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RESEARCH ARTICLE

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Dopaminergic innervation of the rat globus pallidus characterized by microdialysis and immunohistochemistry

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Abstract The present study examined the dopaminergic innervation of the rat globus pallidus by in vivo microdialysis and immunohistochemistry in more detail. Using tyrosine hydroxylase immunohistochemistry, two classes of dopaminergic fibers were distinguished morphologically in the globus pallidus. Unilateral infusion of 6-hydroxydopamine into the substantia nigra produced a loss of dopaminergic fiber density in the globus pallidus which was correlated with the nigral extent of the lesion. These findings are in line with the notion that a degenerative loss of nigral dopaminergic cell bodies might also affect the dopamine input of extrastriatal structures such as the globus pallidus. Using in vivo microdialysis, we tested whether dopamine measured in the globus pallidus is of neuronal origin. Perfusion of tetrodotoxin induced a strong and transient decrease of pallidal dopamine. The tetrodotoxin-sensitivity of pallidal dopamine demonstrates the functional significance of the nigropallidal dopaminergic innervation.

Keywords Substantia nigra · Dopamine · 6-hydroxy dopamine · Tyrosine hydroxylase · Tetrodotoxin · Fibers

Introduction

In current models of the functional organization of the basal ganglia, the globus pallidus (GP) is considered as a major relay nucleus of the indirect pathway linking the caudate-putamen with the output structures of the basal ganglia, i.e., the nucleus entopeduncularis and the substantia nigra pars reticulata in the rat (Chesselet and Delfs 1996). The GP sends prominent GABAergic projections to the entopeduncular nucleus, substantia nigra pars reticulata, subthalamic nucleus and reticular thalamic nucleus as well as to the pedunculopontine nucleus (Moriizumi and Hattori 1992; Parent and Hazrati 1995a, b). Major pallidal afferents using GABA as transmitter originate in the caudate-putamen, while glutamatergic afferents arise from the subthalamic nucleus (Parent and Hazrati 1995a, b) and the parafascicular nucleus (Feger 1997).

The GP also receives a dopaminergic innervation by collaterals of nigrostriatal fibers as shown in rats (Fallon and Moore 1978; Lindvall and Bjorklund 1979), primates (Sato et al. 2000) and humans (Cossette et al. 1999). There is recent evidence for two morphologically different types of nigrostriatal axons, one that arborizes profusely in the striatum and poorly in extrastriatal nuclei as the GP and another that behaves conversely (Cossette et al. 1999). In the GP tyrosine hydroxylase immunoreactive (TH-ir) fibers form possible synaptic contacts with pallidal neurons (Arluison et al. 1984; Zaborszky and Cullinan 1996). Furthermore, dopamine (DA) D_1 and D_2receptors are expressed in the GP of several species (Richfield et al. 1987; Camps et al. 1990). D_2 receptors are located postsynaptically on pallidal neurons (Mansour et al. 1990). In addition, D_2 receptors are found on striatal axons in the rat GP, suggesting that they may also act as pre-synaptic receptors (Mansour et al. 1990; Yung et al. 1995). Electrophysiological and neurochemical data provide consistent evidence that the DAergic innervation of the GP is functional. DA is believed to play a primary role among monoamines in modulating firing rates and patterns of pallidal neurons (Ruskin et al. 1999, 2001). According to their electrophysiological response to DA receptor stimulation, pallidal neurons can be subdivided in two types (Kelland et al. 1995). Data from slice preparations of the rat GP reveal that DA modulates the release of pallidal GABA acting through D_1 (Floran et al.

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1990) and D_2 (Floran et al. 1997) receptors. Moreover, intra-GP infusion of the D_2 receptor antagonist sulpiride induced c-fos expression in pallidal neurons (Marshall et al. 2001) and in nuclei downstream the GP (Hauber and Lutz 1999a). In addition, DA in the GP might play an important role in motor control as local infusions of D_1 or D_2 receptor antagonists produce akinesia in rats (Hauber and Lutz 1999b). Intra-GP DA infusions, in turn, were able to partially restore motor deficits in a rat model of Parkinsons disease (Galvan et al. 2001). Likewise, glial-cell-line-derived neurotrophic factor (GDNF) induced sprouting of DAergic axons in the external GP and substantia nigra of Parkinsonian primates have been correlated with functional recovery of motor symptoms (Gash et al. 1996). Accordingly, in the external GP of Parkinsons patients there is a massive reduction of TH-ir fibers (Jan et al. 2000), a reduced pallidal tissue content of DA and its main metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) (Ploska et al. 1982; Hornykiewicz 1998) and a decreased vesicular monoamine transporter (VMAT2) immunoreactivity (Miller et al. 1999). Thus, DA dysfunction in the external GP has been suggested to contribute to hypokinesia in this disease (Hornykiewicz 1998).

The present experiments were designed to analyze the DAergic innervation of the rat GP by in vivo microdialysis and immunohistochemistry in more detail. Using TH-immunohistochemistry, the DA innervation to the GP was analyzed in order to i) characterize the DAergic innervation pattern with regard to possible morphological differences along the rostrocaudal axis of the GP and ii) investigate effects of nigral cell loss on tyrosine hydroxylase immunoreactive fiber density at the pallidal level after unilateral infusion of 6-hydroxydopamine (6-OHDA) into the substantia nigra. Using microdialysis, we already showed that extracellular DA in the rat GP is calcium-dependent, depolarization-inducible and responsive to salient stimuli, e.g., food (Hauber and Fuchs 2000). The present in vivo microdialysis experiment was performed to ascertain that pallidal DA is of neuronal origin. To this end, we measured pallidal DA levels during a local blockade of the fast voltage-gated Na^+-channels with tetrodotoxin (TTX).

