Brain Struct Funct (2008) 213:247–253
DOI 10.1007/s00429-008-0174-4

ORIGINAL ARTICLE

A functional dissociation of the anterior and posterior pedunculopontine tegmental nucleus: excitotoxic lesions have differential effects on locomotion and the response to nicotine

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Received: 18 July 2007 / Accepted: 16 January 2008 / Published online: 12 February 2008

Abstract Excitotoxic lesions of posterior, but not anterior pedunculopontine tegmental nucleus (PPTg) change nicotine self-administration, consistent with the belief that the anterior PPTg (aPPTg) projects to substantia nigra pars compacta (SNC) and posterior PPTg (pPPTg) to the ventral tegmental area (VTA). The VTA is a likely site both of nicotine's reinforcing effect as well as its actions on locomotion. We hypothesized that pPPTg, but not aPPTg lesions, would alter locomotion in response to repeated nicotine administration by virtue of the fact that pPPTg appears to be more closely related to the VTA than is the aPPTg. Following excitotoxic lesions of aPPTg or pPPTg, rats were habituated to experimental procedures. Repeated (seven of each) nicotine (0.4 mg/kg) and saline injections were given following an on-off procedure. Measurement of spontaneous locomotion during habituation showed that aPPTg but not pPPTg lesioned rats were hypoactive relative to controls. Following nicotine, control rats showed locomotor depression for the first 2 days of treatment followed by enhanced locomotion relative to activity following saline treatment. Rats with aPPTg lesions showed a similar pattern, but the pPPTg lesioned rats showed no locomotor depression following nicotine treatment. These data confirm the role of the pPPTg in nicotine's behavioural effects—including the development of sensitization—and demonstrate for the first time that excitotoxic lesions of the aPPTg but not pPPTg generate a deficit in baseline activity. The finding that anterior but not posterior PPTg affects motor activity has significance for developing therapeutic strategies for Parkinsonism using deep brain stimulation aimed here.

Keywords Deep brain stimulation · Parkinsonism · Rat · Substantia nigra · Ventral tegmental area

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Introduction

Nicotine alters locomotion in a dose-dependent manner that changes with repeated administration (Clarke and Kumar 1983a,b). The functional integrity of the ventral tegmental area (VTA) is critical for these effects, which are mediated, at least in part, by nicotinic acetylcholine receptors located on dopamine (DA) neurons in the VTA. Intra-VTA infusion of nicotine increases locomotion, an effect blocked by pre-treatment with the nicotinic receptor antagonist mecamylamine (Panagis et al. 1996). Direct interference with the integrity of the VTA by 6-hydroxydopamine (6-OHDA) lesion also blocks nicotine's locomotor effects (Louis and Clarke 1998). Acetylcholine, the natural ligand of VTA nicotinic receptors, is provided by neurons of the Ch5 and Ch6 cell groups in the pedunculopontine tegmental (PPTg) and laterodorsal tegmental nuclei (LDTg). We have shown that indirect interference with the functional integrity of the VTA—by making excitotoxic lesions in LDTg—alters the effects on locomotion of repeated nicotine administration (Alderson et al. 2005).

The VTA also receives direct cholinergic innervation from the PPTg, a structure known to be involved in mediating the reinforcing effects of nicotine (Alderson et al. 2006; Corrigall et al. 2002). Inputs from PPTg to the VTA come from the posterior region (pPPTg), while the anterior region (aPPTg) innervates DA neurons in the

substantia nigra pars compacta (SNC) (Oakman et al. 1995). Previous work has provided evidence of a role for the PPTg in the behavioural effects of nicotine. For example, recent work from our lab has shown that, while lesions of the SNC-projecting aPPTg do not affect nicotine self-administration, pPPTg lesions increase it, an effect likely to be the result of altered regulation of VTA DA neurons (Alderson et al. 2006). One aim of the present study, therefore, was to investigate the involvement of pPPTg and aPPTg on the locomotor effects of repeated nicotine. We hypothesized that if PPTg connections with the VTA are of significance in the actions of nicotine, lesions in the posterior (VTA projecting) portion would have effects on nicotine-induced locomotion while lesions in the anterior (SNC projecting) portion would not.

