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Intrastriatal botulinum toxin abolishes pathologic rotational behaviour and induces axonal varicosities in the 6-OHDA rat model of Parkinson's disease

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ABSTRACT

Central pathophysiological pathways of basal ganglia dysfunction imply a disturbed interaction of dopaminergic and cholinergic circuits. In Parkinson's disease (PD) imbalanced cholinergic hyperactivity prevails in the striatum. Interruption of acetylcholine (ACh) release in the striatum by locally injected botulinum neurotoxin A (BoNT-A) has been studied in the rat 6-hydroxydopamine (6-OHDA) model of PD (hemi-PD). The hemi-PD was induced by injection of 6-OHDA into the right medial forebrain bundle. Motor dysfunction provoked by apomorphine-induced contralateral rotation was completely reversed for more than 3 months by ipsilateral intrastriatal application of 1–2 ng BoNT-A. Interestingly, BoNT-A injected alone into the right striatum of naïve rats caused a slight transient ipsilateral apomorphine-induced rotation, which lasted only for about one month. Immunohistochemically, large axonal swellings appeared within the striatum injected with BoNT-A, which we tentatively named BoNT-A-induced varicosities. They contained either choline acetyltransferase or tyrosine hydroxylase. These findings suggest a selective inhibition of evoked release of ACh by locally applied BoNT-A. Intrastriatal application of BoNT-A may antagonize localized relative functional disinhibited hypercholinergic activity in neurodegenerative diseases such as PD avoiding side effects of systemic anti-cholinergic treatment.

Introduction

A hallmark of Parkinson's disease (PD) is diminished dopaminergic signaling in the striatum (caudate putamen, CPu), which leads to increased release of acetylcholine (ACh) by disinhibited tonically active interneurons with the consequence of a disturbed network function and consecutive motor dysfunction. Current therapeutic strategies are based on two major approaches in order to correct the disturbed circuits of involuntary movement control, i.e. stimulation of dopamine (DA) receptors on GABAergic medium spiny neurons and inhibition of hypercholinergic activity of tonically active interneurons (Day et al., 2006; Obeso et al., 2008). However, both approaches are compromised by adverse side effects due to systemic drug application (Whitney, 2007). In the pre-L-DOPA era, the first drugs that turned out to have clinically significant anti-Parkinson activity were anti-cholinergics such as atropine and atropine-like derivatives (Duvoisin, 1967; Kaplan et al., 1954; Ordenstein, 1868). Today some of these compounds are still commonly used in clinical practice, e.g. the piperidine derivative Biperiden (Akineton®). Also, the PD-like side effects of anti-psychotics are typically treated with anti-cholinergic drugs, especially in younger patients (Ekdawi and Fowke, 1966; Mamo et al., 1999). However, the most serious problem with these systemically administered anti-cholinergics remains the occurrence of well-known troublesome peripheral and central side effects, such as mydriasis, paresis of accommodation (blurred vision), reduced salivation (dry mouth), parotitis, dry eyes, muscular pain, loss of power, alteration of voice, dysphagia, regurgitation, constipation, urinary retention, prostatism, tachycardia, fever in warm weather, hallucinations, memory problems and confusion. To avoid this disadvantage we tested local anti-cholinergic treatment applying botulinum neurotoxin A (BoNT-A) into the CPu of hemiparkinsonian (hemi-PD) rats, a well established 6-hydroxydopamine (6-OHDA)-induced animal model of PD (Meredith et al., 2008). In this model, it has been shown that intraperitoneally applied atropine antagonizes the profound PD-typical akinesia (Schallert et al., 1978). In combination with L-DOPA it supported the alleviation of excessive bracing reactions and suppressed pathological circling of the PD rats (Schallert et al., 1979). In our study, intrastriatal application of BoNT-A caused complete long-term abolition of pathological apomorphine-induced

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rotation, which indicates a compensatory effect to the dopaminergic destruction. Simultaneously, large axonal swellings were detected immunohistochemically both at the light and electron microscopic levels in the BoNT-A-injected CPu, which were positively stained for either the cholinergic marker enzyme choline acetyltransferase (ChAT) or the dopaminergic marker enzyme tyrosine hydroxylase (TH). We tentatively named these swellings BoNT-A-induced varicosities (BiVs). The findings for the first time describe morphological changes in response to intrastriatal BoNT-A-application and their functional consequence. Thereby, our study serves as a proof-of-principle for a novel type of intervention with local hypercholinergic activity in the central nervous system (CNS), which may contribute to new therapeutic strategies for diseases with local over-activity of cholinergic neurons like in PD.

Materials and methods

Animals

Adult male Wistar rats (strain Crl:WI BR, Charles River Wiga, Sulzfeld, Germany) aged about 3 months were used and housed in standard cages at 22 °C ± 2 °C under a 12 h light/dark cycle, with free access to tap water and a standard diet.