Materials and methods

Experiments were performed according to the German Law on Animal Protection and approved by the proper authorities in Stuttgart, Germany.

Subjects

Male CD rats (Charles River, Sulzfeld, Germany) were used for all experiments and housed in groups of up to five animals in transparent macrolon cages (type IV; 35×55×10 cm; Ebeco, Castrop-Rauxel, Germany). Animals used for microdialysis were housed individually after surgery in macrolon cages (type III; 37×21×30 cm; Ebeco, Castrop-Rauxel, Germany) with raised solid-walled lids. Temperature (20±2°C) and humidity (50±5%) were kept constant in the animal house and a 12:12-h light-dark schedule was maintained. Rats were given ad libitum access to water; food (standard maintenance chow, Altromin, Lage, Germany) was restricted to 15 g per animal per day.

6-OHDA lesion of the substantia nigra and tyrosine hydroxylase (TH) immunohistochemistry

6-OHDA lesion

For stereotaxic surgery, animals were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.) (Sigma-Aldrich, Taufkirchen, Germany) following pretreatment with atropine sulphate (0.5 mg/kg, i. p.) (Sigma-Aldrich, Taufkirchen, Germany) and secured in a Kopf stereotaxic apparatus (Kopf Instruments, Tujunga, USA). Eight animals weighing 240–300 g received unilateral microinfusions into the substantia nigra. 15 µg 6-OHDA (calculated as free base, 6-OHDA hydrobromide, Sigma, Taufkirchen, Germany) dissolved in sterile saline (Fresenius, Taunusstein, Germany) and containing 0.1% ascorbic acid (Sigma-Aldrich, Taufkirchen, Germany) in 2 µl were infused over 4 min with the infusion cannula (26 g stainless steel) and left in position for additional 5 min. The 6-OHDA solution was prepared freshly and kept on ice in the dark prior to use. The coordinates (Paxinos and Watson 1986) (toothbar 3.3 mm below the interaural line) were: AP –5.3 mm, L 2.0 mm either on the left or right hemisphere, V –7.2 mm (from dura).

Histology

Histological analysis was performed on all animals (n=8) bearing nigral 6-OHDA lesions. In addition, untreated animals (n=3) were included for the morphological analysis of different DA fiber types. Lesioned animals were analyzed 4 weeks after 6-OHDA infusion. They were deeply anaesthetized with Ethrane (Abbot, Wiesbaden, Germany) and perfused transcardially with 200 ml PBS containing 100 mg/l heparin (Merck, Darmstadt, Germany) followed by 200 ml fixative (4% paraformaldehyde in PBS). The brains were dissected and postfixed for about 3 h, immersed in 0.2 M phosphate buffer for 1 day and then transferred in 30% sucrose in 0.1 M phosphate buffer for at least 2 days. Brains were processed for TH-immunohistochemistry within 1 week after fixation.

Cryosections (40 µm) were cut on a freezing microtome (Jung Frigocut, Leica, Nussloch, Germany) and processed for TH immunohistochemistry according to a protocol for free floating sections. First they were washed in 0.05 M Tris buffered saline (TBS, 6.1 g Tris Base and 9 g NaCl in 1 l of demineralized water, pH adjusted to 7.6 with HCl), incubated for 20 min in 50% ethanol with 3% H_2O_2 in water and then blocked for 45 min in 12% normal horse serum (Vector Laboratories, Burlingame, USA) in TBS containing 0.5% Triton X-100 (TBS-T). Sections were incubated overnight at 4°C in primary antibody (mouse, anti-TH, antibody code LNC1, DiaSorin, Stillwater, USA, 1:7500 in TBS-T containing 2% normal horse serum), then for 1 h at room temperature in secondary antibody (horse, anti-mouse IgG, Vector, Burlingame, USA, 1:200 in TBS-T containing 2% normal horse serum) and for 1 h at room temperature in ABC complex (1:500 in TBS-T, Vectastain Elite ABC Kit, Vector). Finally, the sections were reacted with DAB. TBS-T washing (3×10–15 min) was performed between each step. The sections were mounted on coated slides, dried overnight, dehydrated in ascending concentrations of ethanol, cleared in xylene, embedded in DPX (Serva, Germany) and coverslipped.

Morphological analysis and data evaluation

Digital images of stained sections at the nigral (approx. −5.3 posterior to bregma) and two pallidal (approx. −1.3 for the rostral

and −1.8 for the caudal portion of the GP) levels were captured using an Olympus AX70 light microscope (Olympus, Tokyo, Japana) at different magnifications and a 3CCD color video camera with a resolution of 752 (horizontal) × 582 (vertical) effective picture elements (Model DXC-950P, Sony, Tokyo, Japan). All measurements were performed using AnalySis 3.0 software (Soft Imaging Systems, Münster, Germany).