A second aim was to investigate the motor effects of aPPTg and pPPTg lesions. One paradoxical feature of the literature relating to PPTg is to do with locomotor activity. An older literature considered the PPTg to be a part of the mesencephalic locomotor region, but research in several laboratories over the last decade making excitotoxic lesions of the whole PPTg had failed to show any significant effects on locomotion (see for example Inglis et al. 1994; Olmstead and Franklin 1994) (though effects relating to (for example) attention, learning and reward related responding have all been demonstrated—see Winn (2006) for review). Nevertheless, motor outflow from the basal ganglia is directed at the PPTg, and there have been recent attempts to alleviate the signs and symptoms of Parkinsonism using deep brain stimulation aimed at the PPTg (Mazzone et al. 2005; Plaha and Gill 2005). As such a second aim of the present study was to determine whether discrete lesions of these different parts of the PPTg affect spontaneous locomotor activity.

Materials and methods

Twenty-one male Lister hooded rats (Harlan Olac Ltd, UK; weighing 295–369 g at the start of the experiment) were individually housed, with ad libitum access to food and water. Lights in the holding room were on from 7 a.m. to 7 p.m. and testing was carried out during the light phase. Compliance was ensured with national (Animals [Scientific Procedures] Act, 1986) and international (European Communities Council Directive of 24 November 1986 [86/609/EEC]) legislation governing the maintenance of laboratory animals and their use in scientific experiments.

Rats were anaesthetized with 1.0 ml/kg sodium pentobarbitone ("Sagatal", Rhône-Mérieux, Harlow UK; 60 mg/ml i.p.) diluted 50:50 with sterile water. Carprofen analgesia (Rimadyl", Pfizer, Sandwich UK; 0.05 ml s.c. to each rat) was given prior to surgery. Infusions of ibotenate (Tocris-Cookson Ltd., Bristol, UK; 0.02 M solution in phosphate buffer [pH 7.4]; final pH adjusted to pH 7.0 using 2 M NaOH) were delivered in a volume of 200 nl to each site by pressure ejection through a glass micropipette (tip diameter 35–40 μm). The micropipettes were left in situ for 300 s after the infusion to allow for diffusion away from the tip. Control rats ($n = 9$) received the same volume of phosphate buffer only, delivered in the same manner as the ibotenate. For the anterior PPTg lesions ($n = 6$), two injections were made at the following co-ordinates: inter-aural line (IAL) +0.6 mm, midline (ML) ±2.0 mm, dura (D) −6.2 mm; IAL +1.3 mm, ML ±2.1 mm, D −7.0 mm. Posterior PPTg lesions ($n = 6$) were made by a single injection at co-ordinates of IAL +0.2 mm, ML ±2.0 mm, D −6.2 mm. All lesions were made with the stereotaxic frame set such that the skull was level at bregma and lambda. Two separate unilateral operations separated by a minimum of 7 days were conducted to produce bilateral lesions in each rat. A high rate of post-surgical fatalities has previously been found when bilateral lesions of the PPTg were carried out in a single surgery.

Locomotor testing took place under red-light illumination in wire photocell cages measuring 26 cm (W) × 18 cm (H) × 38 cm (L), crossed by two photocell beams equidistant along their length. These were controlled by a "Beetle" real-time processor (Paul Fray Ltd., Cambridge, UK), PC-interfaced for data collection. Locomotion was recorded as sequential beam-breaks. Daily testing sessions were 60 min long and began 7 days post-surgery. Rats were given two habituation sessions with no injections (data not shown) followed by seven sessions with control injections of 0.9% saline (1 ml/kg) immediately prior to testing. Nicotine locomotion testing was carried out over 14 consecutive days, with rats receiving nicotine (nicotine hydrogen tartrate, Sigma–Aldrich, UK; 0.4 mg/ml in 0.9% saline; dose refers to salt; 1 ml/kg of this solution administered sc) or saline on alternate days in a day-on-day-off design, such that all received seven nicotine and seven saline injections in total.

