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European Journal of Pharmacology 434 (2002) 117–123

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Atropine reduces raclopride-induced muscle rigidity by acting in the ventral region of the striatum

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Received 28 June 2001; received in revised form 29 September 2001; accepted 2 October 2001

Abstract

Parkinson-like extrapyramidal motor side effects associated with the use of antipsychotic drugs, such as increased muscle rigidity, are thought to result from blockade of striatal dopamine D2 receptors. While anticholinergic medications (muscarinic receptor antagonists) ameliorate extrapyramidal side effects, the mechanisms underlying their effectiveness remain unclear. We investigated the site of action of atropine, a non-selective muscarinic receptor antagonist, in reducing increased muscle rigidity, assessed as increases in tonic electromyographic (EMG) activity, induced by the selective dopamine D2 receptor antagonist, raclopride. Atropine significantly reduced raclopride-induced EMG increases in rat hindlimb muscles, when injected into the ventral striatum, but not the dorsal striatum or the substantia nigra. Atropine's site of action was localised to a small area of muscarinic receptors within the ventral part of the striatum, using quantitative autoradiography. These findings provide new information about the regulation of motor control by muscarinic receptor antagonists and additional evidence about the functional heterogeneity of the striatum.

Keywords: Muscarinic receptor antagonist; Antipsychotic drug; Striatum; Muscle rigidity; (EMG) Electromyogram

1. Introduction

The treatment of schizophrenia with typical antipsychotic drugs is often associated with the appearance of Parkinson-like extrapyramidal motor side effects, including increased muscle rigidity, bradykinesia and akinesia. Blockade of dopamine D2 receptors in the striatum was suggested to underlie the production of these motor side effects (Snyder et al., 1974), a view supported by human and experimental animal studies demonstrating an increased incidence of motor side effects when >70–80% of striatal dopamine D2 receptors are occupied by antipsychotic drugs (Farde et al., 1992; Hemsley and Crocker, 1998; Crocker and Hemsley, 2001a).

Muscarinic receptor antagonists, such as benztropine, are commonly used to ameliorate the Parkinson-like motor side effects associated with antipsychotic drug treatment (Stanilla and Simpson, 1995). In addition, fewer motor side effects have been reported with the use of antipsychotic drugs which exhibit high levels of anticholinergic activity, such as thioridazine and clozapine, so that an inverse relationship between the incidence of motor side effects and the affinity of drugs for muscarinic receptors has been suggested (Miller and Hiley, 1974; Snyder et al., 1974). Despite this evidence, the site of action of muscarinic receptor antagonists remains unclear.

The aim of the present experiments was to investigate the effects of atropine on muscle rigidity, an objective and quantifiable endpoint of dopamine D2 receptor blockade. Muscle rigidity, i.e. increased muscle tone, is a common component of the extrapyramidal side effects elicited by antipsychotic drugs and therefore a clinically relevant and valid endpoint with which to investigate atropine's actions. It was assessed quantitatively as increases in tonic electromyographic (EMG) activity in the hindlimb of conscious unrestrained rats, using a method developed previously in our laboratory (Double and Crocker, 1993). We have used this technique to study the role of dopamine mechanisms within the basal ganglia structures in motor control and showed it to be a valid and objective method of quantify-

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0014-2999/02/$ - see front matter
PII: S0014-2999(01)01441-8

ing changes in muscle rigidity induced by antipsychotic drugs and disruptions of dopamine neurotransmission (Double and Crocker, 1993, 1995; Crocker, 1997; Hemsley and Crocker, 1998, 1999, 2001; Crocker and Hemsley, 2001a).

We used a similar experimental strategy to that described in Alcock et al. (2001). Three intracerebral sites were chosen for injection of atropine and the level of muscarinic receptor occupancy at each site was related to changes in EMG activity. Striatal sites were selected because it has been suggested that anticholinergic medication modulates motor function via action at striatal muscarinic acetylcholine receptors (Klemm, 1983, 1985; Ellenbroek et al., 1986).