For assessing the extent of the nigral lesion, a polygon was used to delimitate manually the area of TH-ir elements in the substantia nigra ipsilateral and contralateral to the lesioned side. The area of TH-ir elements of the unlesioned side served as control (set to 100%) and was compared to the corresponding area of the lesioned side. Quantification of pallidal fiber densities was achieved after converting the color images from the rostral and caudal GP into monochrome images, followed by a threshold setting that separated the stained fibers from the background. A rectangular frame was placed centrally in the GP to exclude striatal staining. A phase analysis was performed which measured the area of detected fibers in proportion to the total area. This ratio was calculated from both the ipsi- and contralateral side and compared subsequently with the contralateral side serving as control (set to 100%). Parameters for threshold setting and frame size remained unchanged for analyzing both sides, but were adapted in different slices to consider different background staining and varying extension of the GP. A linear regression analysis was performed using SigmaPlot 4.0 (SPSS, Chicago, USA) to reveal the relationship between loss of TH-immunoreactivity in the substania nigra and the rostral and caudal GP. Two slices per animal (n=8) and per region (substantia nigra, caudal and rostral GP) were evaluated and the mean was taken to calculate the coefficient of determination (r^2).

In order to reveal morphological differences along the rostrocaudal axis, high power magnification images (160×120 μm) of the caudal and rostral GP at different dorsoventral levels were captured as color images. A total number of 24 and 36 images were evaluated for the caudal and rostral GP, respectively. Grids of 20×20 μm were overlayed digitally and the number of fibers per grid was counted. As already shown by Zaborszky and Cullinan (1996), TH-immunohistochemical analysis revealed different morphological types of DA fibers with different calibres. In the present study, axons were assigned to two separate classes using morphological criteria: thick and thin axons. The reason for using morphological, instead of size, criteria was that the spatial resolution was too low to allow reliable detection of the calibre of thin fibers. Thin varicose-rich and straight axons with moderate staining were classified as being thin. These fibers typically had a calibre of less than 0.4 μm. Thick fibers with intense staining either smooth or with varicosities often curved and sometimes with helical segments were classified as being thick. Occasionally thick fibers were assorted to bundles of three or more fibers. The calibre of thick fibers was about 0.7–1.2 μm. Only separable fibers in focus were included. The mean counts of thick and thin fibers and the total fiber counts per image were calculated from the pooled data for the caudal and rostral GP and compared using a t-test for independent samples.

Microdialysis

Stereotaxic surgery

For stereotaxic surgery, animals were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.) (Sigma-Aldrich, Taufkirchen, Germany) following pretreatment with atropine sulphate (0.5 mg/kg, i. p.) (Sigma-Aldrich, Taufkirchen, Germany) and secured in a Kopf stereotaxic apparatus (Kopf Instruments, Tujunga, USA). Six animals were implanted unilaterally with intracranial guide cannulae (CMA/12, CMA Sweden) aiming to the GP at the following coordinates (Paxinos and Watson 1986) (toothbar 3.3 mm below the interaural line): AP −1.4 mm, L 3.0 mm and V −5.2 mm (from dura). Each rat was given at least 1 week to recover from surgery.

Microdialysis setup

The microdialysis experiment was performed in the home cage of the animal with the lid replaced by a metal frame bearing a counterbalanced arm with the swivel assembly. Attachment to the swivel was achieved by a spring tether connected directly to the head mount via a self-made plug. A microdialysis probe (CMA/12, CMA, Solna, Sweden; exposed membrane length 2 mm) was inserted through the guide cannula and perfused with artificial cerebrospinal fluid (aCSF) (145 mM Na^+, 2.5 mM K^+, 1.2 mM Ca^{2+}, 0.9 mM $Mg^{2+}$and 151.7 mM Cl^-) delivered by a CMA/100 microdialysis pump (CMA, Solna, Sweden) at 2.0 μl/min. Samples were collected every 30 min. Baseline samples were taken at least 5 h after probe insertion to allow for stabilization of dialysate DA and metabolite levels. All perfusion fluids contained 3 μM nomifensine, a catecholamine reuptake inhibitor.

Drugs

Stock solutions of tetrodotoxin citrate (TTX, Tocris, Ellisville, USA) and nomifensine maleate (RBI, Natick, USA) at concentrations of 652 μM and 887 μM, respectively, were prepared in ultra pure water and stored in frozen (−70°C) aliquots. For perfusion, the TTX stock solution was diluted with aCSF to a final concentration of 1 μM and nomifensine was added to all perfusion fluids in a final concentration of 3 μM.

Analytical procedure

Dialysates were analyzed for DA, DOPAC, HVA and 5-HIAA using HPLC with electrochemical detection. The mobile phase consisted of 2 g/l sodium-acetate, 5 g/l citric acid, 200–600 mg/l 1-heptanesulfonic acid (HSA), 60 mg/l Na_2-EDTA and 15% (v/v) methanol with pH adjusted to 3.9 before methanol addition. Minor modifications in the concentrations of HSA and methanol were made to optimize DA peak separation if necessary. The HPLC apparatus consisted of a Flux Rheos 2000 pump (Flux Instruments, Basel, Switzerland), a cooled CMA/200 autosampler (CMA, Solna, Sweden), a Nucleosil C18 column (Bischoff, Leonberg, Germany; 5 μm particles, length × i.d. 125×3 mm) and a dual electrode BAS LC4C amperometric detector (Bioanalytical Systems, Lafayette, USA) with the electrode potential set to 600 mV at high gain to quantify DA and 700 mV at a lower gain to measure the metabolites. Filter setting was 0.1 Hz and the separation was performed at room temperature. This setup allowed quantification of DA and metabolites in the same run. Sample run time was less than 12 min and the detection limit of DA in a standard solution was routinely about 1 pg per injection or lower.