At the end of testing, rats were deeply anaesthetized with 200 mg/ml/kg sodium pentobarbitone ("Dolethal", Univet Ltd., Bicester, UK) and perfused transcardially with phosphate buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and stored in 20% sucrose overnight before 50 μm sections were cut on a freezing microtome. Sections (50 μm) were processed at 100 μm intervals alternately for nicotinamide adenine dinucleotide phosphate diaphorase (NADPH) histochemistry and neuron-specific nuclear protein (NeuN) immunohistochemistry, which stains neurons rather than glia and lesions can be clearly seen in processed tissue. Processing for NeuN immunohistochemistry used a mouse

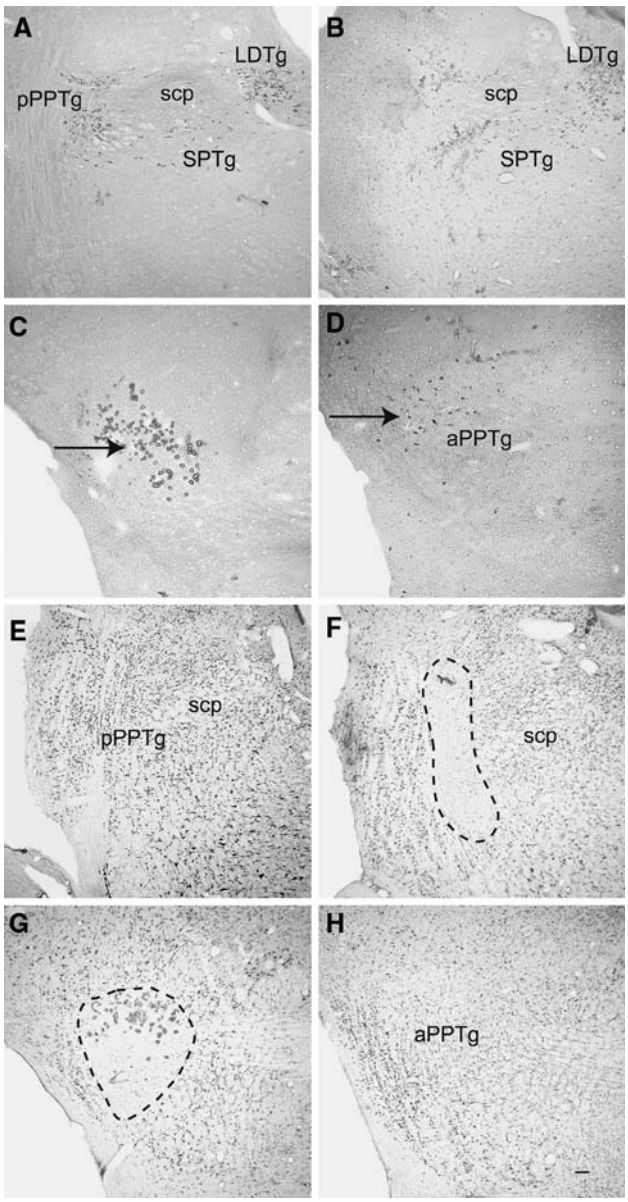

Fig. 1 Panels **a**–**d** present material stained by NADPH diaphorase histochemistry from representative aPPTg lesioned (**a**,**c**) and pPPTg lesioned (**b**,**d**) rats (4×magnification, scale bar [panel **h**] 100 μm). Panel **a** shows an aPPTg lesioned rat with no loss of diaphorase staining—the darkly stained neurons at the lateral tip of the scp—in the pPPTg; Panel **b** shows a pPPTg lesioned rat with considerable loss of diaphorase staining in the pPPTg; Panel **c** shows an aPPTg lesioned rat with loss of diaphorase staining in the aPPTg—the *arrow* indicates the presence of calcification in the tissue, which commonly occurs after excitotoxic lesions; Panel **d** shows a pPPTg lesioned rat with no loss of diaphorase staining in the aPPTg—the *arrow* points to the diffuse cluster of darkly stained diaphorase-positive neurons. Panels **e**–**h** show tissue from the same rats stained using NeuN immunohistochemistry. Panels **e** and **g** are from the aPPTg lesioned rat, panels **f** and **h** from the pPPTg lesioned rat (4× magnification, scale bar [panel **h**] 100 μm). In panels f and g the dashed line surrounds the area of lesion. Panel e shows an aPPTg lesioned rat with no loss of NeuN staining in the pPPTg; Panel **f** shows a pPPTg lesioned rat with considerable loss of NeuN staining in the pPPTg; Panel **g** shows an aPPTg lesioned rat with loss of NeuN staining in the aPPTg; Panel **h** shows a pPPTg lesioned rat with no loss of NeuN staining in the aPPTg. *LDTg* laterodorsal tegmental nucleus; *aPPTg* anterior pedunculopontine tegmental nucleus; *pPPTg* posterior pedunculopontine tegmental nucleus; *scp* superior cerebellar peduncle; *SPTg* subpeduncular tegmental nucleus