However, based on an increasing body of work showing that the striatum is both anatomically and functionally heterogeneous (Ellenbroek et al., 1986; Graybiel, 1990; Carelli and West, 1991), injections were made rostrally into either the ventral or the dorsal striatum. The former site was chosen because previous findings from our group demonstrated that the ventral part of the striatum contains dopamine receptors which mediate dopamine receptor agonist (Cameron and Crocker, 1989) and antagonist induced behaviours (Hemsley and Crocker, 2001). Further, we have shown that muscarinic receptors in the ventral part of the striatum are responsible for regulating behaviours mediated by dopamine receptor agonists (Crocker and Cameron, 1992) and antagonists (Alcock et al., 2001). The dorsal striatal site was chosen because it receives projections from the motor and primary somatosensory cortical regions (Carelli and West, 1991) that control movement of the hindlimbs. The third site was the substantia nigra, which contains small numbers of muscarinic acetylcholine receptors (Gronier and Rasmussen, 1998), and was selected on the basis of studies in experimental animals demonstrating its significance in the regulation of normal muscle tone (Double and Crocker, 1995; Hemsley and Crocker, 1998). This work has been published previously in the form of an abstract (Crocker and Hemsley, 2001b).

2. Materials and methods

2.1. Animals and drugs

Male Sprague–Dawley rats (250–350 g) supplied by the Animal House of the Flinders Medical Centre, were housed in groups of five, maintained under conditions of constant temperature and humidity on a 12-h light/dark cycle and given free access to food and water. All experiments were carried out in accordance with the guidelines of the Australian National Health and Medical Research Council (NHMRC), and were approved by the Flinders University of South Australia Animal Ethics Committee. Animals were closely monitored during surgery and throughout the course of the experiment; however, these procedures did not cause any signs of pain or distress. The minimum number of animals to provide statistically meaningful data was used.

Raclopride (Astra: 2.5 mg/kg) dissolved in isotonic saline (0.9%), was administered in a volume of 1 ml/kg. Raclopride was chosen due to its highly selective actions at dopamine D2 receptors, and the dose on the basis of previous work demonstrating significant dose-dependent increases in EMG activity (Hemsley and Crocker, 1999). Atropine sulfate (Sigma: 5.0 mg/ml), also prepared in isotonic saline, was administered intracerebrally (i.c.) in volumes of 40–50 nl. This dose was chosen as it was the minimum dose that produced a reduction in raclopride-induced catalepsy in a dose–response study (Alcock et al., 2001).

2.2. Implantation of EMG electrodes and guide cannulae

Rats were anaesthetised to surgical depth with Nembutal (sodium pentobarbitone (Boehringer Ingelheim) 45 mg/kg, i.p.) and the head of the rat was secured in a Stoelting stereotaxic apparatus. Local anaesthetic, Xylocaine (10% lignocaine; Astra) was then applied to the scalp. An incision of approximately 30 mm was made along the midline of the scalp, the membranes covering the skull were cleared and burr holes were drilled bilaterally into the skull using a 0.75-mm wide drill bit (Blackwoods, SA) in a pin-vice chuck. The cannulae (constructed from flat-ended 23-gauge needles [1.25 mm diameter, Becton Dickinson]) were lowered vertically into the brain to a position 2 mm above the target injection site, then secured using dental cement (GC Fuji II Glass Ionomer Restorative Cement).

While under surgical depth anaesthesia, animals were implanted with EMG electrodes, introduced via the incision in the scalp, as described by Double and Crocker (1995). A pair of stainless steel electrodes was implanted into the gastrocnemius and anterior tibialis muscles of the right hindlimb and a fifth wire (earth) laid on the surface of the tibialis muscle. The five wires were threaded under the skin and joined to a five-pin socket attached with dental cement to the surface of the skull. Animals were placed under a warm lamp until they recovered from anaesthesia.

