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Activation of midbrain presumed dopaminergic neurones by muscarinic cholinergic receptors: an *in vivo* electrophysiological study in the rat

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1 Extracellular single-unit recording and iontophoresis were used to examine the effects of different cholinoceptor agonists and antagonists on the firing rate and firing pattern of A9 and A10 presumed dopaminergic neurones in the anaesthetized rat.

2 Administration of low currents (1–5 nA) of the selective muscarinic agonists oxotremorine M (Oxo M) and muscarine and of the non-selective muscarinic/nicotinic agonist carbamylcholine (CCh) produced a dose-dependent increase in firing rate in most of the A9 and A10 presumed dopaminergic neurones tested. Oxo M-induced activation could be completely blocked by iontophoretic application of the muscarinic antagonist butyl-scopolamine or systemic administration of the muscarinic antagonist scopolamine (300 μg kg^{-1}, i.v.).

3 Iontophoretic application of the selective nicotinic agonist methylcarbamylcholine (MCCh), but not nicotine, induced a consistent increase in firing rate. Surprisingly, the excitatory effect of MCCh was significantly reduced by the selective muscarinic antagonist scopolamine (300 μg kg^{-1}, i.v.), but not by the selective nicotinic antagonist mecamylamine (2.2 mg kg^{-1}, i.v.). Mecamylamine (3 mg kg^{-1}, i.v.) was also ineffective in reducing the CCh-induced activation of presumed dopamine neurones, suggesting that both CCh and MCCh increased the activity of dopamine neurones via an interaction with muscarinic receptors.

4 Iontophoretic application of the endogenous agonist acetylcholine (ACh) had no or little effect on the firing activity of A10 presumed dopaminergic neurones. However, concomitant application of neostigmine, a potent cholinesterase inhibitor, with acetylcholine induced a substantial activation of these neurones. This activation consisted of two components; one, which was prevalent, was scopolamine (300 μg kg^{-1}, i.v.)-sensitive, and the other was mecamylamine (2 mg kg^{-1}, i.v.)-sensitive.

5 In addition to their effect on firing activity, Oxo M, muscarine and concomitant neostigmine/ACh caused a significant increase in burst firing of A10 neurones, but not of A9 neurones.

6 These data suggest that dopamine cells, both in the A9 and A10 regions, possess functional muscarinic receptors, the activation of which can increase their firing rate and, for A10 neurones, their amount of burst activity. These cholinoceptors would be able to influence the activity of the midbrain dopamine system greatly and may play a role in, and/or be a therapeutic target for, brain disorders in which dopamine is involved (e.g., Parkinson's disease, drug addiction and schizophrenia).

Keywords: Acetylcholine; muscarinic receptors; nicotinic receptors; ventral tegmental area; substantia nigra compacta; microiontophoresis; extracellular recording

Introduction

A great deal of evidence exists for a neuroanatomical relation between the dopaminergic and cholinergic systems in the brain. Tracing studies have shown that dopamine-containing neurones in the rat substantia nigra compacta (SNC) and ventral tegmental area (VTA) receive moderate to dense innervations from mesopontine cholinergic cells (Bolam *et al*., 1991; Henderson & Sheriff, 1991; Oakman *et al*., 1995). High concentrations of choline acetyl transferase and of the degradative enzyme acetylcholinesterase have been found in the vicinity of dopaminergic cells in the two midbrain dopaminergic nuclei (Butcher, 1977a,b; Butcher & Woolf, 1982; Greenfield, 1991). Other cholinergic markers such as the amine base choline (Kobayashi *et al*., 1975) and a sodium-dependent high affinity choline uptake system (Massey & James, 1978) are also present.

Binding studies provide evidence suggestive of a nicotinic-cholinergic innervation of dopaminergic neurones. Nicotinic receptors are present in appreciable density in midbrain dopaminergic nuclei in the rat and man (Clarke *et al*., 1985a). Some of these receptors are likely to be located on the dopaminergic neurones since their density is markedly reduced following a selective lesion of the dopaminergic cells with 6-hydroxydopamine (Clarke & Pert, 1985).

Numerous studies have demonstrated a nicotinic influence on rat midbrain dopaminergic neuronal activity. For example, in *in vivo* experiments, local administration of nicotine directly into the VTA or into the SNC accelerates the efflux of dopamine in dopaminergic projection areas such as the nucleus accumbens and striatum (Blaha & Winn, 1993; Nisell *et al*., 1994a; Blaha *et al*., 1996). Systemic administration of nicotine also increases the neuronal release of dopamine in the nucleus accumbens and frontal cortex, via the stimulation of nicotinic receptors located in the ventral tegmental area (Nisell *et al*., 1994b). In keeping with these neurochemical studies, electrophysiological experiments have shown that acute peripheral administration of nicotine increases both the firing activity and the burst firing of midbrain dopaminergic neurones (Clarke *et al*., 1985b; Grenhoff *et al*., 1986; Mereu *et al*., 1987; Nisell *et al*., 1996). Such an effect is thought to be due in part to

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activation of nicotinic receptors located in the VTA, since *in vitro* intracellular recordings have demonstrated that nicotine induces a tetrodotoxin insensitive depolarization and increase in firing of VTA dopaminergic neurones (Calabresi *et al*., 1989).

On the other hand, a muscarinic influence on the rat dopaminergic system is less well documented. Despite the fact that *in situ* hybridization studies have demonstrated that dopaminergic cells, both in the VTA and the SNC, contain mRNA coding for muscarinic receptors (Weiner *et al*., 1990; Vilaro *et al*., 1990), these two nuclei have a low density of muscarinic receptors (Reisine *et al*., 1979; Cross & Wadington, 1980; Mash & Potter, 1986). However, there is some evidence from microdialysis and voltametry experiments *in vivo* that muscarinic receptor activation in both the SNC and VTA stimulates dopamine turnover in dopaminergic terminal regions (Gongora-Alfaro *et al*., 1991; Grenhoff & Svenson, 1992). Little attention has been paid to a possible physiological role of muscarinic receptors on the firing activity of midbrain dopaminergic neurones. Only one previous *in vitro* electrophysiological study has shown that bath application of muscarine depolarizes midbrain dopaminergic neurones (Lacey *et al*., 1990).

Finally, though the experiments presented above seem to indicate that cholinoceptors, both muscarinic or nicotinic, have excitatory effect on midbrain dopaminergic activity, local application of acetylcholine to midbrain dopaminergic neurones has been found to exert no or little effects in *in vivo* electrophysiological investigations (Aghajanian & Bunney, 1974; Collingridge & Davies, 1981; Pinnock & Dray, 1982; Scarnati *et al*., 1986), with the exception of the study of Lichtensteiger *et al*. (1982).

In the present electrophysiological study, we have undertaken, using *in vivo* extracellular recording and microiontophoresis techniques, a more detailed evaluation on the effects of different muscarinic and nicotinic agonists on the firing activity of presumed midbrain dopaminergic neurones.