anti-NeuN monoclonal antibody (Chemicon International Inc., Temecula, CA, USA) with a Vector Labs "Elite" ABC kit (Peterborough, UK) followed by Sigma Fast™ DAB peroxidase substrate. NADPH diaphorase histochemistry followed a modification of the method of Vincent and his colleagues (Vincent and Kimura 1992; Inglis et al. 1993). Lesion extent was also assessed by the absence of NADPH-diaphorase positive cells in NADPH-diaphorase stained tissue. In the mesopontine tegmentum, NADPH-diaphorase positive cell counts correlate very strongly with counts made of cholinergic cells identified by choline acetyltransferase immunohistochemistry (Vincent et al. 1983).

All data were analyzed by repeated measures ANOVA, followed by planned comparisons where appropriate (Winer 1971), using Statistica (Version 6.0). Data were subject to a square root transform; the factors analyzed were group (aPPTg, pPPTg, control), session (1–7) and drug (saline, nicotine).

Results

Figure 1 shows representative tissue from sham-lesioned control, pPPTg and aPPTg excitotoxin lesion groups. Lesions were judged to be of the pPPTg if they encompassed pedunculopontine neurons posterior to the decussation of the superior cerebellar peduncle, destroying cholinergic and non-cholinergic neurons in this region, but not spreading beyond. Lesions of the aPPTg were those that destroyed both cholinergic and non-cholinergic neurons anterior to the decussation of the superior cerebellar peduncle. All rats given aPPTg and pPPTg lesions were found to have acceptable lesions following histological analysis according to these criteria.

Figure 2 shows the number of beam breaks, square root transformed, measured during each of the seven habituation sessions carried out. It is clear that the aPPTg lesioned group were consistently hypoactive compared to both the pPPTg lesioned rats and the controls, which clearly did not differ from each other. Analysis by repeated measures ANOVA revealed that there was a significant main effect of lesion group ($F_{2,19} = 17.494$, $P < 0.001$) and a significant lesion group × session interaction ($F_{12,114} = 2.037$, $P < 0.05$) but no significant main effect of session. Further analysis of the group × session interaction revealed that