Verification of probe location

After the experiments, animals were killed, the brains removed, fixed for at least 2 h in formalin and immersed in 30% (w/v) sucrose for several days. Cryosections (60 μm) were taken and stained with cresyl violet. Only data from animals with correct probe location, i.e., most of the exposed dialysis membrane located within the GP, were taken for evaluation.

Data expression and statistics

Data are expressed as percentages of control values (± standard error of the mean, S.E.M.). The average concentration of the three samples before TTX perfusion was taken as control and set to 100%. These data were analyzed by a nonparametric one-way ANOVA for

repeated measurements followed by Dunnett´s multiple comparisons test, if appropriate. The last baseline sample was taken as reference.

Results

Effect of 6-OHDA lesions of the substantia nigra lesioning on pallidal TH-ir fiber density

Unilateral application of 6-OHDA (15 μg in 2 μl) into the substantia nigra resulted in a severe loss of TH-ir elements in the substantia nigra (<20% of the TH-ir stained area of the control side) in six of eight treated animals 4 weeks after lesion (Fig. 1). Two animals showed a less severe loss of TH-ir elements (20–60% of the TH-ir stained area of the control side). There is a significant correlation between the extent of substantia nigra lesion and reduction of fiber density in the rostral GP (r^2>0.93) and the caudal GP (r^2>0.59).

Rostrocaudal differences of the TH-ir fibres innervation pattern

The density of thin fibers was significantly higher in the caudal aspects of the GP compared to the rostral GP (P<0.000002), whereas the opposite pattern was found for the thick fibers (P<0.00002) (Table 1). However, the total density of thin and thick fibers did not differ significantly between rostral and caudal levels (P<0.53). Most of the thin fibers were varicose-rich and straight. The thick fibers had a different morphology, as they were either smooth or had varicosities and were straight or curved or even displayed helical segments. Occasionally they were assorted to bundles of three or more fibers, which was rarely observed for the thin fibers. A representative image is shown in Fig. 2.

Basal dialysate levels of DA

Basal dialysate levels of DA in the GP were close to detection limit. Therefore, the catecholamine reuptake inhibitor nomifensine was included in all perfusates. The mean dialysate DA concentration in the reference baseline sample (60 μl) was 13.6±1.6 pg/sample (mean ± S.E.M., n=4). Two animals were excluded due to low or non-detectable DA levels even under reuptake blockade.

Table 1 Comparison of the innervation pattern with regard to the fiber calibre between caudal and rostral GP

	Caudal	Rostral
Thin	27.5±3.5	11.8±0.8*
Thick	16.4±2.1	36.3±3.1*
Total	44.0±3.8	48.0±3.1 n.s.

Mean fiber counts per image ± S.E.M. The counts for thin and thick fibers and the total counts (thin + thick) for the rostral GP were compared with the caudal GP using a *t*-test for independent samples

n.s. not significant

*P<0.0001 vs. caudal

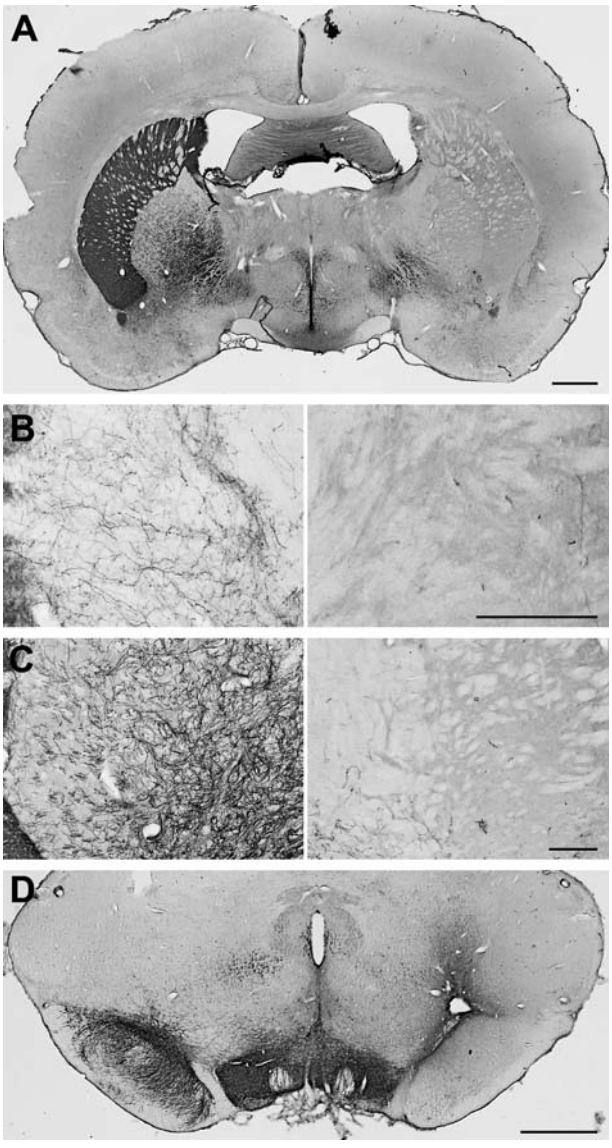

Fig. 1A–D Effect of unilateral nigral 6-OHDA infusion on TH-immunoreactivity in the substantia nigra (**D**), rostral GP (**C**) and caudal GP (**B**). A brain section at the rostral level of the GP is displayed at low power magnification in **A**. Scale bars represent 1 mm (**A, D**) or 250 μm (**B, C**). The lesioned side is shown on the right side