Methods

Male Sprague-Dawley rats were anaesthetized with chloral hydrate (400 mg kg^{-1}) and mounted in a stereotaxic apparatus. Extracellular single unit recordings were made with 5-barrel microiontophoretic pipettes with a central recording barrel, 3 surrounding barrels for drug ejection and a balance channel filled with a 2 M NaCl solution for current neutralization. The recording barrel was filled with a 2 M NaCl solution. Action potentials were screened via a differential amplitude discriminator, which generated square pulses. These pulses were fed to a strip chart recorder (Gould Instruments, Cleveland, OH) and a computer (Macintosh II) which generated firing rate histograms with software for electrophysiology (Spike II, Cambridge Electronic Design, Cambridge, U.K.). The tips of the recording electrodes were broken back under microscope control to a 3–8 mm diameter. Fibreglass strands (100 μm) were added to each barrel including the recording barrel. The side barrels used for drug ejection were filled with the following solutions: acetylcholine 10 or 20 mM, NaCl 150 mM, pH 4; bethanechol 10 or 30 mM, NaCl 200 mM, pH 4.5; butyl-(−)-scopolamine 20 mM, NaCl 150 mM, pH 4.5; carbachol 5 mM, NaCl 200 mM, pH 4.5; DA 0.5 M, NaCl 50 mM, pH 4; L-glutamate 10 mM, NaCl 120 mM, pH 5.5; McN-A-343 10 or 20 mM, NaCl 200 mM, pH 4; methylcarbachol 10 mM, NaCl 200 mM, pH 4.5; (+)-muscarine 5 mM , NaCl 200 mM, pH 4; neostigmine 20 mM, NaCl 150 mM, pH 4; (−)-nicotine 30 mM 120 mM, pH 4; oxotremorine M 5 mM, NaCl 200 mM, pH 4. McN-A-343 ((4-hydroxy-2-butinyl)-1-trimethylammonium-M-chlorocarbanilate chloride, L-glutamate and (−)-scopolamine were purchased from Sigma (St. Louis, MO), the other drugs tested were purchased from Research Biochemical International (Natick, MA).

Presumed dopaminergic neurones were identified by their location within the VTA (or A10 region; 4.8–6.0 mm anterior to bregma and 0.3–1 mm lateral to the medline, 6.5 to 9 mm below the cortical surface) and the SNC (or A9 region; 4.8–6 mm anterior to bregma, 1.8 to 2.4 mm lateral to the medline, 6 to 8.5 mm below the cortical surface), and well established electrophysiological criteria (Grace & Bunney, 1983) including: (1) spontaneous firing rate between 5 and 90 spikes 10 s^{-1} (occurring sometimes in bursts); (2) triphasic or biphasic waveforms, with an initial positive deflection, (usually notched) followed with a prominent negative phase; (3) long action potential (duration 2–4 ms); and, (4) low pitch sound when monitored by an audioamplifier.

Administration of cholinoceptor drugs

Intravenous injections of (−)-scopolamine, physostigmine and mecamylamine (solubilized in saline pH 6) were administered via a lateral tail vein. With the exception of glutamate, which was ejected as an anion, all the drugs were ejected at small positive currents. Ranges of iontophoretic ejecting currents were 1 to 7 nA, but for cholinoceptor agonists such as oxotremorine M and muscarine, currents higher than 3 nA were often found to induce a depolarization of the cell tested. All applications of the excitatory substances were of 60–120 s. A small current (from 3 to 5 nA), of opposite polarity to the ejecting current, was used for retaining each compound between microiontophoretic applications.

Calculations

The degree of activation induced by excitatory substances was calculated by measuring the number of supplementary spikes generated per nC (1 nC being the charge generated by 1 nA applied for 1 s). The effects of the administration of cholinoceptor drugs were assessed by comparing the levels of neuronal activation before and after their i.v. administration, or before and during their long lasting microiontophoretic application. Burst analysis was made by use of a slightly modified version of the Spike II program. The onset of a burst was defined by an interspike interval shorter than 80 ms and the termination of a burst by the next interval longer than 160 ms (Grace & Bunney, 1984). Cells were considered as burst firing cells if they fire at least two bursts of three of more spikes during an analysis period of 300 s. Intervals below 20 ms were automatically ignored in the analysis. For each cell, firing pattern was analysed within a period of 40–200 s before drug ejection and was compared to the firing pattern within the same period during the drug ejection. This period of analysis was adjusted in order to perform an analysis of 220 spikes before the drug ejection.

Statistical analysis

All results are expressed as the mean ± s.e.mean of the number of spikes generated nC^{-1}. Statistical significance was assessed by use of two-tailed Student's *t* test. Probability values smaller than 0.05 were considered significant.

Results

A total of 159 presumed dopaminergic neurones were studied in the VTA (A10 region) and 102 in the SNC (A9 region). Their basal firing rate was in the range of 5–90 spikes 10 s^{-1}. Only neurones which met all the four criteria mentioned in Methods were included in the present study.

Effects of cholinoceptor drugs on spontaneous activity of presumed dopaminergic cells

Figure 1 shows the firing activity of four representative dopaminergic neurones exhibiting different types of excitatory responses upon application of the muscarinic agonist oxotremorine M (Oxo M). In general, the activation induced by this agonist developed progressively and persisted 1 to 3 min after the cessation of the application (Figure 1a–c). The effective current required to induce activation was very low, ranging from 1–3 nA. The activation induced by the agonist was sometimes associated with a decrease in spike amplitude and even with the complete disappearance of extracellular spikes, particularly if currents higher than 3 nA were administered. This apparent depolarization inactivation was transient, since spike amplitudes recovered progressively after iontophoretic ejection was terminated (Figure 1d). To avoid this depolarization process, a standard current range of 1–3 nA was used to eject the different muscarinic agonists. Nevertheless, even at low currents of agonist, some cells were still hypersensitive and depolarized upon drug application (Figure 1d).

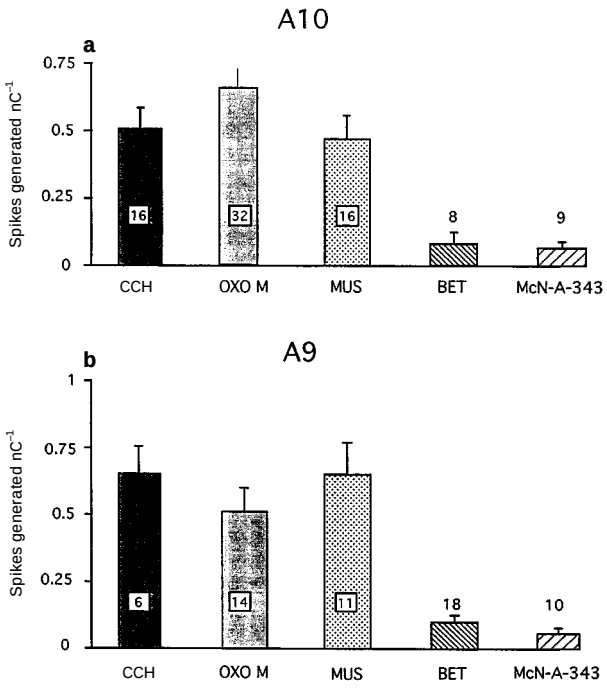

Figure 2 Responsiveness, expressed as the number of spikes generated nC^{-1} (mean ± s.e.mean) of dopaminergic neurones recorded in the A10 (a) and A9 (b) regions to microiontophoretic applications of the muscarinic agonists: carbamylcholine (CCH), oxotremorine M (OXO M), muscarine (MUS), McN-A-343 and bethanechol (BET). Neurones were tested by use of a standard current range of 1–3 nA.