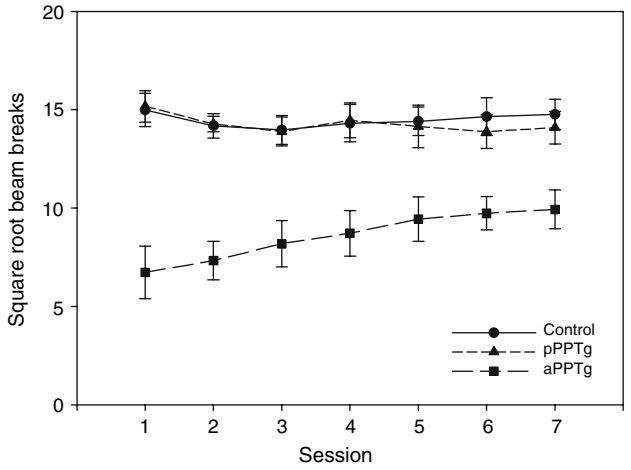

Fig. 2 The mean number of beam breaks (square root transformed) following saline injection over seven daily habituation sessions by sham lesioned control (*circles; solid line*), pPPTg lesioned rats (*triangles; short dashed line*) and aPPTg lesioned rats (*squares; long dashed line*). Error bars = ±SEM

the aPPTg lesion group differed significantly from both the control ($P < 0.01$) and pPPTg ($P < 0.05$) lesion groups on all sessions. There were no significant effects of session for either the control or pPPTg lesion groups. However, planned comparisons demonstrated that for the aPPTg lesion group, session 1 differed significantly from sessions 4 ($P < 0.05$), 5, 6 and 7 (all at $P < 0.01$); session 2 was significantly different from sessions 5 ($P < 0.05$), 6 ($P < 0.01$) and 7 ($P < 0.05$); and session 3 differed significantly from session 7 ($P < 0.05$); there were no significant differences between sessions 4–7.

Figure 3 shows the number of beam breaks, square root transformed, measured following saline and nicotine treatment. The data shown in this figure indicate that the response to nicotine was similar in the control and aPPTg lesioned rats (panels a and c), but different in the PPTg lesioned rats (Panel b). The pPPTg lesioned rats did not show hypoactivity when first given nicotine and showed an increased locomotor response to nicotine before either of the other groups did. Analysis by repeated measures ANOVA revealed that there were significant main effects of lesion group ($F_{2,18} = 17.051$, $P < 0.001$) and of session ($F_{6,108} = 13.143$, $P < 0.001$). There was a significant drug × lesion group interaction ($F_{2,18} = 5.548$, $P < 0.05$), a significant drug × session interaction ($F_{6,108} = 21.685$, $P < 0.001$) and a significant drug × session × lesion group interaction ($F_{12,108} = 2.340$, $P < 0.05$). Planned comparisons revealed that the pattern of locomotor activity in response to nicotine and saline differed over sessions according to lesion group. The control group showed significant locomotor depression in response to nicotine in sessions 1 and 2 ($P < 0.001$), while the difference in

