muscarine: 18 out of 24 (75%) neurones tested were depolarized more

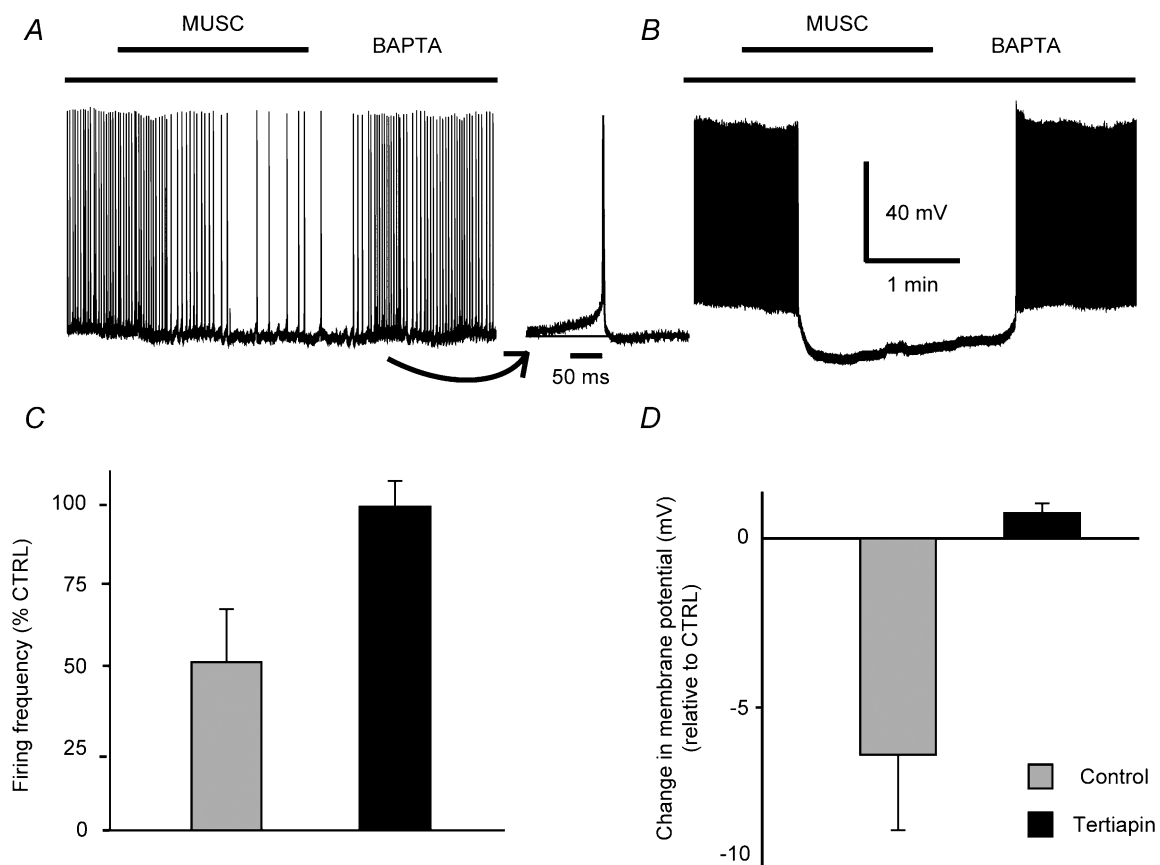


Figure 4. Calcium-dependent action of muscarine on firing rate

A and B, examples of current-clamp recordings performed from two different neurones showing a decrease in firing frequency in response to muscarine (MUSC) ($10 \mu\text{M}$) under conditions where intracellular calcium was chelated by including BAPTA (10 mM) in the patch pipette. Muscarine was applied for 2 min. The trace with an expanded time scale shows the reduced amplitude of AHPs in the presence of BAPTA. A minority of neurones such as the one in B showed a prominent hyperpolarization. C, graph representing the average peak effect of muscarine ($10 \mu\text{M}$) on firing frequency. Data are presented as the percentage change relative to the average of the control and washout periods ($n = 8$). The black columns represent data acquired in the presence of the GIRK blocker tertiapin (100 nM). D, graph representing the average peak effect of muscarine ($10 \mu\text{M}$) on membrane potential. Data are presented as the percentage change relative to the average of the control and washout periods ($n = 8$). The black columns represent data acquired in the presence of tertiapin (100 nM).

than 2 standard deviations (see methods) above the resting potential, compared to 14 out of 29 (48%) in control experiments without tertiapin (see above). When including all of the 24 neurones tested, membrane potential was significantly depolarized in response to muscarine ($n = 24$, $F = 6.58$ (DF = 431), $P < 0.001$) (not shown). The maximal depolarization was on average 4.3 ± 1.0 mV, which was close to double the average depolarization in control experiments without tertiapin (2.4 ± 1.0 mV; see Fig. 3B).