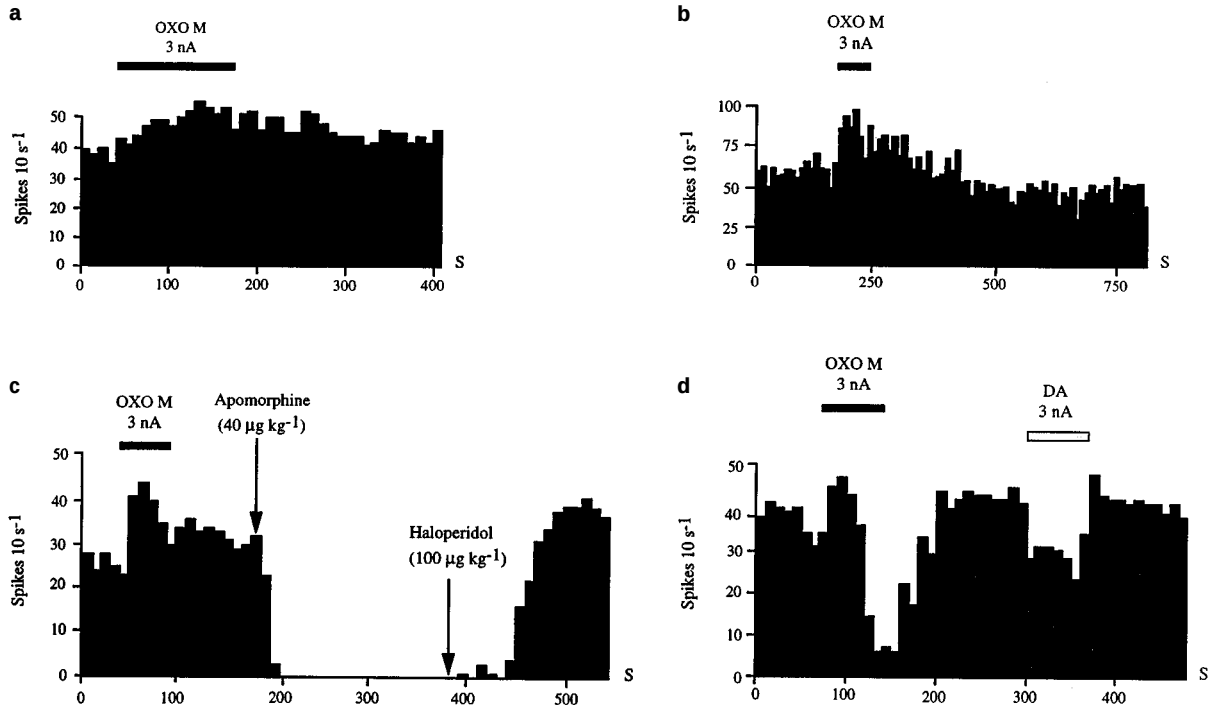

Figure 1 Firing rate histograms showing the individual response of four representative A9 or A10 midbrain dopaminergic neurones to iontophoretic application of low currents of oxotremorine M (OXO M). In (a), (b), and (c) the excitatory response persisted several minutes after the cessation of the application. During its application, oxotremorine M induced an average increase of 11 spikes 10 s^{-1} in neurone a (0.37 spikes generated nC^{-1}), 29 spikes 10 s^{-1} in neurone b (0.97 spikes generated nC^{-1}), and 15 spikes 10 s^{-1} in neurone c (0.5 spikes generated nC^{-1}). In (d), the low current administered was sufficient to induce an apparent transient depolarization inactivation. For this neurone, it was not possible to calculate the number of additional spikes generated by oxotremorine M. The dopaminergic nature of the neurones in (c) and (d) was confirmed by the inhibitory effect induced by apomorphine (40 $\mu g\ kg^{-1}$, i.v.) or iontophoretic dopamine (DA). Bars indicate the duration of the application of a compound for which currents are given in nA.

The mean neuronal response of A9 and A10 dopaminergic neurones to iontophoretic application, in the same short range of currents (1–3 nA), of the 5 muscarinic agonists, muscarine (mean current administered: 2.8±0.1 and 2.7±0.2 nA, in A9 and A10, respectively), oxotremorine M (Oxo M; mean current administered: 2.4±0.1 and 2.4±0.2 nA, in A9 and A10, respectively), carbamylcholine (CCh; mean current administered: 2.6±0.1 and 2.4±0.1 nA, in A9 and A10, respectively), bethanechol (mean current administered: 3±0 nA in A9 and A10) and McN-A-343 (mean current administered: 3±0 nA in A9 and A10) is shown in Figure 2. The responses evoked by muscarine, CCh and Oxo M had very similar characteristics. As observed for Oxo M, the microiontophoretic application of muscarine or CCh produced a progressive activation which decreased slowly after the cessation of the application, and was sometimes associated with a decrease in spike amplitude. Comparison between A9 and A10 neurones showed no difference in their responses to the agonists (Figure 2). In both nuclei, more than 90% of the neurones tested responded with an increase in firing rate (ranging from 25% to 200%) upon application of a low current of Oxo M, CCh and muscarine. Responses were reproducible on subsequent applications. Muscarine, CCh and Oxo M applications produced higher responses than those obtained with the other agonists, though there was a variability in the magnitude of the response between cells. Less than half of the neurones tested, both in the A9 and A10 regions, showed even a slight enhancement of their discharge frequency during McN-A-343 and bethanechol application (Figure 2). Higher ejecting currents were not more effective in promoting cell activation. Higher concentrations of McN-A-343 or bethanechol (20–30 mM) in the ejecting channels only slightly increased the sensitivity of the cells to the agonists (data not shown).

The endogenous agonist acetylcholine (ACh) was also found to have a very poor excitatory effect on A10 dopaminergic neurones (Figure 3). However, during the long lasting application of the cholinesterase inhibitor neostigmine, the neuronal response to iontophoretic application of acetylcholine was dramatically enhanced (Figure 3). Some cells which were insensitive to acetylcholine application were found to be responsive to the co-application of ACh and neostigmine. As observed with other muscarinic agonists, the co-application of ACh and neostigmine produced an apparent depolarization inactivation in some neurones. A similar potentiating effect was observed after the systemic administration of the centrally active cholinesterase inhibitor physos-