Pallidal DA levels after reverse microdialysis of TTX

Perfusion of 1 μM TTX for 60 min produced a strong decrease to about 16% of baseline value in dialysate DA, which was highly significant (P<0.0008; ANOVA for repeated measurements followed by Dunnett´s multiple comparisons test; n=4). After removal of TTX, dialysate DA levels returned and significantly exceeded pre-drug

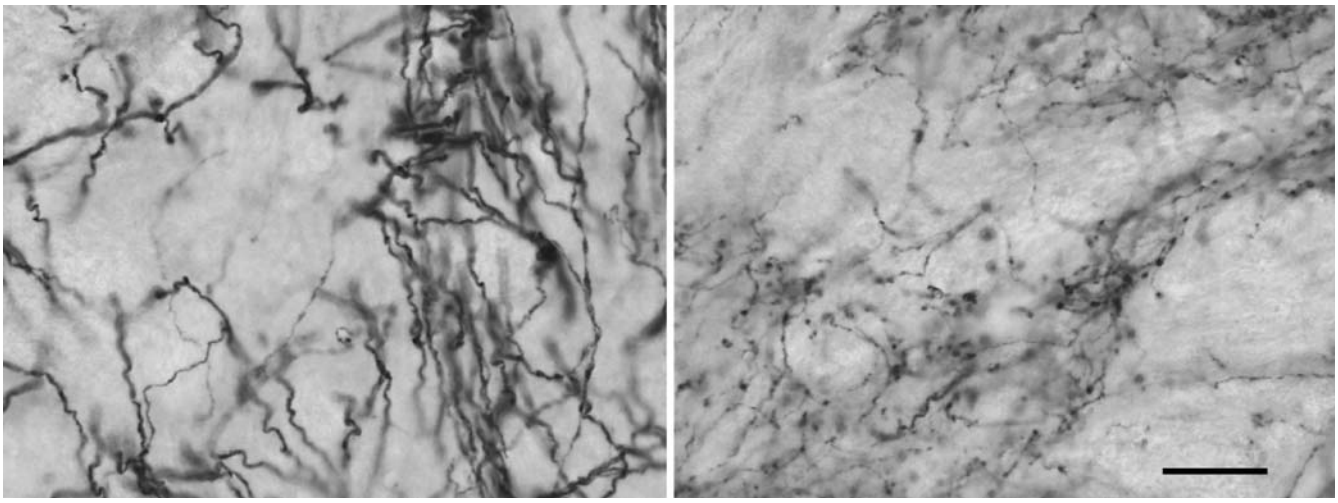

Fig. 2 Innervation pattern of the rostral (*left*) and caudal (*right*) GP as visualized with TH-immunohistochemistry. In the caudal GP the number of thin fibers was significantly higher compared to the number of thick fibers. The opposite pattern was found within the rostral GP. The scale bar represents 25 μm

levels starting 120 min after termination of perfusion with TTX (Fig. 3).

Pallidal DOPAC, HVA, and 5-HIAA levels after reverse microdialysis of TTX

Perfusion with TTX also decreased DOPAC and HVA levels to about 57% and 69% of baseline value, respectively. The decrease was significant for DOPAC (P<0.05 ANOVA for repeated measurements followed by Dunnett´s multiple comparisons test; n=4), but not for HVA. After removal of TTX, both DOPAC and HVA exceeded pre-drug levels significantly and remained elevated during the observation period (Fig. 3). Furthermore, TTX did not produce changes in 5-HIAA levels during application, but resulted in a significant post-application increase of dialysate 5-HIAA levels (data not shown).

Behavior

Reverse microdialysis of TTX produced lacrimation in all animals, salivation in two out of four animals and contralateral turning in three out of four animals.

Probe placement

In all animals used for microdialysis, the probes were placed in the medio-caudal portion of the GP with most of the total dialysis membrane in the GP (Fig. 4). In no case was dialysis membrane of probes located in striatal tissue.