Following recovery from surgery, rats were connected to a Grass polygraph (Model 7D), and a baseline EMG was recorded from the right hindlimb. Recordings were made in conscious, unrestrained animals housed in circular plastic beakers that accommodated the animal comfortably, while restricting excessive locomotor activity, and thus phasic activity in the EMG signal. The EMG signal was amplified, filtered (10 Hz–10 kHz), rectified and integrated over 10-s periods and the resultant signal recorded at 10 Hz for 20-min periods on a computerised recording system (CODAS, Dataq, USA). EMG is expressed as

mean tonic EMG activity (mV/10 s). Phasic activity resulting from animal movement was excluded from analysis. Movement was detected by both observation of the rats and inspection of the corresponding EMG signal that exhibited large, irregular spikes, easily discernible from the characteristically regular nature of the tonic EMG signal.

Following the recording of a baseline EMG trace, rats received bilateral intracerebral injections of either atropine or vehicle (saline) via the implanted cannulae. The coordinates for injections were as follows: dorsal portion of the striatum (A + 1.3 mm, L ± 2.5 mm, V − 4.0 mm), ventral part of the striatum (A + 1.3 mm, L ± 2.5 mm, V − 6.5 mm), substantia nigra (P − 5.2 mm, L ± 2.4 mm, V − 7.8 mm) with respect to bregma, according to the atlas of Paxinos and Watson, (1986). Injection needles were constructed from 30-gauge dental needles (0.3 mm diameter; Halas Dental), attached to clear polyethylene tubing (Critchley Electrical). The tubing was attached to a water-filled 5-μl Hamilton syringe (Scientific Glass Engineering, Australia) held horizontally on a motor-driven pump (Sage Instruments). Animals were gently restrained and the injection needle was lowered through the cannulae to the brain region of interest. Bilateral injections of atropine were made over 30 s and the needle remained in place for a further 30 s to reduce back-flow up the injection track. The rat was returned to the beaker and EMG activity was recorded for 2 h.

2.3. Quantitative autoradiography

Rats were killed by decapitation at 2 h post-injection, the brains were removed and sagittal sections (20 μm) were cut by cryostat. Sections were cut in this plane to allow assessment of receptor occupancy within the striatum and substantia nigra simultaneously. The left hemisphere was used for subsequent assessment of receptor occupancy because this side is involved in motor control of the right hindlimb, from which EMG recordings were made. Thaw mounted sections were incubated in 50 mM Tris buffer (pH 7.4), containing either [^{125}I]iodosulpiride (0.3 nM) for dopamine D2 receptors or the muscarinic receptor ligand, [^{3}H]quinuclidinyl benzilate (QNB) (1 nM: both Amersham, Australia) at room temperature according to the protocol of Schotte et al. (1993). These authors acknowledged that the calculated receptor occupancy using the ex vivo method is likely to be lower than that formed in vivo, due to diffusion of drug during the assay. Therefore pre-incubation washes were not performed, the incubation time was reduced to 10 min and the procedure incorporates a reduction in washing steps to limit diffusion of drug–receptor complexes. Non-specific binding was determined in adjacent sections using sulpiride (1.7 μM) or atropine (1 μM), respectively.

After washing and air drying, sections were exposed to tritium sensitive film (Hyperfilm, Amersham) for up to 1 week for dopamine D2 receptors or 1 month for muscarinic receptors, together with [^{125}I] or [^{3}H] standards (Amersham). The resulting autoradiographs were analysed using a computerised densitometry system (MD20: Flinders Imaging) and optical density values of selected brain regions were converted to nCi/mg of tissue, after subtraction of non-specific binding, with reference to the appropriate standards. Receptor concentration was calculated from the average optical density of 2–3 sections from each rat. Ex vivo receptor binding labelled all receptors in saline injected rats but only receptors not occupied by drug in the experimental groups (Schotte et al., 1993). Thus, receptor ligand binding was inversely proportional to the receptor occupancy of the test drug administered in vivo.