Muscarine does not increase firing rate by affecting the AHP or voltage-dependent K^+ currents

Considering that activation of muscarinic receptors enhances cell firing together with minimal membrane depolarization, one obvious hypothesis is that the enhanced firing rate induced by muscarine might, at least in part, result from a decrease in the amplitude and/or the kinetics of spike after hyperpolarizations (AHP). To test this hypothesis, we evoked single spikes under whole cell current-clamp mode by injecting a short (1 ms) depolarizing current pulse in single GABAergic neurones. For each neurone, V_m was set to -55 mV at the beginning of the experiment to prevent spontaneous firing. The amplitude of the AHP was not affected by muscarine. The peak amplitude of the AHP was $100 \pm 3\%$ of the control period ($n = 10$) (not shown).

Variations of AHP amplitude were also examined in the presence of the GABA_A receptor antagonist SR95531. This was done to control for the fact that all experiments were done on isolated GABAergic neurones that establish autapses, conditions under which autaptic synaptic responses can contribute to the amplitude of AHPs and regulate firing rate (Bacci *et al.* 2003). Block of GABA_A receptors induced a significant decrease of $26.2 \pm 8.6\%$ ($n = 9$, $P < 0.05$) in AHP amplitude compared to the control period. This decrease of the AHP amplitude induced a substantial increase in firing (not shown). However, under these conditions muscarine still induced a significant increase in firing rate and the amplitude of AHPs remained unchanged by muscarine in the presence of SR95531 ($n = 9$, $F = 6.751$ (DF = 27), $P = 0.995$, compared to SR95531 alone).

Finally, we determined whether muscarine caused a change in outward voltage-dependent K^+ currents recorded in whole-cell mode. GABAergic neurones were first identified electrophysiologically and switched to saline containing $0.5 \mu\text{M}$ TTX to block voltage-dependent Na^+ currents. Current-voltage relationships were established before, during and after muscarine. No significant change

in the early or late phase of outward K^+ currents was detected ($n = 6$) (not shown).

Muscarine inhibits the synaptic release of GABA in isolated neurones

Considering previous work showing that activation of muscarinic M3 receptors depress unidentified GABAergic synapses established onto midbrain dopamine neurones in slices (Grillner *et al.* 2000), we decided to directly evaluate the capacity of muscarine to regulate the amplitude of GABAergic autaptic currents evoked by single action potentials. Brief depolarizing voltage steps evoked a fast inward sodium current followed by a slow outward GABA_A receptor-mediated IPSC (autaptic IPSC) in isolated neurones. The GABAergic nature of the evoked autaptic current was confirmed by its reversal potential close to -50 mV and its sensitivity to SR95531 ($5 \mu\text{M}$) (Fig. 5A). Muscarine ($10 \mu\text{M}$) inhibited autaptic IPSC amplitude by $45.9 \pm 3.5\%$ ($n = 25$; $P < 0.05$) (Fig. 5C). Washout of the effect of muscarine was essentially complete after 5 min (Fig. 5C) if one takes into account the habitual run-down of synaptic currents in control experiments (approximately 20% over 10 min). To confirm the presynaptic nature of this effect, we determined the effect of muscarine on membrane currents evoked by rapid puff-application of exogenous GABA (10 ms at $500 \mu\text{M}$) (Fig. 5B and C). Muscarine had no effect on puff-evoked GABA currents ($104 \pm 2\%$ of control, $n = 4$; $P = 0.122$). These results show that muscarine inhibits GABA release through some pre-synaptic mechanism.