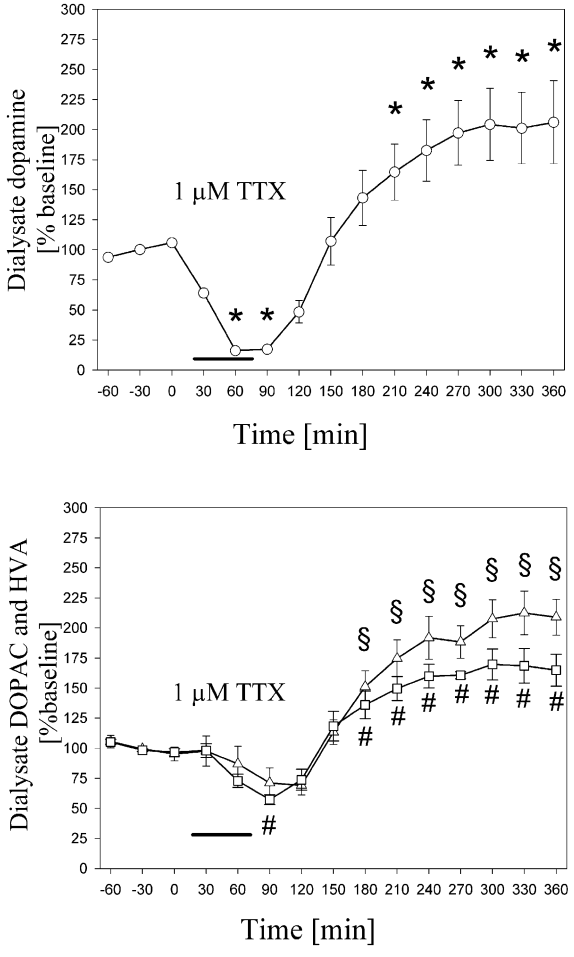

Fig. 3 Effect of perfusion with 1 μM TTX on dialysate DA (*upper panel*) and main metabolites DOPAC (*squares*) and HVA (*triangles*) (*lower panel*) in the GP. The black bar indicates time of drug delivery. P<0.05 as compared to control value (T=0 min) for DA (*), DOPAC (#) and HVA (§); ANOVA for repeated measurements followed by Dunnetts multiple comparisons test

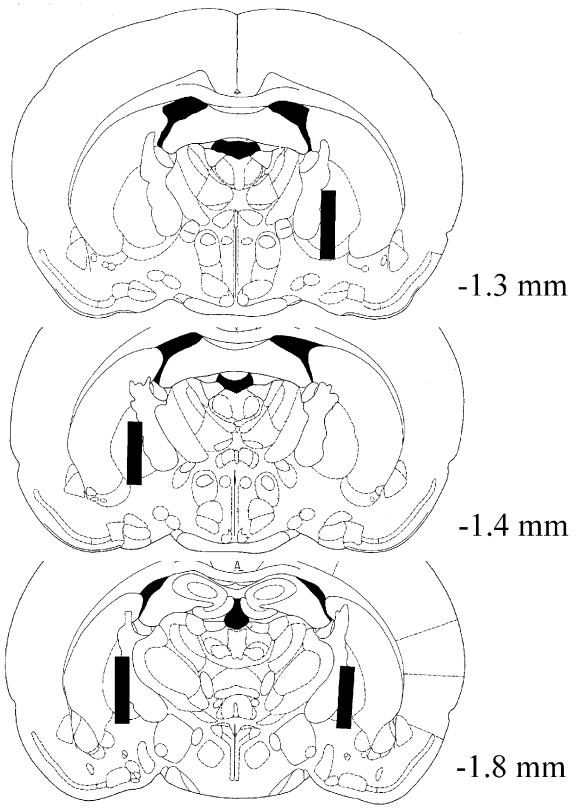

Fig. 4 Schematic drawing of the probe locations according to the atlas of Paxinos and Watson (1986). The black lines represent the position of the dialysis membrane in a representative plane (from top to bottom: 1.3 mm, 1.4 mm and 1.8 mm posterior to bregma)

Discussion

Using in vivo microdialysis in awake rats, we demonstrate that extracellular DA release in the GP depends on neuronal activity, as it is TTX-sensitive. As pallidal DA release is also calcium-dependent and responds to potassium stimulation (Hauber and Fuchs 2000), it fulfills the most stringent criteria to prove neuronal transmitter release (for reviews see Ungerstedt 1991; Di Chiara 1991; Westerink 1995). The data provide further support for the notion that DA inputs to the GP are functional and subserve a role in basal ganglia signal processing (Chesselet and Delfs 1996).

Effect of substantia nigra 6-OHDA lesion on pallidal TH-immunoreactivity

The DAergic innervation of the GP is brought about by collaterals from nigrostriatal fibers arising from the substantia nigra pars compacta (Fallon and Moore 1978; Lindvall and Bjorklund 1979; Rodrigo et al. 1998). DAergic fibers bear varicosities (Zaborszky and Cullinan 1996) and are supposed to form en passant synapses with pallidal cells (Arluison et al. 1984). To characterize the density and extent of the GP DA innervation in our preparation, we analyzed TH immunoreactivity in rats with unilateral 6-OHDA lesions of the substantia nigra. As expected, unilateral destruction of nigral DAergic cell bodies with a relatively high dose of 6-OHDA produced a massive reduction of TH-ir fibers in the ipsilateral GP. The ipsilateral GP was almost devoid of fibers reacting for TH in those animals bearing a near complete lesion of substantia nigra pars compacta DA neurons. Correspondingly, a massive loss of TH-ir fibers was detected in the rat GP after intrastriatal 6-OHDA infusion (Rosenblad et al. 2000). Likewise, in monkeys systemically treated with MPTP, TH-ir was reduced in the external pallidum (Jan et al. 2000); however, a relative sparing of the nigropallidal projection as compared to the nigrostriatal pathway has been observed as well (Parent et al. 1990).