The site of action of atropine was mapped using the technique described in Hemsley and Crocker (2001) and Alcock et al. (2001). Sagittal sections from rats killed 2 h after intrastriatal injections of atropine were examined using a computerised image analysis system. This system consisted of a video camera (attached to a computer), suspended over a light source on which the autoradiographic film was placed. The site and area of atropine's occupancy of muscarinic receptors was traced from the computer screen onto transparent sheets, along with appropriate anatomical landmarks, which could be overlaid on maps in the brain atlas of Paxinos and Watson (1986).

2.4. Statistical analysis

Parametric statistics (one-way analysis of variance (ANOVA), multivariate regression analysis/ANOVA for multiple dependent variables, with post-hoc Bonferroni or Ryan, Einot, Gabriel, and Welsch multiple *F*-test) were employed to compare EMG activity in drug-treated rats with the EMG activity of the control group. A P value of < 0.05 was taken as significant. Data shown in the figures represents the mean ± standard error of the mean.

3. Results

3.1. Effect of intracerebral injection of atropine on increases in EMG activity resulting from injection (s.c.) of raclopride

Atropine (40–50 nl of a 5 mg/ml solution) or vehicle was injected into one of the three selected brain sites, at the same time an injection of raclopride (2.5 mg/kg; s.c.) or saline was administered. The effects of the i.c. injections on EMG activity were monitored for up to 2 h post-injection. Following injection of raclopride (s.c.) + vehicle (i.c.), significant increases in EMG activity in both the tibialis and gastrocnemius muscles were observed from 1 to 2 h post-injection compared with saline (s.c.) + vehicle (i.c.) controls (Fig. 1).

Raclopride not only significantly increased EMG activity but also elicited changes in cataleptic behaviour, as reported previously (Alcock et al., 2001). In contrast, rats that received raclopride (s.c.) + atropine (50 nl) in the ventral part of the striatum showed no significant increases in EMG activity at any time point and demonstrated very little change in behaviour. EMG activity at 2 h in animals receiving raclopride (s.c.) + atropine (i.c.) in the ventral part of the striatum was not significantly different from control animals (Fig. 1).

However, when rats received injections of raclopride (s.c.) + atropine into either the dorsal part of the striatum (50 nl) or substantia nigra (40 nl), significant increases ($P < 0.05$) in EMG activity of a magnitude similar to those seen following raclopride + vehicle (i.c.) were observed. Accompanying these effects were similar changes in behaviour to those seen in rats receiving raclopride (s.c.) + saline (i.c.). Rats receiving saline (s.c.) + atropine (i.c.) did not differ significantly from those injected with saline (s.c.) + saline (i.c.), either with respect to the EMG signal or behaviourally (data not shown).

3.2. Dopamine D2 receptor occupancy at 2 h post-injection

The level of occupancy of dopamine D2 receptors by raclopride was determined for the whole of the striatum and substantia nigra at 2 h post-injection. Fig. 2 shows that raclopride occupied 73.4–88.2% dopamine D2 receptors in both regions, in all experimental groups.