Lack of effect of muscarine on miniature IPSCs

To determine whether muscarine inhibits synaptic GABAergic currents through some direct inhibition of the release process, we measured the frequency of spontaneous miniature inhibitory postsynaptic currents (mIPSCs). Recordings were performed on standard VTA/SN cultures in the presence of CNQX ($10 \mu\text{M}$) to block glutamate-mediated synaptic currents and TTX ($0.5 \mu\text{M}$) to isolate impulse-independent GABAergic synaptic events. The holding potential was set between -10 mV and 10 mV to yield outward synaptic currents (Fig. 6A). Muscarine affected neither the frequency nor the amplitude of mIPSCs (Fig. 6A), as shown by the absence of a shift in the cumulative probability distribution of mIPSC frequency (Fig. 6B) or amplitude (Fig. 6C). Across all experiments, the average frequency and amplitude of mIPSCs was not significantly changed (Fig. 6D). These results show that

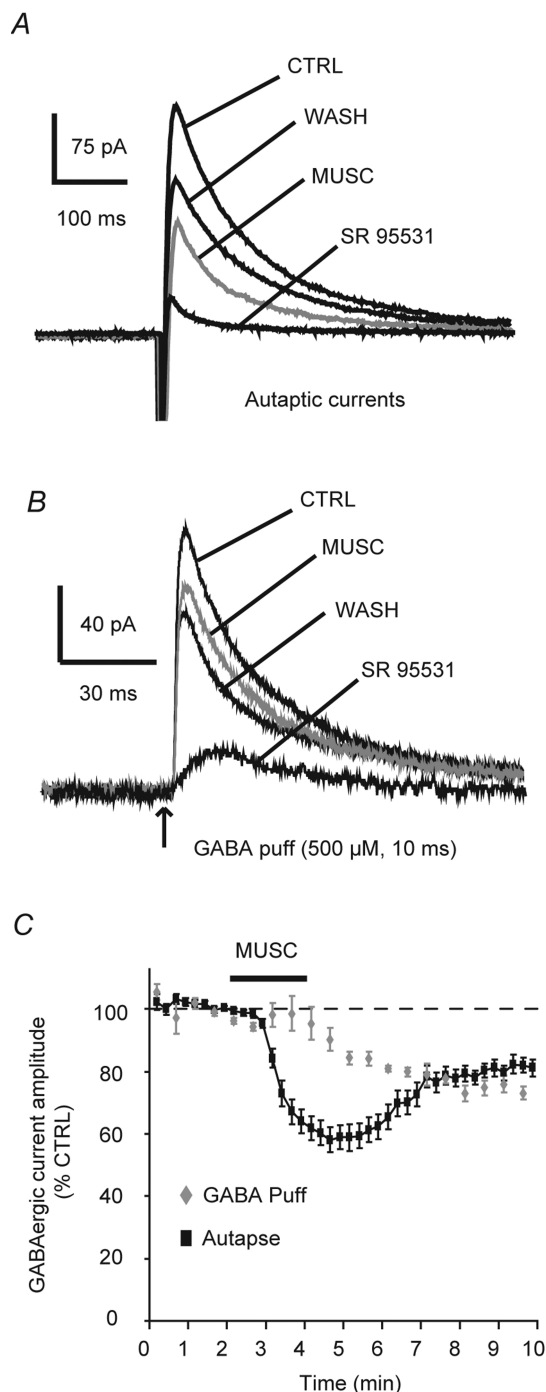


Figure 5. Presynaptic inhibition of GABA release through muscarinic receptors

A, autaptic IPSC recorded in an isolated GABAergic neurone at a holding potential of -40 mV. The IPSC is preceded by a sodium 'action current'. Action current waveforms have been clipped for clarity. The IPSC was blocked by SR95531 ($5 \mu\text{M}$), a selective GABA_A receptor antagonist. Muscarine (MUSC) $10 \mu\text{M}$ caused a reversible inhibition of the amplitude of IPSCs. In these panels, each trace is an average of 3 sweeps. B, whole-cell recordings of membrane currents evoked in the same neurone as in A in response to puff-application of exogenous GABA ($500 \mu\text{M}$). Muscarine did not affect the amplitude of GABAergic currents. Note that a small residual current persisted after GABA_A

presynaptic inhibition mediated by muscarinic receptor activation does not involve a direct inhibition of the secretory process.