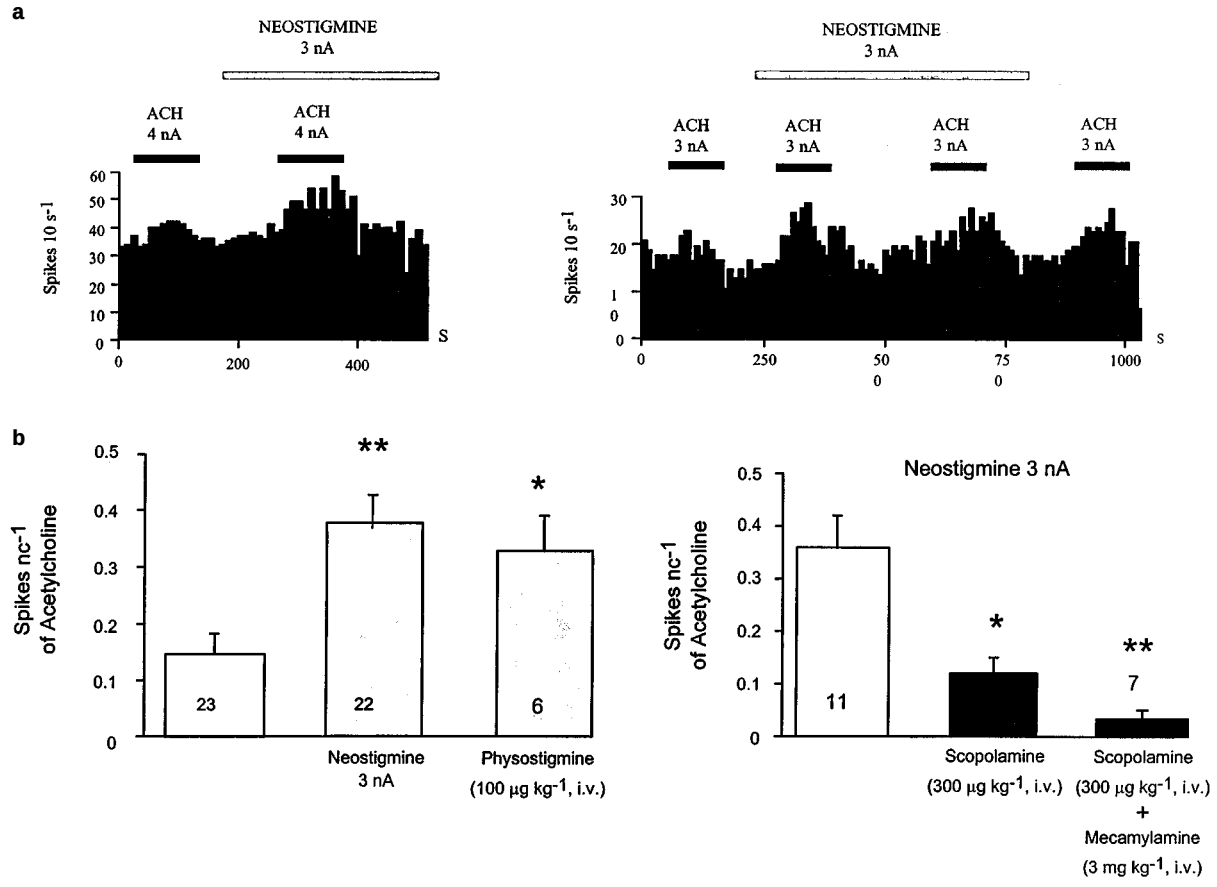

Figure 3 (a) Firing rate histograms showing the response of two A10 dopaminergic neurones to microiontophoretic applications of acetylcholine (ACH), before and during the long-lasting microiontophoretic application of the cholinesterase inhibitor neostigmine. Bars indicate the duration of the application of a compound for which currents are given in nA. (b) Left: responsiveness, expressed as the number of spikes generated nC^{-1} (mean±s.e.mean), of VTA dopaminergic neurones to the application of acetylcholine, with or without the concomitant application of the cholinesterase inhibitor neostigmine, and after the i.v. administration (100 $\mu g\ kg^{-1}$) of the brain-penetrating cholinesterase inhibitor physostigmine. $^{**}P<0.001$; $^{*}P<0.03$, compared to the value obtained with acetylcholine alone; unpaired Student's t test. Right: responsiveness, expressed as the number of spikes generated per nC (mean±s.e.mean), of VTA dopaminergic neurones to the concomitant application of acetylcholine and neostigmine, before and after the subsequent administration of the muscarinic antagonist scopolamine (300 $\mu g\ kg^{-1}$) and the nicotinic antagonist mecamylamine (3 mg kg^{-1}). $^{*}P<0.01$, compared to the control value; paired Student's t test; $^{**}P<0.001$, compared to the value obtained after scopolamine administration, paired Student's t test. In this series of experiments, the same neurones were recorded during the complete sequence.

tigmine (100 μg kg^{-1}, i.v., Figure 3b). Neostigmine application and physostigmine administration by themselves were found to have no significant effect on the firing rate of the dopaminergic neurones (before and after physostigmine administration: 43.3$\pm$4.1 and 43.8$\pm$4.17 spikes 10 s^{-1}, $n=6$, respectively; before and during neostigmine application: 27.6$\pm$3.9 and 28.6$\pm$4.1 spikes 10 s^{-1}, respectively). The activation induced by the co-application of ACh and neostigmine dramatically reduced following the i.v. administration of the muscarinic antagonist scopolamine (300 μg kg^{-1}, i.v., Figure 3b). The scopolamine-insensitive remaining component of this activation was suppressed following the administration of the nicotinic antagonist mecamylamine (3 mg kg^{-1}, i.v., Figure 3b).

In both nuclei, the neuronal activation induced by Oxo M was abolished following the administration of the selective muscarinic antagonist (−)-scopolamine (300 μg kg^{-1}, i.v., Figure 4). This blocking effect was observed at a dose that did not affect the glutamate-induced activation (Figure 4). By analysing the firing activity immediately after the scopolamine administration, a depression of the firing activity was sometimes observed (Figure 4a). However, this depression occurred when the firing rate was still activated as a result of the protracted response induced by the muscarinic agonists. By comparing the firing rate preceding the first application of the agonist with the

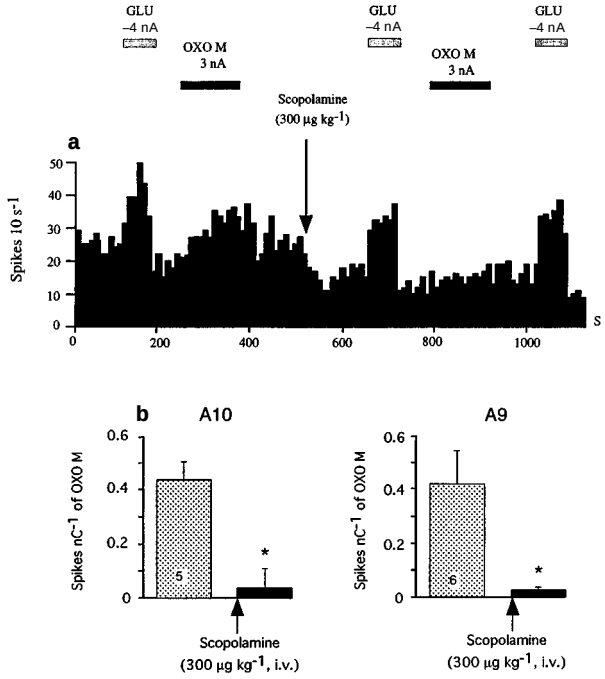

Figure 4 (a) Firing rate histogram showing the response of one A10 dopaminergic neurone to microiontophoretic applications of oxotremorine M (OXO M) and glutamate (GLU), before and after the i.v. administration of the muscarinic antagonist (−)-scopolamine (300 μg kg^{-1}). Bars indicate the duration of the application of a compound for which currents are given in nA. (b) Responsiveness, expressed as the number of spikes generated nC^{-1} (mean$\pm$s.e.mean), of VTA (A10) and SNC (A9) dopaminergic neurones to the application of oxotremorine M, before and after the i.v. administration of the muscarinic antagonist (−)-scopolamine (300 μg kg^{-1}). In this series of experiments, the same neurones were recorded during the complete sequence of drug administration. *$P<0.01$, compared to the corresponding value before the administration of (−)-scopolamine; paired Student's t test.