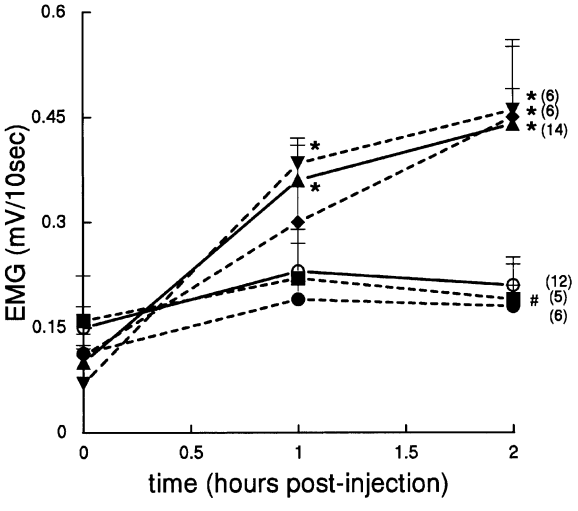

Fig. 1. Effect of subcutaneous injection of raclopride (2.5 mg/kg) + intracerebral injection of atropine (5 mg/ml) or vehicle (saline) into various brain regions on EMG activity in the gastrocnemius muscle over time. The number of animals used per treatment group is given in parentheses. – ▲ – Raclopride (s.c.) + saline (50 nl, i.c.), – ○ – saline (s.c.) + saline (50 nl, i.c.), - - -■- - - raclopride (s.c.) + atropine (50 nl, i.c.) ventral striatum (VSTR), - - -◆- - - raclopride (s.c.) + atropine (50 nl, i.c.) dorsal striatum (DSTR), - - -▼- - - raclopride (s.c.) + atropine (40 nl, i.c.) substantia nigra (SN), - - -●- - - saline (s.c.) + atropine VSTR (50 nl, i.c.). *$P < 0.05$ c.f. saline (s.c.) + saline (i.c), #$P < 0.05$ c.f. raclopride (s.c.) + saline (i.c.).

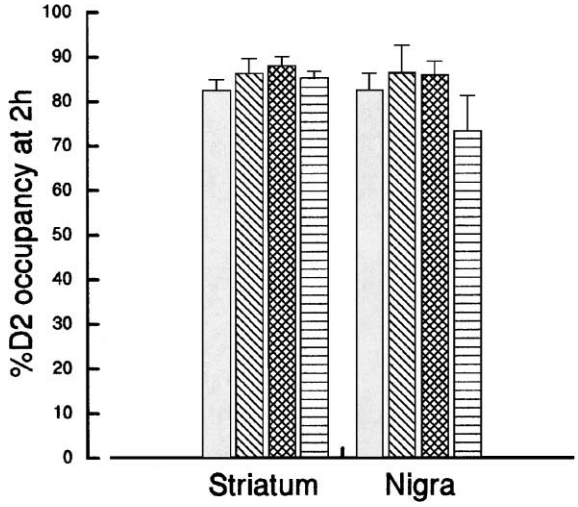

Fig. 2. Percentage occupancy of dopamine D2 receptors in the whole striatum and substantia nigra at 2 h following subcutaneous raclopride administration (2.5 mg/kg) + atropine (5 mg/ml) or saline by intracerebral injection. The number of animals used per treatment group is given in parentheses. □ Raclopride (s.c.) + saline, (50 nl, i.c.) (mean of all groups), ▨ raclopride (s.c.) + atropine (50 nl, i.c.) dorsal striatum (DSTR), ⊠ raclopride (s.c.) + atropine (50 nl, i.c.) ventral striatum (VSTR), ▤ raclopride (s.c.) + atropine (40 nl, i.c.) substantia nigra (SN).

3.3. Determination of the striatal site of action of atropine in reducing raclopride-induced increases in EMG activity

The site of action of atropine was mapped as described in the Materials and methods. The areas where atropine occupied muscarinic acetylcholine receptors, thereby competing for [^{3}H] QNB binding sites, had lower optical density and are visible as paler areas in the representative autoradiographs shown in Fig. 3(B–F). Examples of the location of areas of atropine receptor occupancy associated with decreased levels of EMG activity are shown in Fig. 3(B–D). Initially, areas of occupancy were assessed in brain sections from all rats in which atropine injection reduced EMG activity. When these areas of occupancy were overlapped, a common area of intersection was identified in the ventral part of the striatum, which contained the muscarinic receptors whose blockade was associated with increased muscle rigidity. We have termed this common area the 'atropine' site. Next, areas of atropine occupancy from rats in which intrastriatal injections of atropine failed to reduce EMG activity were mapped, and examples of these are shown in Fig. 3(E,F). None of these areas was found to overlap with the 'atropine site' shown diagrammatically in Fig. 4.