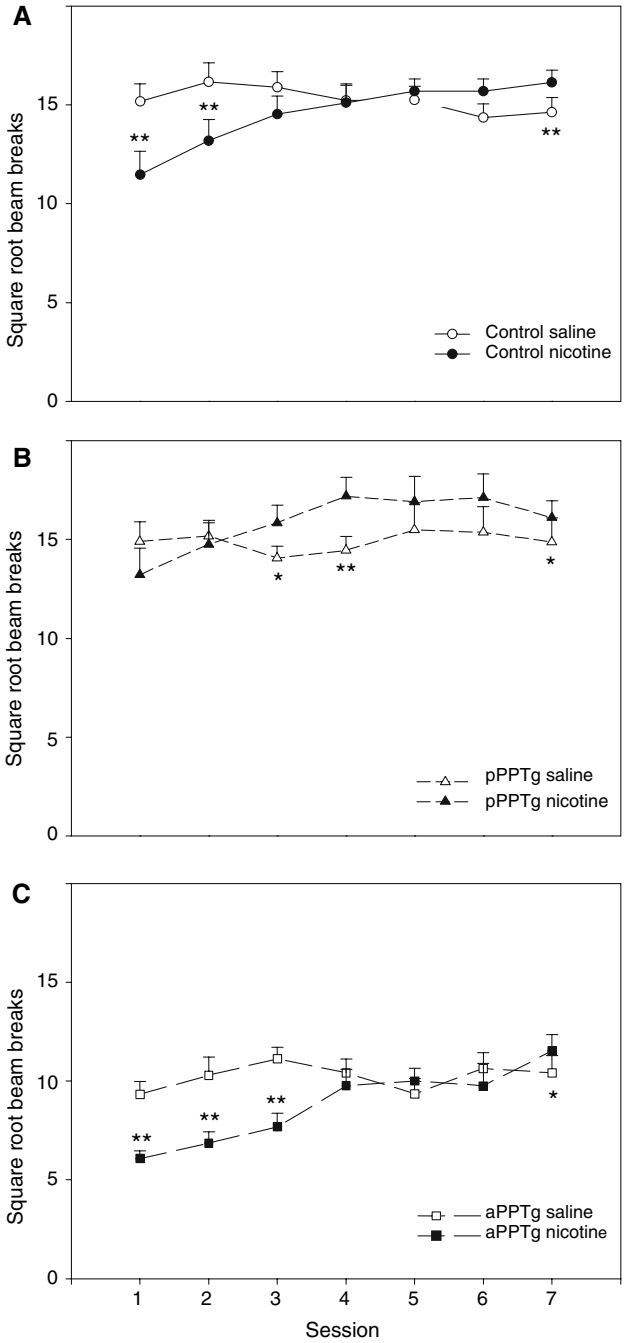

Fig. 3 Shows the number of beam breaks, square-root transformed, during the 14 alternating 1 h nicotine/saline treatment sessions. **a** Mean number of beam breaks elicited by saline (*open circles*) and nicotine (*filled circles*) by sham lesioned control rats. **b** Mean number of beam breaks elicited by saline (*open triangles*) and nicotine (*filled triangles*) over treatment sessions (seven drug, seven saline) by pPPTg lesioned rats. **c** Mean number of beam breaks elicited by saline (*open squares*) and nicotine (*filled squares*) over treatment sessions (seven drug, seven saline) by aPPTg lesioned rats. Error bars = ±SEM; *$P < 0.05$, ** $P < 0.01$

session 3 approached significance ($P = 0.054$); locomotion in response to nicotine was significantly greater than following saline in session 7 ($P < 0.01$). The aPPTg lesion

group showed a similar pattern of locomotor depression and elevation over the seven sessions tested: these rats showed significant locomotor depression in response to nicotine in sessions 1–3 ($P < 0.01$); locomotion in response to nicotine was significantly greater than following saline in session 7 ($P < 0.05$). The pPPTg lesion group however showed a different pattern of responding: there was no difference between the responding to saline or nicotine on session 1 or 2, but the response to nicotine was significantly elevated on sessions 3 and 4 ($P < 0.05$). On sessions 5 and 6 the increase in the response to nicotine did not reach statistical significance (session 5: $P = 0.085$; session 6: $P = 0.084$) but the increase was again statistically significant on session 7 ($P < 0.05$).

Discussion

Our hypothesis was that if PPTg connections with the VTA are important for the actions of nicotine, then lesions in the posterior (VTA projecting) portion would change the behavioural response to nicotine while lesions in the anterior (SNC projecting) portion would not. This hypothesis is unambiguously supported by the present data: pPPTg but not aPPTg lesions changed the locomotor response to nicotine, despite the fact that the pPPTg lesions did not change baseline levels of locomotion during habituation, in contrast to the aPPTg lesions. When given nicotine, pPPTg but not aPPTg lesioned rats showed an altered response, in that the initial locomotor depressant effect of nicotine was not seen at all in pPPTg lesioned rats. The locomotor effects of nicotine were similarly changed after lesions of the LDTg (Alderson et al. 2005) and, given that the LDTg projects to VTA but not SNC (Oakman et al. 1995), it is likely that in the present experiments it is loss of VTA innervation by pPPTg that is critical.