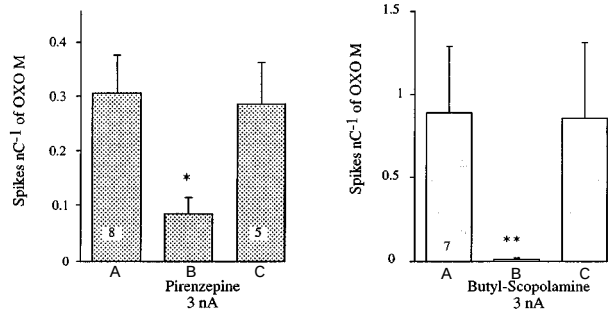

Figure 5 Responsiveness, expressed as the number of spikes generated nC^{-1} (mean$\pm$s.e.mean), of VTA dopaminergic neurones to applications of oxotremorine M (OXO M) before (A), during (B) and after (C) the iontophoretic application of the antimuscarinic agents butyl-(−)-scopolamine and pirenzepine. In this series of experiments, the same neurones were recorded during the complete sequence of drug administration. **$P<0.001$, *$P<0.04$, compared to the corresponding values before the application of the antagonists; paired Student's t test.

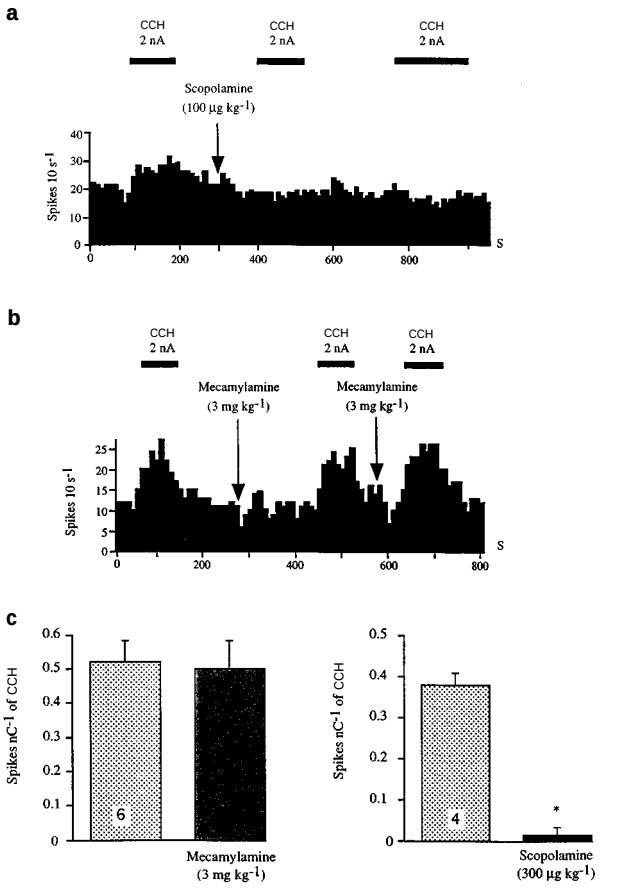

Figure 6 (a) Firing rate histogram showing the response of an A10 dopaminergic neurone to microiontophoretic application of carbamylcholine (CCH) before and after the i.v. administration of the muscarinic antagonist (−)-scopolamine (100 μg kg^{-1}). (b) Firing rate histogram showing the response of an A9 dopaminergic neurone to microiontophoretic application of carbamylcholine (CCH) before and after the i.v. administration of the nicotinic antagonist mecamylamine (6 mg kg^{-1}). (c) Responsiveness, expressed as the number of spikes generated nC^{-1} (mean$\pm$s.e.mean), of midbrain dopaminergic neurones to applications of carbamylcholine (CCH) before and after the i.v. administration of mecamylamine (3 mg kg^{-1}) (left), and before and after the i.v. administration of (−)-scopolamine (300 μg kg^{-1}) (right). Bars indicate the duration of the application of a compound for which currents are given in nA. In this series of experiments, the same neurones were recorded during the complete sequence. *$P<0.01$, compared to the corresponding values before the administration of scopolamine.

firing rate occurring after the administration of scopolamine, there was in fact no evidence for a significant decrease in basal firing activity (before and after scopolamine administration: 34.2 ± 3.1 and 31.3 ± 2.6 spikes $10\ s^{-1}$, $n=24$, respectively for A10 neurones; 33.3 ± 6.2 and 30.7 ± 6.5 spikes $10\ s^{-1}$, $n=11$, respectively for A9 neurones). Furthermore, firing activity recorded from a single-barrel recording electrode of five dopaminergic neurones remained absolutely unchanged, even after the systemic administration of cumulative doses of (−)-scopolamine up to 1 mg kg^{-1} (data not shown). Concomitant application of butylscopolamine with Oxo M reversibly abolished the excitatory effect of the agonist (Figure 5). In addition, the preferential M_1 muscarinic antagonist pirenzepine partially reduced the level of activation induced by Oxo M (Figure 5).

To test whether the carbachol-induced activation of dopaminergic cells is mediated by muscarinic receptors, we compared the effects of (−)-scopolamine with those of mecamylamine, a selective nicotinic antagonist. In contrast to (−)-scopolamine (300 μg kg^{-1}, i.v.), which completely abolished CCh-induced activation of dopaminergic neurones, the nicotinic antagonist mecamylamine, administered at a dose largely adequate to reach central nicotinic receptors (3 mg kg^{-1}, i.v.) was without any significant effect (Figure 6). Furthermore, an additional dose of 3 mg kg^{-1} (i.v.) tested in 4 neurones was also ineffective in reducing carbachol-induced activation (Figure 6b).

Iontophoretic application of (−)-nicotine at low currents, slightly accelerated the firing activity of some A9 and A10 neurones (Figure 7a). This neuronal response was very