It cannot be ruled out that 6-OHDA infusion into the substantia nigra damaged ascending noradrenergic fibers. As TH immunoreactivity is present in both DAergic and noradrenergic fibers, reduced TH staining in the GP might be in part due to damage of noradrenergic fibers in the GP. However, as there are only a small number of noradrenergic fibers in the basal ganglia (Gaspar et al. 1985; Lavoie et al. 1989), TH might be a reliable marker of DAergic fibers in our preparation. Our data further indicate that there is a significant correlation between nigral and pallidal loss of TH-ir. Remarkably, the caudal GP seems to be more severely affected by a nigral lesion as compared to the rostral GP. This might give a clue for a heterogeneous innervation pattern; however, this idea requires more detailed analysis. In line with the findings of Rodrigo et al. (1998), our data show that the fiber appearance varies along the rostrocaudal axis. There is evidence that nigrostriatal and nigropallidal DAergic fibers differ in their arborization pattern (for review see Smith and Kieval 2000). Gauthier et al. (1999) revealed that nigropallidal, but not nigrostriatal, DA fibers arborize profusely in the globus pallidus. Thus, it is very unlikely that the thick fibers in rostral GP form en passant synapses. These fibers are very likely thick axons that travel through the GP to reach the striatum. The thin fibers could represent a distinct nigropallidal projection. In addition, nigropallidal and nigrostriatal DAergic fibers might have different neurochemical characteristics. For instance, Parent et al. (1990) found in MPTP-treated primates that the nigropallidal projection is relatively spared compared to the nigrostriatal pathway. Our data showed that the loss of TH-ir was particularly high in the caudal GP. Given that thin DAergic fibers predominantly innervating the caudal GP represent the nigropallidal DA projection as suggested above, these findings implicate that in rats the nigropallidal DA fibers are more sensitive to the neurotoxic effects of 6-OHDA than nigrostriatal DA fibers. However, this notion is preliminary and needs further analysis.

Microdialysis: methodological considerations

The spatial resolution of the microdialysis technique deserves consideration when sampling from small brain

nuclei as the GP. To exclude DA spillover from the striatum, we positioned the dialysis membrane into the medio-caudal aspects of the GP to ensure maximum distance to the DA rich striatum. Our immunohistochemical analysis confirmed that the pallidal target area of the dialysis membrane receives a prominent DA innervation. Though our immunohistochemical data suggest rostrocaudal differences in the pattern of the pallidal DAergic innervation, we found no relationship between individual DA release characteristics measured by microdialysis and respective probe placements.

The sampling area for DA around the dialysis probe has been suggested to be smaller than 1 mm because this is the maximum distance radioactively labeled DA delivered from a second probe could be detected (Hoistad et al. 2000). Also, dialysate DA changes measured in core and shell subdivisions of the nucleus accumbens differed after physiological and pharmacological challenges, although the lateral distance of the microdialysis probes was only 0.9 mm (Sokolowski et al. 1998) or 1.2 mm (Hedou et al. 1999). Ewing and Wightman (1984), using in vivo voltammetry, assumed that the effective tissue diffusion distance for DA is even shorter, i.e., below 100 μm. These data suggest that DA measured here is mainly of pallidal origin; however, most analyses on DA diffusion were made in striatal tissue, which might have a diffusion dynamics different from that of the GP. Of course, one has to take into account that reuptake inhibition prolongs the effective diffusion distance of DA (Nicholson and Sykova 1998). On the other hand, the reuptake inhibitor was applied via reverse microdialysis and was supposed to act primarily around the probe, as its concentration markedly decreases with increasing distance from the probe. Furthermore, the density of striatal DA reuptake sites is high as compared to the GP (Mennicken et al. 1992). Therefore, it is unlikely that DA sampled by pallidal microdialysis probes is merely a spillover from the striatum.

Basal DA levels and nomifensine

Reliable measurement of basal dialysate DA levels was achieved using nomifensine (3 μM) and a calcium concentration close to physiological conditions (1.2 mM) (Moghaddam and Bunney 1989) in the perfusion fluid. The mean basal dialysate dopamine concentration was considerably lower (about 43%) than the values of our previous study on pallidal DA (Hauber and Fuchs 2000). This reduction might be primarily due to a decreased nomifensine concentration (from 30 μM to 3 μM). Corresponding nomifensine-induced changes in DA basal levels were found in the striatum, as a decrease from 10 μM to 1 μM nomifensine resulted in 2–3-fold reduction in dialysate DA levels (Nomikos et al. 1990). Together, these data suggest a comparable efficacy of pallidal and striatal DA reuptake, although quantitative autoradiographic analysis revealed a much lower density of DA uptake sites in the GP as compared to the striatum (Mennicken et al. 1992).

Effects of TTX on pallidal DA levels

A major finding of the present study is that extracellular DA in the rat GP is TTX-sensitive, implicating that the release is a result of neuronal activity. There is consistent evidence that chronic nomifensine application via reverse microdialysis does not alter major pharmacological and physiological characteristics of extracellular DA release (Di Chiara 1990; Feenstra and Botterblom 1996). Hence, TTX sensitivity and calcium-dependency of prefrontal DA release can be demonstrated under conditions of reuptake inhibition with a comparable nomifensine concentration (5 μM) (Santiago et al. 1993b). However, chronic reuptake blockade might elevate extracellular DA levels, resulting in an unphysiologically high DA tone, e.g., on DA D_2 autoreceptors. Therefore, it is conceivable that the magnitude of changes in extracellular DA after pharmacological challenge is difficult to compare quantitatively to conditions without reuptake inhibition. The levels of DA and metabolites significantly increased about 120 min after cessation of TTX administration. Likewise, rebound DA release was observed after perfusion with cocaine (Lee et al. 2001), which acts on DA neurons in part through inhibition of Na^+ channels (Kiyatkin and Rebec 2000). However, the mechanisms underlying rebound DA release as observed here are unknown, as long-term effects of TTX have been rarely investigated.