Several intriguing microinjection and neurophysiological studies have emphasized the role of VTA in mediating the behavioural effects of nicotine. The present data, taken together with the intravenous self-administration studies of Alderson and her colleagues (2006), strengthen the belief that the pPPTg has a role to play in mediating the behavioural effects of nicotine through interaction with the VTA. One mechanism that might underlie this is the up-regulation of cholinergic receptors in the midbrain following lesions in PPTg. This has not been examined in the VTA, but is known that unilateral lesions of the whole PPTg result in an increase in striatal DA efflux after microinjection of nicotine into the substantia nigra (Blaha and Winn 1993). While upregulation of receptors might be a likely mechanism, it is still important to determine which receptors are primarily responsible for these effects. It has been proposed that the primary effect of nicotine in the VTA is on GABA and glutamate containing terminals, with a direct action on DA neurons themselves being of lesser importance (Mansvelder et al. 2002). There seems little doubt that nicotinic activation of receptors on glutamate terminals (Jones and Wonnacott 2004) can stimulate release of this transmitter and consequently drive DA neurons. Likewise, nicotinic activation of GABA neurons (either local inhibitory interneurons or collaterals of projection neurons (Garzon et al. 1999 and possibly GABA neurons that project back to the PPTg, Laviolette and Van der Kooy 2004) will generate inhibitory activity, though because of rapid desensitization this is likely to be only a relatively brief event (Mansvelder et al. 2002). Neither of these processes however precludes the possibility of direct nicotinic activation of DA neurons. The cholinergic innervation of both the SNC and VTA is well documented: electron microscopy studies show that cholinergic fibres penetrate the SNC, each one making multiple synaptic contacts with the dendrites of DA neurons (Bolam et al. 1991), and in the VTA, some 40% of cholinergic terminals are in apposition to tyrosine hydroxylase containing neurons (Garzon et al. 1999). In addition, it is important to remember that, regardless of the local synaptology, the endogenous ligand for all the nicotinic receptors on midbrain DA neurons is provided by the Ch5 and Ch6 neurons of the mesopontine tegmentum. In the present experiments pPPTg lesions clearly alter the locomotor response to nicotine. Identifying precisely how these lesions influence the dynamics of neuronal interactions in the VTA—do they affect a direct nicotinic activation of DA neurons or is the effect mediated through GABA?—is an obvious next question to address, as is the question as to whether or not specific nicotinic subunits are involved in these effects. One further possibility needs to be borne in mind. While it might be parsimonious to explain the present data in terms of differential PPTg innervation of the VTA, it must also be recognized that changed activity within the mesopontine tegmentum might directly account for all or part of the effects. Recent work has demonstrated that inactivation of the LDTg significantly reduces the ability of PPTg stimulation to generate burst firing in VTA DA neurons (Lodge and Grace 2006), with the clear implication that the PPTg and LDTg are not independent units. In this context it is intriguing to note that excitotoxic lesions of the LDTg (Alderson et al. 2005) produce a changed response to repeated nicotine similar to that of pPPTg lesioned and a baseline hypoactivity similar to that of aPPTg lesions.

In contrast to the effects of pPPTg lesions, aPPTg lesions did not change the locomotor response to repeated nicotine: an almost identical pattern to the controls of initial locomotor depression followed later by enhancement

was found. However, aPPTg lesions did affect baseline levels of locomotion, both in the habituation sessions and during drug testing. This effect was not shown by rats bearing pPPTg lesions. The level of spontaneous locomotor activity by the aPPTg rats increased over the first days of habituation but stabilized to a constant level through the later sessions, and through the 7 saline/nicotine injection regime. (Compare Fig. 2 and the saline sessions for the aPPTg rats shown in Fig. 3.) A reduction in baseline levels of locomotor activity might be considered predictable, given that the aPPTg is better connected to the SNC than to the VTA. Loss of SNC DA neurons has long been associated with the development of akinesia in 6-OHDA lesioned rats and, of course, with Parkinsonism. The removal of an excitatory drive to the SNC would not be expected to have profound effects on movement, but a small reduction in locomotion is not unexpected. One curious point to note however is that the effect on baseline locomotion of aPPTg lesions is very similar to that seen after LDTg lesions (Alderson et al. 2005) despite the observation that the LDTg projects overwhelmingly to the VTA, not SNC. One possible explanation for this apparent paradox is that the LDTg also makes extensive contacts (bilaterally) with the PPTg. It is possible that these intrapontine connections account for the similarity of the locomotor effects of LDTg and aPPTg lesions. The prediction would have to be that the LDTg reduced spontaneous locomotor activity by virtue of eliminating an excitatory drive on the SNC via the PPTg, though precisely how this might be mediated is not clear. Another possibility is that connections of the LDTg, other than those with the VTA or PPTg, independently affect spontaneous locomotor activity.