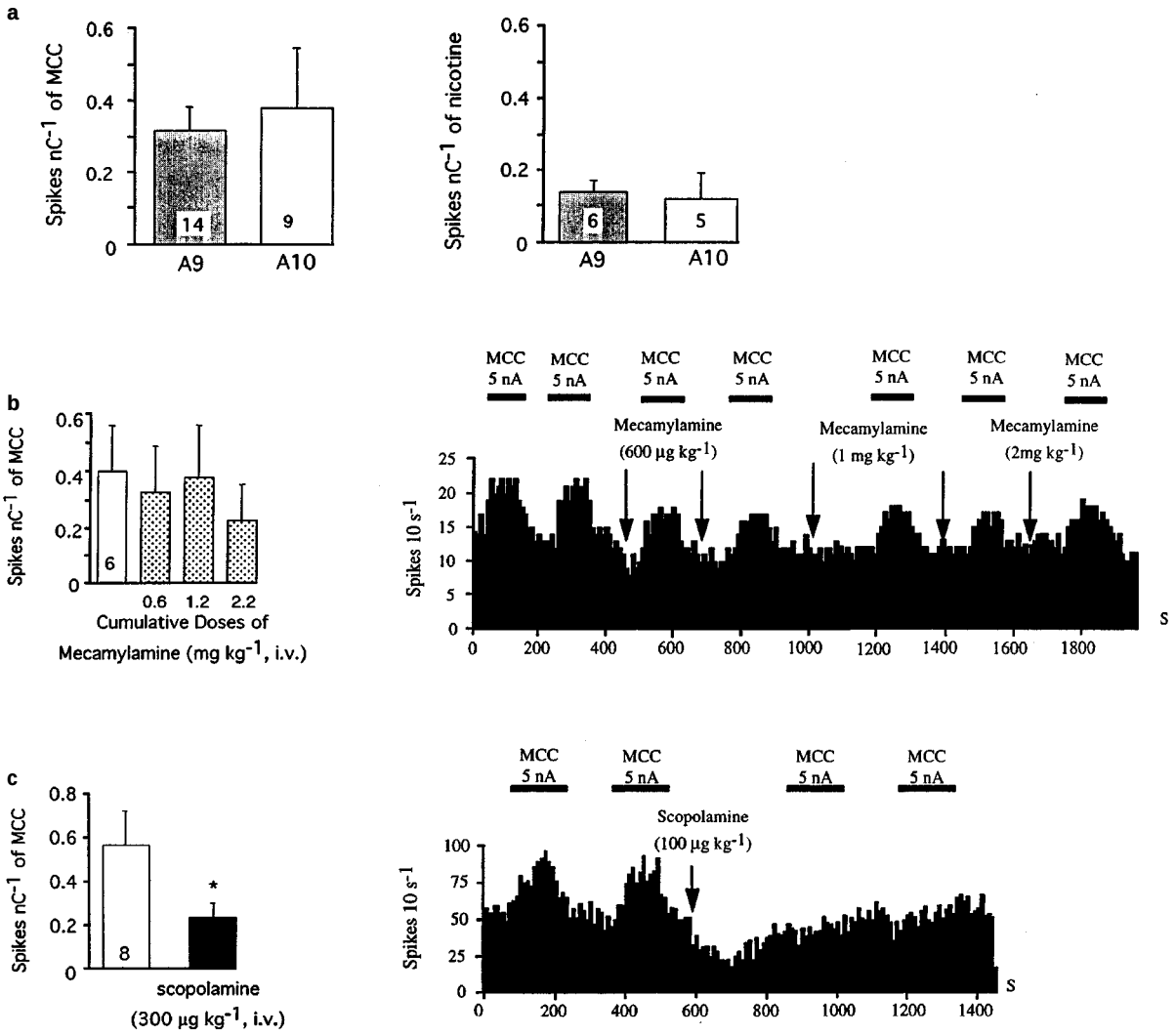

Figure 7 (a) Responsiveness, expressed as the number of spikes generated nC^{-1} (mean ± s.e.mean), of A10 and A9 dopaminergic neurones to applications of the selective nicotinic agonists (−)-nicotine and methylcarbamylcholine (MCC). (b) Left: responsiveness, expressed as the number of spikes generated nC^{-1} (mean ± s.e.mean), of midbrain dopaminergic neurones to applications of the nicotinic agonist methylcarbamylcholine (MCC), before and after three subsequent i.v. administrations of the nicotinic antagonist mecamylamine (cumulative dose: 2.2 mg kg^{-1}). In this series of experiments, the same neurones were recorded during the complete sequence of drug administration. Right: firing rate histogram of an A9 dopaminergic neurone showing the weak inhibitory effect of mecamylamine on the neuronal activation induced by methylcarbamylcholine. (c) Left: responsiveness, expressed as the number of spikes generated nC^{-1} (mean ± s.e.mean), of midbrain dopaminergic neurones to applications of the nicotinic agonist methylcarbamylcholine, before and after i.v. administration of the muscarinic antagonist (−)-scopolamine (300 μg kg^{-1}). Right: firing rate histogram of an A10 dopaminergic neurone showing the suppressive effect of (−)-scopolamine on the neuronal activation induced by methylcarbamylcholine. In this series of experiments, the same neurones were recorded during the complete sequence. Bars indicate the duration of the application of a compound for which currents are given in nA. $^*P<0.01$, compared to the corresponding values obtained before the i.v. administration of (−)-scopolamine, paired Student's t test.

low compared to that induced by the muscarinic agonists, and was not reproducible upon repeated applications. More consistent activation was obtained when the nicotinic agonist methylcarbamylcholine (MCCh) was applied (Figure 7a). However, the i.v. administration of cumulative doses (2.2 mg kg^{-1}) of mecamylamine failed to reduce significantly the MCCh-induced activation (Figure 7b). On the other hand, this activation was dramatically reduced following a single administration of (−)-scopolamine (300 μg kg^{-1}, i.v., Figure 7c). An interaction of (−)-scopolamine, at the relatively low dose it was administered, on nicotinic receptors is unlikely, since we observed that in neurones pretreated with (−)-scopolamine the systemic administration of (−)-nicotine (30 and 300 μg kg^{-1}) still exerted its known stimulating effect (data not shown). Mecamylamine by itself, was found not to alter significantly the firing activity of A9 and A10 dopaminergic neurones (before and after mecamylamine administration: 18.6±3.2 and 18.5±3.1 spikes 10 s^{-1}, $n=11$, respectively for A9 neurones; 26.1±2.8 and 24.6±2.5 spikes 10 s^{-1}, $n=14$, for A10 neurones).

Effects of cholinoceptor agonists on the firing pattern of presumed dopaminergic cells

Seventy three neurones in the VTA and twenty six neurones in the SNC were analysed for their firing pattern. The dopaminergic cells tested fired between 0 and 90% of their spikes in bursts. Oxo M, muscarine and ACh/neostigmine applications induced a significant increase in burst firing of A10 dopaminergic neurones (Figure 8). Some non-bursting neurones were brought into a burst-firing mode during the application of Oxo M (7 out of 11 cells), muscarine (3 cells out of 6) and ACh/neostigmine (6 cells out of 9). The elevation in the percentage of spikes occurring in bursts following muscarine and Oxo M applications was associated with an increase in burst frequency (the interburst interval decreased from: 5.2±1.1 to 2.3±0.5 s during muscarine application, $n=11$, $P<0.02$; and from 8.3±2.0 to 2.1±0.4 s during Oxo M application, $n=18$, $P<0.001$) and an increase in spikes per burst (from 2.3±0.5 to 2.9±0.2, $n=11$, $P<0.01$, during muscarine application and from 2.5±0.1 to 4.1±0.5, during Oxo M application, $n=18$, $P<0.01$). On the other hand, the increase in burst firing of A10 cells induced by CCh was not significant, despite four cells showing an increase in burst firing of more than 50% during local application of the agonist. In contrast to A10 dopaminergic neurones, the A9 neurones tested did not respond to Oxo M, muscarine or CCh with a significant increase in burst firing activity (before and during Oxo M application: 14.0±7.6% and 20.0±7.0%, $n=9$, respectively; before and during muscarine application: 2.7±0.8% and 6.8±3.7%, $n=11$, respectively; before and during CCh application: 4.6±2.0% and 6.3±2.5%, $n=6$, respectively). Analysis of interspike interval histograms revealed that the increase in firing induced by the application of Oxo M was characterized by a large increase in short interspike intervals (ISI; <100 ms, within the same period of analysis, short ISIs varied from 35.6±7.8 to 97.1±15.5 during Oxo M application, $n=26$, $P<0.0001$, Figure 8b), while the number of long ISIs (>250 ms) did not vary significantly (within the same period of analysis, long ISIs varied from 90.8±8.6 to 89.0±10.6 during Oxo M application, $n=26$, NS, Figure 8b). Figure 8c, obtained with raw data of discharge activity, shows that the persisting presence of long ISIs during Oxo M application is often indicative of post-burst inhibitory periods. In this figure, as found in numerous traces from other neurones, the switch to a burst firing mode is clearly visible.