Effects of the M3 muscarinic receptor antagonist 4-DAMP

To date, only M3 and M5 muscarinic receptor mRNAs have been detected in the mesencephalon (Weiner *et al.* 1990; Zubieta & Frey, 1993). Although pharmacological tools cannot easily distinguish between M3 and M5, we have used 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP), which is recognized as one of the most selective M3 receptor antagonist available (Michel *et al.* 1989; Araujo *et al.* 1991). This antagonist blocked completely the effect of muscarine on sIPSC frequency (Fig. 7A). In these experiments, muscarine was applied three times. A first application induced an increase in sIPSC frequency to $157.1 \pm 17.0\%$ of control. A second application of muscarine in the presence of 4-DAMP (100 nM) failed to induce a significant change in sIPSC frequency ($109.6 \pm 12.3\%$ of control). Finally, after washout of 4-DAMP, a final application of muscarine increased sIPSC frequency to $167.7 \pm 38.1\%$ of control, showing complete recovery (non-parametric Friedman ANOVA, $n = 4$ (DF = 2), $P < 0.05$). The ability of muscarine to inhibit autaptic IPSC amplitude was also abolished by 4-DAMP (100 nM) ($100.7 \pm 4.9\%$ of control compared to $65.0 \pm 9.9\%$ with muscarine alone) (Fig. 7B) ($n = 4$, $P < 0.05$).

Compatible with the involvement of M3 muscarinic receptors, mesencephalic GABAergic neurones were found to be immunopositive for this receptor. A clear M3-like labelling was detected both in dopamine and non-dopamine neurones (presumed GABAergic) (Fig. 7C), but not in astrocytes (not shown). This finding is in agreement with our pharmacological experiments with 4-DAMP. The lack of a suitable M5 receptor antibody precluded a similar evaluation of the presence of M5 receptors.

Discussion

Functional and anatomical evidence suggests that the cholinergic system should have a major influence on

receptor blockage, suggesting a minor component probably mediated by GABA_B receptors. C, graph illustrating the average effect of muscarine on the amplitude of autaptic GABAergic currents ($n = 25$, in black) and on puff-evoked GABAergic currents ($n = 4$, in grey). Note the presence of a gradual 20% rundown of both responses over the 10 min recording period.