Taken together, our finding that dialysate DA levels are highly responsive for TTX, even during partial reuptake inhibition, suggests that extracellular DA levels in the GP depend on neuronal activity. Otherwise, TTX would not be effective in blocking pallidal DA efflux transiently. In our earlier study we demonstrated that GP extracellular DA is also calcium-dependent and responds to potassium stimulation (Hauber and Fuchs 2000). Therefore, pallidal DA fulfills the most stringent criteria to prove neuronal transmitter release (for reviews see Ungerstedt 1991; Di Chiara 1991; Westerink 1995) and our data add further support to the view that DAergic inputs to the GP are functional.

Effects of TTX on pallidal metabolite levels

Changes in dialysate levels of the major extracellular DA metabolites DOPAC and HVA also respond to TTX perfusion with a delay of at least one sample (30 min). Initially, TTX induced a decrease in DOPAC levels, as already shown by Westerink et al. (1987), followed by a long lasting increase. The biochemical mechanisms underlying changes of DA metabolites are difficult to delineate. DOPAC is a product of extracellular DA degradation by monoamine oxidase, an enzyme known to be present in the GP (Saura et al. 1992). The diminished DOPAC levels might be simply due to the reduced DA

release after TTX and thus to a reduced substrate availability. However, this explanation is complicated by the fact that extracellular DOPAC is assumed to be mainly a marker of DA synthesis originating from a cytoplasmatic pool of newly synthesized DA not being recently released (Zetterstrom et al. 1988; Soares-da-Silva and Garrett 1990). Therefore, an alternative explanation is that decreased DA release after TTX could lead to an intracellular accumulation of DA in the vesicular and cytoplasmatic pool (Arbuthnott et al. 1990). In turn, DA synthesis would be down-regulated, thereby decreasing DOPAC formation through fast inhibitory feedback mechanisms.

The time course of HVA and DOPAC levels after TTX perfusion were very similar in our experiment. This might reflect the fact that most extracellular DOPAC is rapidly cleared by the catechol-O-methyltransferase to HVA (Cumming et al. 1992).

Taken together, the fact that changes of DA, DOPAC and HVA were time-locked after TTX perfusion add support to the notion that pallidal DA innervation is functional as DA metabolizing systems are operative in a way shown for other nuclei receiving DAergic input (Westerink et al. 1987; Santiago et al. 1993a).

Implications for the role of pallidal DA in the basal ganglia

Immunocytochemical findings revealed that TH-positive fibers were in close proximity to cholinergic neurons in ventromedial aspects of the GP, suggesting that DAergic afferents might influence forebrain cholinergic neurons (Zaborszky and Cullinan 1996, Rodrigo et al. 1998, Zaborszky and Duque 2000). In line with this notion, the basal forebrain was found to play a complex role in amphetamine-stimulated cortical acetycholine release (Arnold et al. 2001). Thus, DA in the GP measured here might act on forebrain cholinergic neurons, which are involved in control of cognitive aspects of behavior (Sarter and Bruno 1994). Furthermore, DA in the GP modulates GABAergic projection neurons, which are part of the indirect basal ganglia pathway (Chesselet and Delfs 1996). There is evidence that DA, by acting on D_2 receptors in the GP, control gene expression in pallidal neurons (Marshall et al. 2001) as well as depolarization-induced pallidal GABA release (Floran et al. 1997) and intra-GP administration of DA or DA ligands changed activity in subsets of pallidal neurons (Napier et al. 1991; Querejeta et al. 2001). In addition, pallidal DA subserves motor functions, as blockade of intra-GP DA receptors resulted in akinesia in rats (Hauber and Lutz 1999b). Further evidence for a major role of pallidal DA in control of motor behavior has been provided in studies showing that intra-GP DA infusions were able to partially restore motor deficits in rats with unilateral lesion of the medial forebrain bundle (Galvan et al. 2001). Likewise, glial-cell-line-derived neurotrophic factor (GDNF) induced sprouting of DAergic axons in the GP and substantia nigra of Parkinsonian primates have been correlated with functional recovery from motor symptoms (Gash et al. 1996). Furthermore, the massive reduction of TH-ir fibers (Jan et al. 2000) and of tissue DA levels (Ploska et al. 1982; Hornykiewicz 1998) in the external pallidum in Parkinsonian patients most likely contributes to hypokinesia in this disease (Hornykiewicz 1998). In line with these latter studies, the present data confirm that lesions of substantia nigra DA neurons produced a massive impairment of the DAergic input in the rat GP. Our finding that pallidal DA release is of neuronal origin provides further support to the view that DAergic inputs to the GP are functional and play a significant role in basal ganglia motor control (Chesselet and Delfs 1996).

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