Discussion

Data obtained from the first series of experiments showed that local application of different selective muscarinic agonists directly onto midbrain presumed dopaminergic neurones potently stimulated the firing of these neurones. This excitatory effect was selectively abolished by the selective

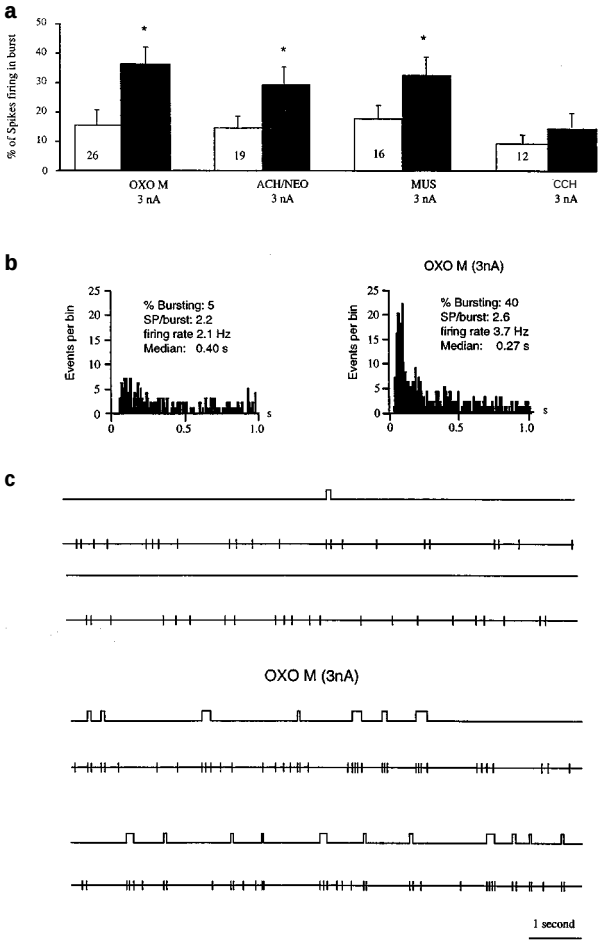

Figure 8 (a) Effect of oxotremorine M (OXO M), acetylcholine/neostigmine (ACh/NEO), muscarine (MUS) and carbamylcholine (CCH) on the burst activity of A10 dopaminergic neurones expressed as the % of spikes firing in bursts (mean±s.e.mean). *$P<0.01$, compared to the corresponding values obtained before the application of the agonists, paired Student's t test. (b) Interspike interval histograms of one representative dopaminergic cell, before (left) and during (right) the application of oxotremorine M at a low current. Histogram was constructed from time-intervals between spikes within 100 s periods, before and during oxotremorine M application. Note that during oxotremorine M application, short interspike intervals (<100 ms) increased dramatically, while long interspike intervals (>250 ms) were still present. % bursting: % of spikes firing in burst. SP/burst: average number of spikes per burst. (c) Example of firing pattern of the same neurone before and during oxotremorine M application. Before oxotremorine M application, the neurone demonstrates a typical slow, single firing pattern. During its application, the pattern changes into a burst-firing pattern. Note that the long interspike intervals (>250 ms) are often associated with post-burst silences. The notches on the upper lines indicate the presence of burst firing.

muscarinic antagonist (−)-scopolamine, which in turn was ineffective in modifying the glutamate-induced activation, confirming the specificity of the muscarinic action of the drugs tested. The apparent occurrence of depolarization inactivation observed in numerous cases indicates a marked sensitivity of the cells to the muscarinic agonists. These data are in accordance with a previous *in vitro* electrophysiological study in which intracellular recording demonstrated that (±)-muscarine depolarized and increased the firing of VTA and SNC dopaminergic neurones (Lacey *et al*., 1990). Our results also indicate that the control exerted by muscarinic receptors on the neuronal activity of midbrain dopaminergic neurones appears to be, at least in the anaesthetized rat, phasic rather than tonic, as shown by the lack of effect of scopolamine on the basal firing activity of the neurones. To our knowledge, our present data constitute the first *in vivo* experimental evidence for the presence of functional muscarinic receptors on midbrain dopaminergic neurones. This is consistent with the recent studies of Weiner *et al*. (1990) and of Villaro *et al*. (1990) which demonstrated, by use of *in situ* hybridization and fluorescence techniques, the presence of significant amounts of muscarinic receptor mRNA in VTA and SNC, with a unique localization inside the dopaminergic neurones. Paradoxically, in binding and autoradiographic studies, the density of muscarinic receptors has been found to be low in midbrain dopaminergic nuclei, with only a fraction located on dopaminergic neurones (Cross & Waddington, 1980).

In our study, no differences were observed between cells located in the VTA or in the SNC with respect to the magnitude of their response to the different muscarinic agonists. This suggests that, although SNC and VTA dopaminergic cells receive distinct cholinergic innervation from different mesopontine nuclei (Oakman *et al*., 1995), they are exposed to the same excitatory influence through cholinoceptors located on their soma. Our study concurs with some previous findings providing indirect behavioural and neurochemical evidence suggesting that muscarinic receptor activation has an excitatory influence on dopaminergic neurones, both in SNC and VTA. For example, administration of muscarinic agonists into the VTA and SNC has been shown to stimulate the metabolism of dopamine in dopaminergic projection areas (Gongora-Alfaro *et al*., 1991; Grenhoff & Svensson, 1992). In addition, muscarinic agents, microinjected in the VTA, alter some reward behaviours associated with dopaminergic stimulation (Yeomans *et al*., 1993; Yeomans, 1995). Further, the effects of microinjection of muscarinic agonists into the rat SNC on food consumption and oral stereotypy were abolished following systemic administration of low doses of haloperidol, or following the lesion of SNC dopaminergic neurones by 6-OHDA (Taha & Regrave, 1979; Parker *et al*., 1991).

On the other hand, our results indicate a weak sensitivity of midbrain presumed dopaminergic neurones to iontophoretic application of the endogenous cholinoceptor agonist acetylcholine (ACh). This result is in keeping with most previous *in vivo* electrophysiological investigations showing a lack of effect of local application of ACh on SNC dopaminergic neurones (Aghajanian & Bunney, 1974; Collingridge & Davies, 1981; Pinnock & Dray, 1982; Scarnati *et al*., 1986). The concomitant administration of the cholinesterase inhibitor neostigmine with ACh markedly increased the sensitivity of VTA dopaminergic cells to ACh, suggesting a very efficient inactivation of ACh by extraneuronal acetylcholinesterase. These data are compatible with studies demonstrating high levels of acetylcholinesterase in midbrain dopaminergic nuclei, which appeared to be released directly from dopaminergic neurones (Mash & Potter, 1986; Greenfield, 1991; Bernard *et al*., 1995). It has even been demonstrated that this enzyme exerts a modulatory effect on the activity of dopamine, which occurs independently of its catalytic site (Greenfield, 1991).