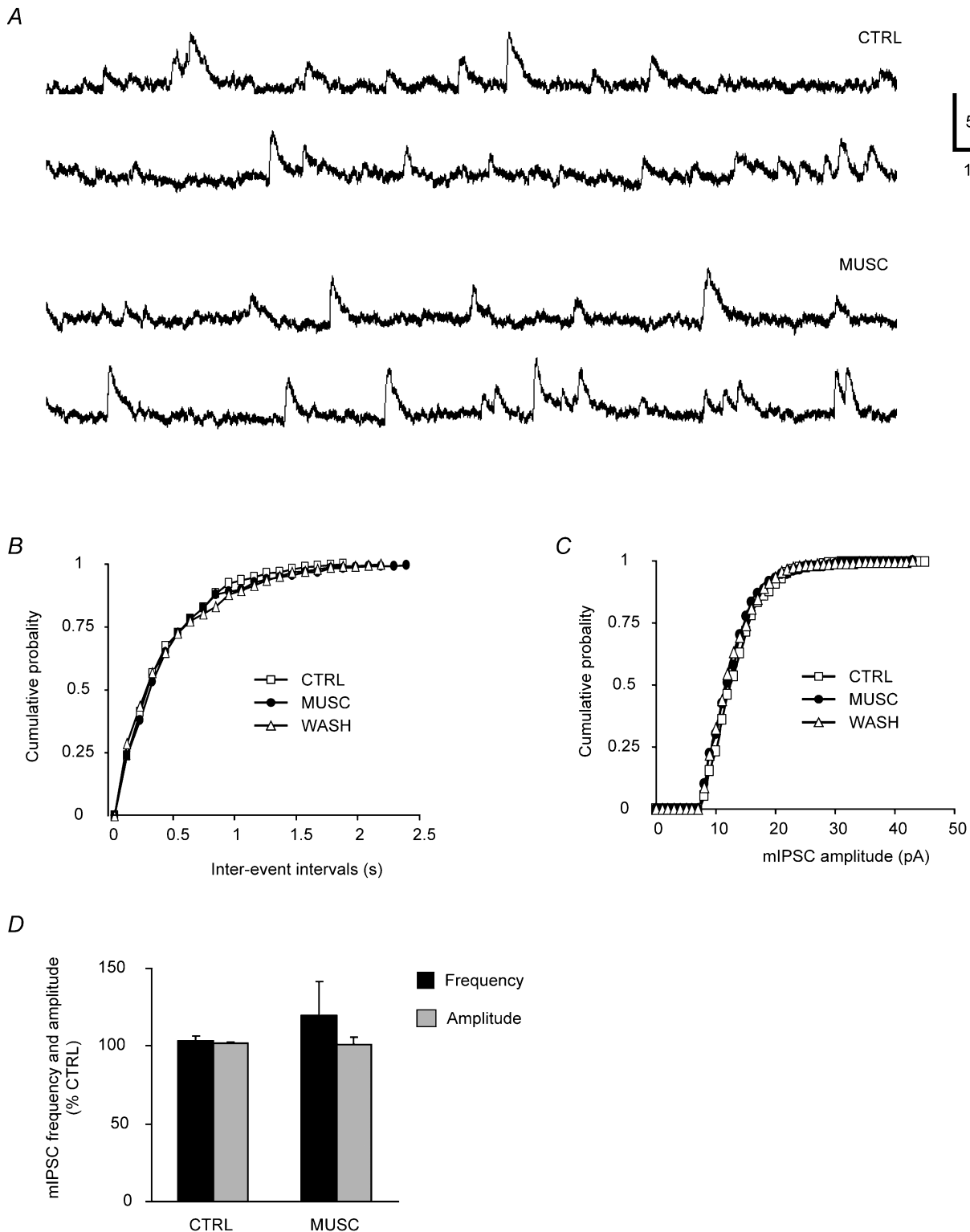


Figure 6. Muscarinic receptor activation does not affect miniature IPSCs

A, miniature IPSCs recorded in the presence of TTX under control conditions (CTRL) (upper pair of traces) or in the presence of $10 \mu\text{M}$ muscarine (MUSC) (lower pair of traces). B, cumulative probability distribution of interevent intervals from a representative experiment. Muscarine (MUSC) had no effect of event frequency. C, cumulative probability distribution of mIPSC amplitudes from the same experiment as in B. Muscarine did not cause any change in mIPSC amplitude. D, bar graph illustrating the average effect of muscarine on mIPSC amplitude and frequency ($n = 12$).