Stereotactic intervention of four animal groups

Hemi-PD was induced in adult male Wistar rats by injection of 6-OHDA into the right MFB. Rats weighing 280–300 g were anaesthetized by intraperitoneal injection of ketamine (50 mg/kg body weight) and xylazine (4 mg/kg body weight) for stereotactic surgery. Four experimental groups were constituted: (1) 6-OHDA-lesioned animals receiving BoNT-A (6-OHDA-lesion + BoNT-A group), (2) 6-OHDA-lesioned animals receiving BoNT-A-vehicle substance (6-OHDA-lesion + sham-BoNT-A group), (3) sham-6-OHDA-lesioned animals receiving BoNT-A-vehicle substance (sham-6-OHDA-lesion + sham-BoNT-A group), and (4) naïve animals receiving BoNT-A (BoNT-A only group).

Lesions were made in the right MFB by injection of 4 µl of 6-OHDA (24 µg) dissolved in 0.1 M citrate buffer and delivered over 4 min each via a 26 gauge 5 µl Hamilton syringe. The coordinates with reference to bregma were: anterior–posterior = −2.3 mm, lateral = −1.5 mm and ventral = −9.0 mm, respectively (Paxinos and Watson, 2007). Successful lesions were evaluated with apomorphine-induced rotations 28 days after surgery. Six weeks after 6-OHDA-lesioning animals received injections of 2 × 1 µl BoNT-A solution (Lot #13028A1A, List, Campbell, USA, purchased via Quadratech, Surrey, UK) containing a total of 100 pg, 1 ng or 2 ng BoNT-A into the right CPu delivered over 4 min for each 1 µl aliquot (6-OHDA-lesion + BoNT-A group). The coordinates with reference to bregma were: anterior = +1.3/−0.4 mm, lateral = −2.6/−3.6 mm and ventral = −5.5 mm, respectively. The first control group received BoNT-A-vehicle solution containing phosphate-buffered saline with 0.1% bovine serum albumin (PBS–BSA 0.1%) instead of BoNT-A (6-OHDA-lesion + sham-BoNT-A group). The remaining two animal groups received either only BoNT-A solution without previous lesioning (BoNT-A only group) or both vehicle solutions instead of 6-OHDA and BoNT-A, respectively (sham-6-OHDA-lesion + sham-BoNT-A group).

Apomorphine-induced rotation test

The DA receptor agonist apomorphine stimulates the supersensitive DRD2 (and to a lesser degree DRD1) receptors on the injured DA depleted hemisphere more than the normal DRD2 and DRD1 receptors on the intact side causing a net rotation away from the side of the lesion, i.e. anti-clockwise (Ungerstedt et al., 1969). In our study apomorphine was applied subcutaneously at a concentration of 0.25 mg/kg body weight in normal saline. Rotations were measured using a self-constructed automated rotometry device over 40 min modified according to Ungerstedt and Arbuthnott (1970). They were defined as complete 360° ipsilateral turns and reported as net differences between the two directions per minute. The rotation test was performed 28 days after 6-OHDA-lesioning and at different time points after BoNT-A or vehicle application (see Results section and Fig. 1).

Histochemistry

For analysis of the consequences of BoNT-A-treatment on the morphology and neurotransmitter expression of affected brain regions investigations were performed applying the following stainings or immunohistochemical reactions: (1) cresyl violet according to Nissl (for vital neurons), (2) ChAT (for cholinergic neurons), (3) TH (for dopaminergic neurons), and (4) glutamic acid decarboxylase-67 (GAD-67) (for GABAergic neurons). In detail, after finishing of rotation tests animals were killed with an overdose of ketamine/xylazine and perfused with 3.7% paraformaldehyde solution for fixation. The fixed brains were cryoprotected, frozen and stored at −80 °C. Coronal brain sections of 30 µm thickness were prepared by cryo-cutting and consecutive sections were stained according to Nissl or immunostained for ChAT, TH and GAD-67, respectively, using the avidin–biotin complex immunoperoxidase method (Vector Laboratories, Burlingame, CA, USA). The following primary antibodies were applied: polyclonal goat anti-ChAT affinity purified antibody (Millipore, Schalbach, Germany), monoclonal mouse anti-TH antibody (clone TH2, Sigma-Aldrich, St. Louis, MO, USA) and monoclonal mouse anti-GAD-67 antibody (Chemicon International, Millipore, Temecula, CA, USA). Endogenous peroxidase was blocked with 3% H_2O_2 in 0.1 M PBS for 15 min followed by three washings with PBS for 5 min. Nonspecific binding of proteins was blocked by incubation with 3% rabbit serum (for ChAT staining) or with 3% goat serum and 1.5% horse serum (for TH and GAD-67 staining) at room temperature for 60 min. Sections were then incubated (1) in 10 µg/ml of primary antibody overnight at 4 °C. After subsequent washing with PBS, sections were (2) incubated with either biotinylated rabbit anti-goat IgG (for ChAT staining) or with biotinylated horse anti-mouse IgG (for TH and GAD-67 staining) overnight at 4 °C before color development with diaminobenzidine and ammonium nickel sulfate for enhancement.