The level of muscarinic receptor occupancy in the 'atropine' site was 73.7 ± 3.7% ($n = 4$) following intracerebral injections of atropine directed at the ventral part of the

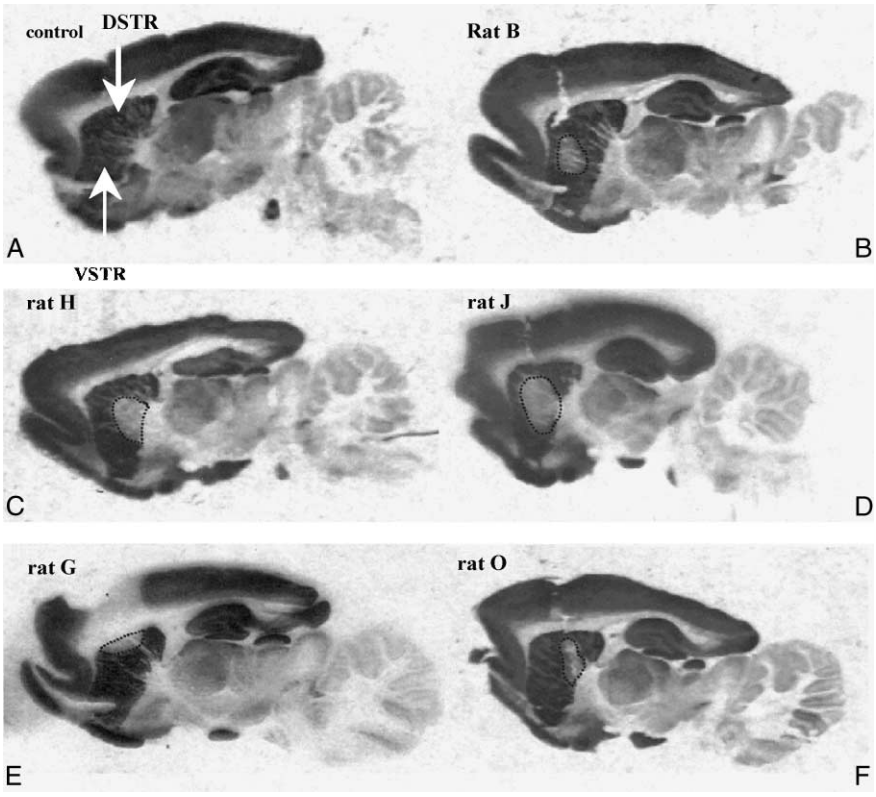

Fig. 3. Autoradiographs depicting muscarinic cholinergic receptor occupancy by atropine at 2 h following injection into the striatum. (A) Control rat (saline s.c. +50 nl, i.c.); (B, C, D) raclopride (s.c.)+atropine (50 nl, i.c.) injections associated with no increase in EMG activity (E, F) raclopride (s.c.)+atropine (50 nl, i.c.) injections associated with significant increases in EMG activity. Dotted lines indicate the extent of muscarinic receptor occupancy, appearing as paler grey areas in the striatum.

striatum, which reduced EMG activity as shown in Fig. 1. Following atropine injections into the dorsal region, however, only 4.2 ± 2.6% (7) of receptors in the atropine 'site' were occupied and no reductions in EMG activity were observed in any animal. The level of muscarinic receptor occupancy in the dorsal part of the striatum ranged from 35% to 81% (6) with a mean value of 58.9 ± 13.3%; however, none of the dorsal injections reduced EMG activity. Injection of atropine into either dorsal or ventral parts of the striatum resulted in 8.3–15.6% (11) occupancy of nigral muscarinic receptors at 2 h, indicating a small degree of spread of atropine to extra-striatal sites. At 2 h after atropine injections into the substantia nigra, 21.9 ± 5.5% (6) of nigral muscarinic receptors were occupied and 2.6 ± 2.4% (6) of striatal receptors.