The increase in firing rate induced by Oxo M, muscarine and the ACh/neostigmine association was accompanied by a significant increase in burst firing in the VTA. This increase in burst firing was characterized by an elevation in the percentage of spikes occurring in bursts, an increase in burst frequency and an increase in the number of spikes occurring in each burst. However, it is difficult to determine whether these changes in firing pattern are simply due to an acceleration of the firing activity, since for individual neurones, increases in firing rate have been found to be highly correlated with increases in bursts (Grace & Bunney, 1984). Nevertheless, the switch from a single firing pattern to a burst firing pattern during the application of Oxo M, was often clearly visible on traces of single unit discharge, particularly in neurones showing a moderate firing activity (Figure 8b and c). In addition, the persistence of long interspike intervals during Oxo M application, despite the acceleration of firing, also appears to be indicative of an increase in burst activity (see Figure 8b and c). However, it is not clear why the muscarinic agonist carbachol was less efficacious in inducing burst firing of A10 dopaminergic cells.

Our data do not provide any clear indication of the muscarinic receptor involved in the neuronal activation of midbrain dopaminergic neurones. Molecular cloning of muscarinic receptors has demonstrated the presence of five distinct muscarinic receptors (Hulme *et al*., 1990). According to *in vitro* hybridization studies, both VTA and SNC dopaminergic cells express only M5 muscarinic receptors (Vilaro *et al*., 1990; Weiner *et al*., 1990). However, Lacey *et al*. (1990) have suggested that the muscarinic receptor responsible for the activation of dopaminergic cells recorded *in vitro* may belong to the M_1 receptor family because bath application at nanomolar concentrations of pirenzepine, a muscarinic antagonist which has a high affinity for M_1 and a relatively low affinity for M_2 receptors, inhibited this activation. However, pirenzepine is not selective enough to distinguish between receptors with intermediate affinities like the M_3, M_4 and M_5 receptors. Therefore, additional experiments are necessary to determine if the M_5 receptor mediates the cholinergic excitation of VTA and SNC dopaminergic cells.

In our experimental conditions, muscarinic receptors, more than nicotinic receptors, effectively stimulated the firing activity of presumed A10 dopaminergic neurones. As a matter of fact, we observed that nicotinic receptor stimulation, evaluated by application of ACh following scopolamine treatment, or by the direct application of nicotine, poorly activated dopaminergic neurones. Further, the activation of dopaminergic neurones induced by CCh, a mixed muscarinic/nicotinic agonist, and methyl-carbamyl choline (MCCh), a compound which has been shown to be a selective nicotinic agonist (Abood & Grassi, 1986; Boksa *et al*., 1989; Marks *et al*., 1993), did not appear to be mediated by nicotinic receptor stimulation. These excitatory effects were unchanged by the brain-penetrating nicotinic antagonist mecamylamine. This drug, in addition, did not modify significantly the firing rate of dopaminergic neurones, an observation which confirms the lack of effect of cholinergic tone on dopaminergic activity in our experimental conditions. On the other hand, MCCh- and CCh-induced activation were dramatically reduced, if not abolished, by scopolamine, indicating that MCCh and CCh produce their physiological response on dopaminergic cells

mainly through muscarinic receptor activation. However, we cannot rule out the possibility of a minor participation of the nicotinic receptor in the stimulant effect induced by MCCh, since MCCh-induced activation was slightly reduced by high doses of mecamylamine and not completely abolished by scopolamine (Figure 7). The apparent lack of nicotinic effect of CCh may be due to its relatively low affinity for nicotinic receptors (contrary to nicotine itself and to MCCh) (Rapier *et al*., 1990; Punzi *et al*., 1991; Glenon & Dukat, 1996), combined with the small contribution of nicotine receptor stimulation to the cholinergic activation of dopaminergic cells. The muscarinic character of MCCh-induced neuronal activation is unexpected in view of a variety of biochemical, physiological, and behavioural studies that conclude MCCh is a selective nicotinic agonist devoid of muscarinic activity (Abood & Grassi, 1986; Araujo *et al*., 1988; Boksa *et al*., 1989). However, a recent study has shown that, in the rat, the central effect of MCCh on drinking behaviour is mediated by muscarinic neurotransmission (Yang *et al*., 1994). In addition, despite its low affinity for brain muscarinic receptors *in vitro*. MCCh has been shown to promote a muscarinic-mediated response in cell lines transfected with muscarinic receptors (Wang *et al*., 1994). Therefore, in keeping with these two latter studies, our results indicate that MCCh should be considered as a nonselective cholinoceptor agonist.

The relatively modest effects of iontophoretic application of nicotinic agonists on the firing activity of A10 presumed dopaminergic neurones differs from the robust depolarizing effect of nicotine obtained *in vitro* on these cells (Calabresi *et al*., 1989). However, when systemically administered, nicotine is found to have only a slight activating effect on A10 dopaminergic neurones (Nisell *et al*., 1996), while it produced a more pronounced effect on the A9 neurones (Clarke *et al*., 1985b; Grenhoff *et al*., 1986; Mereu *et al*., 1987; Nisell *et al*., 1996). Nevertheless, peripheral administration of nicotine strongly modified the burst firing pattern of both A9 and A10 neurones (Clarke *et al*., 1985b; Grenhoff *et al*., 1986; Mereu *et al*., 1987; Nisell *et al*., 1996). Some explanations can be envisaged to account for the relatively modest effect of nicotine obtained in our study. For example, nicotinic receptors are known to desensitize rapidly (Ochoa *et al*., 1990; Wonnacott *et al*., 1990). Such a phenomenon may occur in our study, since we found a lack of reproducibility of the weak excitatory action of nicotine on dopaminergic neurones. This would imply that in the course of the application, higher numbers of nicotinic receptors must be constantly recruited to promote cell activation, indicating that higher ejecting currents of nicotine or MCCh are required. However, in our experimental conditions it was not possible to eject a high current of nicotine. The nature of the tissue preparation may also interfere. One electrophysiological study has demonstrated that the excitatory effect of systemic administration of nicotine on VTA dopaminergic neurones is greater in unanaesthetized paralyzed animals than in chloral hydrate-anaesthetized animals (Mereu *et al*., 1987).

In conclusion, our results provide evidence for a direct muscarinic influence on midbrain dopaminergic activity. It is as yet uncertain under what physiological conditions the cholinergic activation of VTA and SNC dopaminergic cells occurs. Several lines of evidence indicate that the brain cholinergic input to dopaminergic neurones may play a critical role in the expression of rewarding brain stimulation (Yeomans *et al*., 1993). Further, it has been hypothesized that hyperactivity of the cholinergic input to dopaminergic neurones may play a critical role in the pathophysiology of schizophrenia (Garcia-Rill *et al*., 1995; Yeomans, 1995). Therefore, cholinoceptors on midbrain dopaminergic neurones could constitute a new therapeutic target for brain disorders in which dopamine plays a role, such as Parkinson's disease, drug addiction, schizophrenia and related psychoses.

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