Image analysis of immunohistochemical stainings

For quantification of immunohistochemical findings an optical grid was applied and at least 1000 total cells per CPu section were counted manually for ChAT-stained cells (see Fig. 2). In both ChAT- and TH-stained sections of BoNT-A-injected CPu peculiar axonal varicosities, intentionally named BoNT-induced varicosities (BiVs), were found. They were counted in areas of equal size and localization at both sides of the brain thereby comparing the density of BiVs between the lesioned and the intact sides. For quantitation of BiVs, sections were digitized at a resolution of 0.23 µm using the virtual slide scanner Mirax (Zeiss, Jena, Germany) (Mikula et al., 2007). Virtual slides were converted to a multiresolution image pyramid to allow the interactive definition of the region of interest (ROI), i.e. the CPu complex as illustrated by a TH-stained section in Fig. 3. BiVs were segmented using a multiscale (gray level classes) multifeature (roundness, local BiV staining intensity, and BiV area) approach implemented in Matlab (Mathworks). Due to the large amount of data (Gigapixel images) a parallelization was performed on Linux 64Bit systems. The result of a typical segmentation is shown in Figs. 3E and F. The segmented BiVs were mapped in 2D (Fig. 3C) to judge the spatial distribution of BiVs. The local density of BiVs was color coded (Fig. 3D).

In the ChAT-stained sections the evaluation is more complex due to quantification of ChAT-positive neurons and BiVs (Figs. 2A–C). Due to Gauss-distributed noise anisotropic noise filtering need to be performed for successful segmentation of nerve fibres (Figs. 3B and C). The total number of neurons was estimated using the optical fractionator in