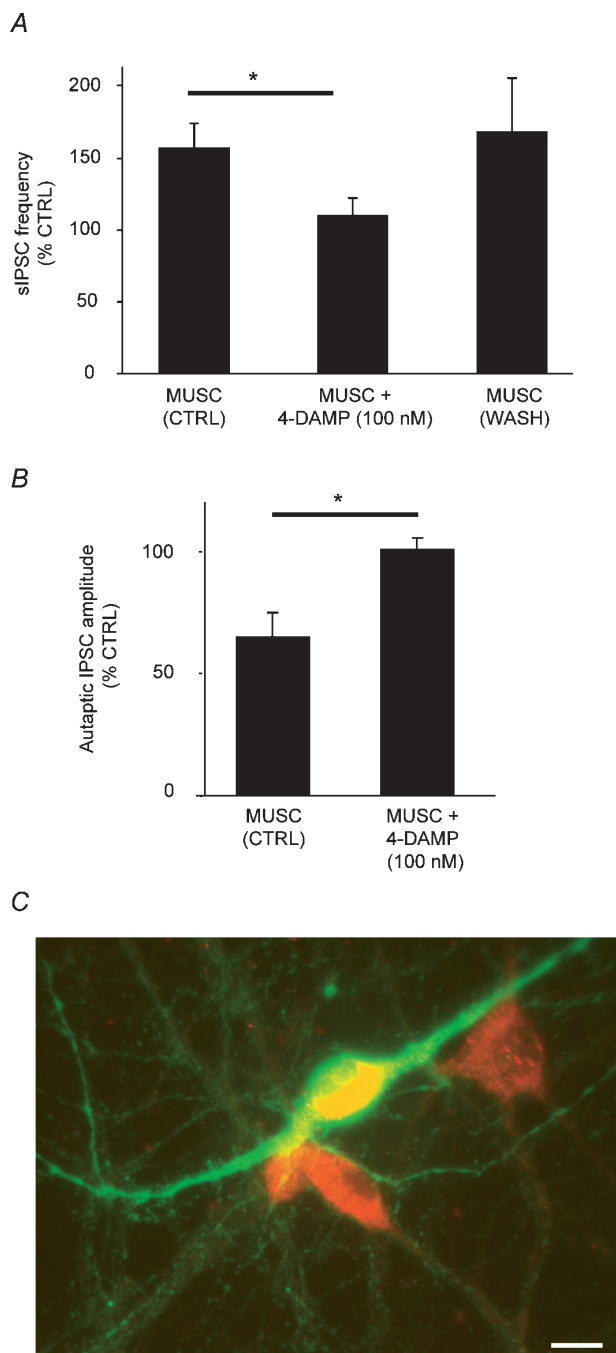


Figure 7. The effects of muscarine implicate M3 muscarinic receptors

A, bar graph showing the effect of muscarine (MUSC) ($10 \mu\text{M}$) on sIPSC frequency ($n = 4$). Data were normalized to the control period. The preferential M3 receptor antagonist 4-DAMP (100 nM) blocked the effect of muscarine. The effect was reversible. **B**, bar graph showing the ability of 4-DAMP (100 nM) to block the reduction of autaptic IPSC amplitude induced by muscarine ($n = 4$). Data were normalized according to the control period. **C**, image showing the result of a double-labelling immunofluorescence experiment performed using an anti-M3 muscarinic receptor antibody (red) and a tyrosine hydroxylase (TH) antibody (green). Note that M3-like immunoreactivity is observed in non-TH (presumed GABAergic) neurones. Scale bar: $15 \mu\text{m}$.

the activity of VTA/SN neurones and in particular on GABAergic interneurons (Garzon *et al.* 1999; Forster & Blaha, 2000; Fiorillo & Williams, 2000). GABAergic neurones located within the ventral mesencephalon represent a minority and are thus difficult to study in intact tissue. In the current work, we took advantage of a primary culture model to determine how ACh acts on VTA/SN GABAergic neurones.

We found that stimulation of cholinergic receptors had a general excitatory effect on GABAergic neurones. The natural neurotransmitter ACh induced a major increase in sIPSC frequency reflecting a general increase in the firing of spontaneously active GABAergic neurones. Both nicotinic and muscarinic receptor pathways were involved in this effect. Additional experiments focused on characterizing the muscarinic component. We found that muscarine increased the firing frequency of isolated GABAergic neurones. This effect was accompanied by mild depolarization. The increase in firing rate induced by muscarine was completely Ca^{2+} dependent since it was blocked by BAPTA. Finally, 4-DAMP (100 nM) inhibited the effects of muscarine, arguing for the involvement of muscarinic M3 receptors. Somatodendritic immunolabelling for M3 receptors supports the hypothesis of an expression of this receptor in GABAergic neurones and its probable participation in the excitatory effects of muscarine. In this report, we have also shown that muscarine inhibits the amplitude of IPSCs evoked by single action potentials in isolated GABAergic neurones, suggesting that muscarinic receptors also act at the terminal level to inhibit GABA release.

Excitatory action of muscarinic receptors

Although some heterogeneity was present, muscarine generally caused an increase in firing rate. The mild depolarization induced by muscarinic receptor activation probably underlies this increase. Other mechanisms regulating cell excitability such as modulation of spike AHP or voltage-dependent K^+ channels appear not to be involved under our experimental conditions. Muscarinic receptors have been found to regulate a large number of different conductances such as several K^+ currents including GIRK-type K^+ currents, M current, S_{K} -type Ca^{2+} -activated K^+ currents and leak-type currents (Adams *et al.* 1982; Cole & Nicoll, 1984; Madison *et al.* 1987; Uchimura & North, 1990). In addition, muscarinic receptors are known to activate non-selective cationic currents (NSCCs) mediated by receptor-operated channels (ROCs) or store operated channels (SOCs) (Colino & Halliwell, 1993; Guerineau *et al.* 1995).