The effects described here emphasize functional dissociations within PPTg. There are two points of particular interest to note. (1) It is worth reiterating that several labs have shown that bilateral excitotoxic lesions of the entire PPTg do not affect spontaneous locomotor activity (for example, Inglis et al. 1994; Olmstead and Franklin 1994) whereas in the present data it is abundantly clear that there is a reduction in locomotion after aPPTg but not pPPTg lesions. The clarity of the effect is difficult to match with clarity of explanation. Hypotheses might be couched in terms of the effects of pPPTg lesions adding with those of aPPTg when they were combined as "whole PPTg" lesions, though if this were the case one might expect pPPTg lesions to have the opposite effect to aPPTg on spontaneous locomotion, rather than no effect. Alternatively, it might be explained in terms of the effects on DA systems: lesions of the whole PPTg might uniformly effect midbrain DA neurons, while those in the aPPTg or pPPTg alone unbalance DA neurons. Here, it would be the relative inactivity of the SNC that was critical. Recent hypotheses concerning the organization of DA neurons have emphasized the interconnectedness of SNC and VTA, in a spiral looped system (Haber et al. 2000) and it is therefore possible that selective interference with a subset of this system has a different effect to uniform interference across the whole. (2) Recent clinical studies have demonstrated that deep brain stimulation of the PPTg alleviates the akinesia shown by Parkinsonian patients (Mazzone et al. 2005; Plaha and Gill 2005). All previous data concerning the effects of excitotoxic lesions of the whole PPTg in rats indicate that there are no motor impairments (see Winn 2006). The present data however suggest that lesions within a restricted portion of the PPTg in rats do produce motor deficits. Whether these are achieved through effects on descending motor projections, or on re-entrant circuits into the basal ganglia, is not yet clear. The notion of a re-entrant circuit into the basal ganglia from PPTg is an intriguing one. It has been recognized for many years that basal ganglia structures, including pallidum, subthalamic nucleus and substantia nigra zona reticulata all project to PPTg. However, it is also the case that structures in the extended amygdala (Heimer 2003) also project to PPTg (see for example Zahm et al. 2001; Winn 2006 for review). It is possible therefore that the PPTg is a structure through which basal ganglia and extended amygdala outflow can be synthesized in order to shape ongoing behaviour. This recalls the notion of a limbic-motor interface (see Heimer 2003) but set here in the mesopontine tegmentum rather than forebrain.

In conclusion these data demonstrate functional dissociations within the PPTg, consistent with the effects already observed with self-administration of nicotine (Alderson et al. 2006). The data are consistent with the hypothesis that the pPPTg is more strongly associated with VTA function rather than SNC, unlike the aPPTg which has the reverse pattern. The data are also consistent with the hypothesis that the anterior PPTg—analogous to the pars dissipatus of Olszewski and Baxter—have functions related to motor processes, while the posterior PPTg—analogous to the pars compactus of Olszewski and Baxter—are less concerned with motor control processes. This is consistent with an emerging body of data regarding the effectiveness of deep brain stimulation in Parkinson's disease.

Acknowledgements This work was supported by a Wellcome Trust project grant (066281/Z/01/Z) to PW. We wish to thank the School of Psychology technical staff for their help, Anna Jermyn and Susanne Monka for their involvement with behavioural testing and David Roche for his assistance with photography.

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