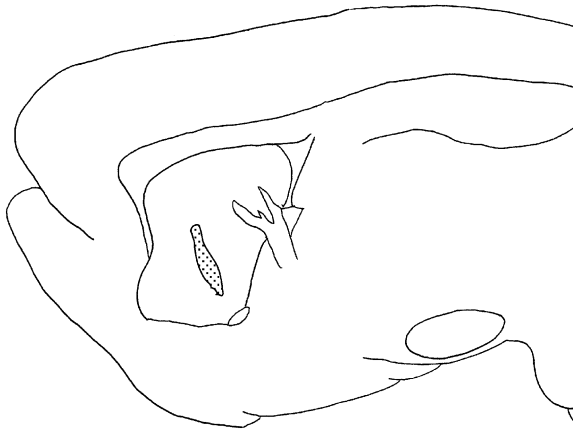

Fig. 4. The site of ventral striatal muscarinic receptors that mediate the effects of atropine on raclopride-induced increases in EMG activity, mapped as described in the text. Approximate co-ordinates of site, A+0.45 mm to P−0.2 mm, L~2.4 mm, V−5.2 to −6.6 mm, according to Paxinos and Watson (1986).

4. Discussion

This study demonstrated that atropine prevents raclopride-induced increases in EMG activity by occupying muscarinic receptors in a ventral part of the striatum. No evidence was found for an action of atropine in the dorsal part of the striatum or substantia nigra, the latter area previously shown by us to be of key importance in regulating changes in muscle tone (Double and Crocker, 1995; Hemsley and Crocker, 1998). The importance of the current observations is that, by using a small injection volume (50 nl) and measuring receptor occupancy, it has been possible, for the first time, to localise the site of the muscarinic receptors involved in reducing raclopride-induced increases in muscle tone. These observations are consistent with those of

our recent study (Alcock et al., 2001), which demonstrated that muscarinic receptors located in the ventral part of the striatum were responsible for ameliorating raclopride-induced catalepsy. Overall, these findings extend the work of Ellenbroek et al. (1986), who showed that injections of scopolamine (1 μg/0.5 μl) into the rostral striatum reduced increased EMG activity, resulting from rostral injection of haloperidol (500 ng/0.5 μl). Our findings also support the view that the striatal mechanisms mediating catalepsy and muscle rigidity are similar, as suggested by Ellenbroek et al. (1985).

The level of muscarinic receptor occupancy observed in the ventral part of the striatum to be associated with reduced EMG activity was similar to that found in the dorsal part of the striatum, where no reduction in raclopride-induced increases in EMG activity were observed. These findings do support the site-specific nature of atropine's effects. However, the maximum level of muscarinic receptor occupancy assessed in the substantia nigra was much less than the striatal values and may well have been insufficient to modify EMG activity. This view is supported by reports that nigral muscarinic receptors mediate changes in muscle rigidity (Turski et al., 1984) and catalepsy (De Montis et al., 1979). Experiments are in progress to determine the threshold level of muscarinic receptor occupancy associated with reductions in raclopride-induced EMG activity. However, findings from a previous study (Alcock et al., 2001), which investigated the dose-dependent effects of atropine on catalepsy, showed that catalepsy was significantly reduced when occupancy of striatal muscarinic receptors exceeded approximately 69%.

Atropine did not reduce EMG activity indirectly by reducing the level of dopamine D2 receptor occupancy by raclopride, as no significant differences in occupancy were seen in the groups receiving saline (i.c.) compared with those receiving atropine (i.c.) (Fig. 2). Dopamine D2 receptor occupancies in both the striatum and the substantia nigra were in excess of the threshold level previously reported by us to be associated with significantly increased EMG activity (Hemsley and Crocker, 1998, 1999).