Although our experiments did not allow us to demonstrate significant changes in whole-cell membrane resistance in the presence of muscarine, this finding does not discount the implication of cationic currents because NSCCs such as those mediating Ca^{2+} release-activated Ca^{2+} currents are known to have very small unitary conductances (Zweifach & Lewis, 1993). Our failure to detect an overall change in membrane resistance in response to muscarine could thus simply be due to insufficient signal to noise ratio, as previously noted in previous work on the excitatory action of muscarinic receptors on neurones (Shalinsky *et al.* 2002). In addition, we cannot exclude that muscarinic receptor stimulation excites GABAergic neurones by some simultaneous increase in a cationic conductance coupled with the closure of some leak-type K^+ channels, leading to a lack of overall change in membrane resistance. We have not directly characterized the biophysical properties of ionic conductances mediating the excitation of VTA/SN GABAergic neurones by muscarine. However, our finding that this excitation is fully Ca^{2+} dependent provides other evidence in favour of the hypothesis that NSCCs are involved. Additional supporting evidence comes from our recent observation that muscarine induces Ca^{2+} influx independently of membrane depolarization in GABAergic neurones in our primary culture model (F. Michel, P. Martel and L.-E. Trudeau, unpublished observations). This influx was found to be insensitive to the Ca^{2+} -store depleting drug thapsigargin, and could thus be mediated through ROC-type channels.

Recent work has shown that muscarinic receptors activate Ca^{2+} -dependent NSCCs in entorhinal cortex neurones and in CA1 hippocampal pyramidal neurones (Fraser & MacVicar, 1996; Klink & Alonso, 1997a,b; Kuzmiski & MacVicar, 2001; Shalinsky *et al.* 2002). In entorhinal cortex neurones, muscarinic receptor activation causes physiological effects that are essentially the same as those found in the current report: increase in firing rate, Ca^{2+} dependency, depolarization and virtually no change in input resistance.

Pharmacological and molecular characterization of NSCCs is currently very partial. Recent reports from a number of groups have proposed that mammalian homologues of 'transient receptor potential' (TRP) channels, a new family of channels cloned from *Drosophila*, could contribute to the assembly of NSCC-type channels (Minke & Cook, 2002; Zitt *et al.* 2002). Interestingly, channels assembled from TRP subunits form Ca^{2+} -permeable cationic channels that are regulated by muscarinic receptors such as M1, M3 or M5 (Boulay *et al.* 1997; Zhu *et al.* 1998; Zhang & Saffen, 2001). Although speculative at

the moment, a reasonable hypothesis is that the excitatory effects mediated by muscarinic receptors in VTA/SN GABAergic neurones are mediated by channels containing TRP subunits.

Multiple mechanisms of action through muscarinic receptors

Chelation of intracellular Ca^{2+} with intrapipette BAPTA not only inhibited the excitatory action of muscarine, but actually revealed an inhibitory effect. This finding suggests that muscarinic receptors have multiple targets in GABAergic neurones leading to parallel regulation of different conductances. In our experiments, the net effect was usually excitatory. However, the existence of simultaneous inhibitory and excitatory mechanisms could provide a partial explanation of the heterogeneous response profile found in our preparation. GIRK-type channels are good candidates to explain the inhibitory effect of muscarine in the presence of BAPTA. Indeed, muscarinic receptors are known to regulate such K^+ channels (Sui *et al.* 1999; Fernandez-Fernandez *et al.* 1999). Our finding that tertiapin prevented the membrane hyperpolarization and decrease in firing rate caused by muscarine provides strong support for this hypothesis. Furthermore, our observation that the proportion of GABAergic neurones that were depolarized by muscarine increased in the presence of tertiapin also supports the idea that activation of GIRK channels limits the ability of an independent depolarizing mechanism to excite GABAergic neurones.

Although the simplest hypothesis is that M3-like receptors activate in parallel NSCC-like channels, leading to excitation, and GIRK-type channels, leading to inhibition, an alternative explanation is that the tertiapin-sensitive inhibitory response is mediated through a second muscarinic receptor, such as M2 or M4. Indeed, M2, M3, M4 and M5 proteins have previously been detected in the ventral mesencephalon by Levey (1993). This author hypothesized that M2 and M4 receptors are expressed in the mesencephalon on afferent fibres from pontine nuclei. However, it is not impossible that a subpopulation of GABAergic neurones express these receptors, even though their mRNAs have not been detected. We have thus not formally excluded the possibility that the GIRK-mediated inhibitory response to muscarine revealed in the presence of BAPTA is mediated by M2 or M4 receptors in VTA GABAergic neurones. However, considering the lack of residual inhibitory response in the presence of 4-DAMP, the involvement of non-M3 receptors remains less likely.

Considering that M3 and M5 muscarinic receptors signal through phospholipase C, IP₃, DAG, Ca²⁺ and PKC, a number of other ionic channels in addition to NSCCs could be regulated by muscarinic receptors at the somatodendritic and terminal level. The inhibition of GABA release identified in our experiments on autaptic IPSCs could perhaps be caused by activation of some K⁺ conductance at the terminal level. This would lead to a reduced activation of voltage-gated Ca²⁺ channels in nerve terminals and to a reduction in action potential-evoked GABA release. Such a model would be compatible with our finding of a lack of reduction in the frequency of spontaneous miniature synaptic events, which would not be expected to be influenced by terminal polarization. A more direct, negative regulation of voltage-gated Ca²⁺ channels in nerve terminals by muscarinic receptors could also explain our observations. Additional experiments will be necessary to explore these possibilities.

Role of M3 receptors

In our experimental model, the majority of GABAergic neurones were immunopositive for M3-like receptors. Together with our finding that the preferential M3 receptor antagonist 4-DAMP completely blocked muscarine-induced cell firing and presynaptic inhibition, these data suggest that M3 receptors mediate, at least in part, the physiological effects of ACh on VTA/SN GABAergic neurones. Our results are compatible with previous data showing that M3-like receptors mediate inhibition of IPSCs evoked in a VTA slice preparation by extracellular stimulation (Grillner *et al.* 2000). However, the presence of M3 receptors has not been conclusively demonstrated in VTA/SN neurones by *in situ* hybridization in intact tissue. If this isoform is predominantly expressed in the numerically less numerous GABAergic neurones in the VTA/SN region, confirmation of its presence by *in situ* hybridization or immunostaining in intact tissue may require careful double-labelling studies. The possible contribution of M5 receptors cannot be excluded at present. This isoform has been shown to be abundant in the mesencephalic region by *in situ* hybridization (Weiner *et al.* 1990). Because pharmacological tools are not sufficiently selective to clearly discriminate between M3 and M5 receptors, additional experiments with M5 or M3 knockout mice or with the single-cell RT-PCR technique will be required to determine the relative contribution of these receptors to the effects observed in the present report.

Functional considerations

VTA/SN GABAergic neurones exert continuous tonic inhibition on dopamine neurones (Westerink *et al.* 1996) and are likely to play a role in fine tuning of their firing rate. The important role of these neurones is highlighted by previous work showing that opiates exert their stimulatory effect on the firing of dopamine neurones at least partly by inhibiting local GABAergic interneurones in the VTA/SN (Johnson & North, 1992a), thus leading to disinhibition.

The LDT and PPT send dual projections containing ACh and glutamate to the ventral mesencephalon. Dopamine neurones receive direct glutamatergic input while a majority of the cholinergic innervation apparently innervates local GABAergic neurones (Garzon *et al.* 1999). Cholinergic agonists or electrical stimulation of cholinergic afferents that project to the VTA and SN induce an increase in the activity of dopamine and GABAergic neurones in brain slices, together with a long-lasting elevation of extracellular DA *in vivo* which has been shown to require M5 muscarinic receptors (Forster & Blaha, 2000; Yeomans *et al.* 2001; Forster *et al.* 2002). This elevation in dopamine level is multiphasic and involves nicotinic, glutamatergic and muscarinic receptors. The excitatory muscarinic part of the response is delayed and begins several minutes after stimulation. This notable time delay argues for the involvement of a complex integrated response that may implicate local GABAergic interneurones. Nonetheless, the specific physiological contribution of an increase in the activity of GABAergic neurones in response to cholinergic activation is at present unclear. It is possible that coactivation of GABAergic neurones, possibly through M3-like receptors, may prevent dopamine neurones from reaching a state of 'depolarization block' (Grace & Bunney, 1986), thus allowing them to maintain their ability to release DA tonically in projection areas. A formal test of such hypotheses will require the use of M3 knockout mice or the use of novel and more selective M3 receptor antagonists.

In conclusion, we have demonstrated that the activation of cholinergic receptors induces a strong excitatory effect on VTA/SN GABAergic interneurones in culture. In particular, we found that muscarinic receptor stimulation increases the firing rate of these neurones through a Ca²⁺-dependent mechanism. This enhancement in firing is accompanied by a decrease in the amplitude of IPSCs evoked by single action potentials. Both of these effects appear to depend on M3-like muscarinic receptors. Together, our results provide the first direct physiological evidence in support of the hypothesis of a regulation

of mesencephalic GABAergic neurones by cholinergic receptors.

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