Dopamine and acetylcholine appear to play an important role in regulating motor control through actions in the striatum. On the basis of work characterising the striatal neurons expressing muscarinic receptor genes (Bernard et al., 1993) and other experimental studies (reviewed by Di Chiara et al., 1994), it appears that striatal projection neurones are differentially regulated by acetylcholine acting via different muscarinic receptor subtypes. Thus, it has been suggested that acetylcholine opposes the action of dopamine in the striatum by inhibiting striatonigral neurones, possibly via muscarinic M_4 receptors, while stimulating muscarinic M_1 receptors located on striatopallidal neurones (Di Chiara et al., 1994).

In terms of the experiments reported here and based on models of motor control by basal ganglia circuitry (DeLong, 1990; Gerfen, 1992; Crocker, 1997), we hypothesise that raclopride increased muscle rigidity by blocking inhibitory D2 receptors on striatopallidal neurones, which are part of two indirect pathways connecting the striatum and substantia nigra. As a consequence of raclopride disinhibiting these pathways, there will be an increase in the activity of nigrothalamic efferents and ultimately a reduction in the activation of the motor cortex, resulting in increased muscle rigidity and hypokinesia (DeLong, 1990; Crocker, 1997). At the same time, blockade of dopamine D2 receptors located on the terminals of cholinergic interneurones (Foley and Crocker, 1993; Di Chiara et al., 1994) will increase striatal acetylcholine, resulting in inhibition of the direct striatonigral pathway and further activation of nigrothalamic efferents, culminating in increased muscle rigidity. The non-selective muscarinic receptor antagonist atropine, in the presence of the dopamine D2 receptor blockade and the resulting increase in acetylcholine release, will block acetylcholine's inhibitory effects at muscarinic M_4 receptors on the striatonigral neurons of the direct pathway, resulting in their activation. In addition, blockade of the stimulatory effects of acetylcholine at muscarinic M_1 receptors on striatopallidal neurones will also reduce activity in the two indirect pathways. Both of these actions will facilitate movement and reduce muscle tone.

While the information presented above relates to striatal function generally, the current findings suggest a specific role for muscarinic receptors in the ventral part of the striatum in reducing dopamine D2 receptor antagonist-mediated EMG increases in hindlimb muscles. This was somewhat unexpected, given the large body of research demonstrating that topographical projections of motor and supplementary motor cortical areas terminate in the dorsal part of the striatum, and the knowledge that regions of the dorsal part of the striatum are involved in motor control of the hindlimbs (McGeorge and Faull, 1989; Carelli and West, 1991). However, the current findings are consistent with a growing body of studies supporting an important functional role for the rostroventral part of the striatum in dopamine-mediated behaviour and motor control (Arnt, 1985; Cameron and Crocker, 1989; Crocker and Cameron, 1992; Ossowska et al., 1990; Wardas et al., 1995, Eagle et al., 1999; Alcock et al., 2001; Hemsley and Crocker, 2001).

In conclusion, muscarinic receptor antagonists are the mainstay of current treatments to reduce extrapyramidal side effects induced by antipsychotic drugs, emphasising the importance of muscarinic receptors in motor regulation (Snyder et al., 1974). The current study has identified a site in the rostroventral part of the striatum, where interactions between dopamine and acetylcholine mechanisms occur to regulate muscle rigidity. The site is the same as that found for the cholinergic modulation of raclopride-induced catalepsy (Alcock et al., 2001), and is located close to those identified previously for the regulation of stereotyped behaviour and its modulation by cholinergic mechanisms

(Cameron and Crocker, 1989; Crocker and Cameron, 1992) and muscle tone (Hemsley and Crocker, 2001). These findings, which further confirm the functionally heterogeneous character of the striatum, have important implications for the understanding of how the basal ganglia controls motor function and behaviour.

Acknowledgements

We thank the Flinders Medical Centre Foundation for their financial support of this study.

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