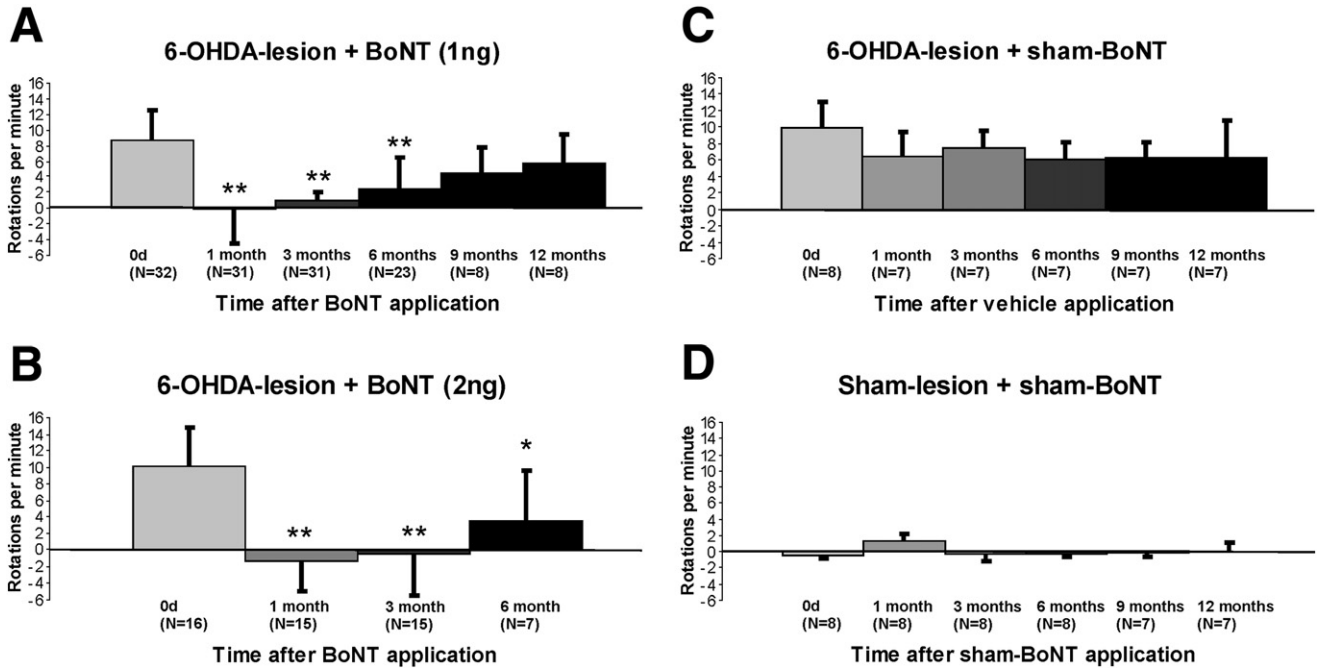

Fig. 1. Apomorphine-induced rotation of hemi-PD rats treated with intrastriatal BoNT-A. In order to induce a toxic model of PD, Wistar rats received 6-OHDA injections into the right MFB, which leads to an apomorphine-induced asymmetric rotation away from the site of lesion, i.e. anti-clockwise, of about 9 rotations per minute (positive values). Six weeks after 6-OHDA-application rats received injections of 1 ng or 2 ng BoNT-A (A, B) or vehicle (C) into the ipsilateral CPu (0 d). (A, B) BoNT-A abrogated the apomorphine-induced rotation completely for 3 months. Subsequently, the pathologic rotation was slowly restored, but even after 6 months there was still a significant reduction seen. (C) Vehicle treatment had a slight non-significant reducing effect up to 12 months. (D) For control, naïve rats were injected with vehicle substance of 6-OHDA solution (0.1 M citrate buffer) into the right MFB and subsequently with vehicle substance of BoNT-A (PBS–BSA 0.1%) into the ipsilateral CPu. This double-sham treatment had no effect on apomorphine-induced rotation. All results are presented as mean values ± SD. Asterisks indicate significant changes in comparison to initial values (0 d) according to one way ANOVA and post-hoc Holm–Sidak test (A, B) and Kruskal–Wallis test (C, D), respectively. P-values: $^{*}<0.01$, $^{**}<0.001$.

Stereoinvestigator 8.0 using the BX51 (Olympus, Hamburg, Germany) three axes motorized videomicroscope. In each case (serial sections of an animal) the coefficient of error was smaller than or equal to 0.05. Visualization of immunofluorescent stainings was performed on the confocal laser scanning microscope Eclipse E400 (Nikon, Düsseldorf, Germany).

Immunoelectron microscopy

For ultrastructural analysis of BiVs, striatal sections were immunoreacted with antibodies against TH and ChAT, embedded in epoxy resin and evaluated with a transmission electron microscope. In detail, animals were perfused with PBS containing 3.7% paraformaldehyde and 0.05% to 0.1% glutaraldehyde (pH 7.2). Brains were dissected out after 1 h and postfixed overnight, cryoprotected and processed as described for light microscopy with the following exceptions: 50–60 µm floating sections were cut and collected in 0.1 M PBS and treated with 3% H_2O_2 for 15 min. No permeabilizing agents were used. All following steps were performed in a 12-well cell culture plate. Incubation times of primary antibodies were extended to 2 days. Secondary antibodies were biotinylated and visualized with standard ABC/DAB methods. No Nickel amplification was performed. After the DAB reaction the sections were washed in PBS and postfixed in cacodylate buffer (pH 7.6) containing 2.5% glutaraldehyde overnight, then washed and postfixed in 1% osmium tetroxide for 30 min. After dehydration in a series of graded ethanols and infiltration with a mixture of 100% ethanol/Epon 812 resin (v/v) and pure Epon overnight, the sections were embedded between two Aclar sheets (Ted Pella, Redding, CA, USA), fixed between glass slides and polymerized at 60 °C for 24 h. After light microscopic evaluation of the immunoreaction, regions of interest were cut out, one Aclar sheet removed, and the specimen glued on an Epon cylinder. Ultrathin sections were mounted on Formvar-coated slot copper grids contrasted with 2% aqueous uranyl acetate (8 min) and lead citrate (2 min) and analyzed with a Zeiss EM 906 transmission electron microscope at 80 kV (Zeiss, Oberkochen, Germany).

Statistics

Data of time-kinetics of the apomorphine-induced rotation test were compared by one way analysis of variance (ANOVA) for Gaussian distributed values. Significant results were derived from the post-hoc test according to Holm–Sidak (Figs. 1A and B). Non-Gaussian distributed values were compared by the Kruskal–Wallis test (Figs. 1C and D). For comparison of the rotations of animals treated with BoNT-A with the corresponding rotations of vehicle-treated animals 2-tailed Student's t-test for unpaired observations was applied. Likewise the results of quantitative immunohistochemistry were compared by 2-tailed Student's t-tests for unpaired observations. The level of significance was set at $p<0.05$ for all statistical analyses.

Ethics

All animal experiments were approved by the local Animal Research Committee of the state Mecklenburg-Western Pomerania (LALLF M-V/TSD/7221.3-1.1-019/08).

Results

In order to determine the effective and tolerated dose of BoNT-A to be applied intrastriatally, we first injected BoNT-A into naïve rat

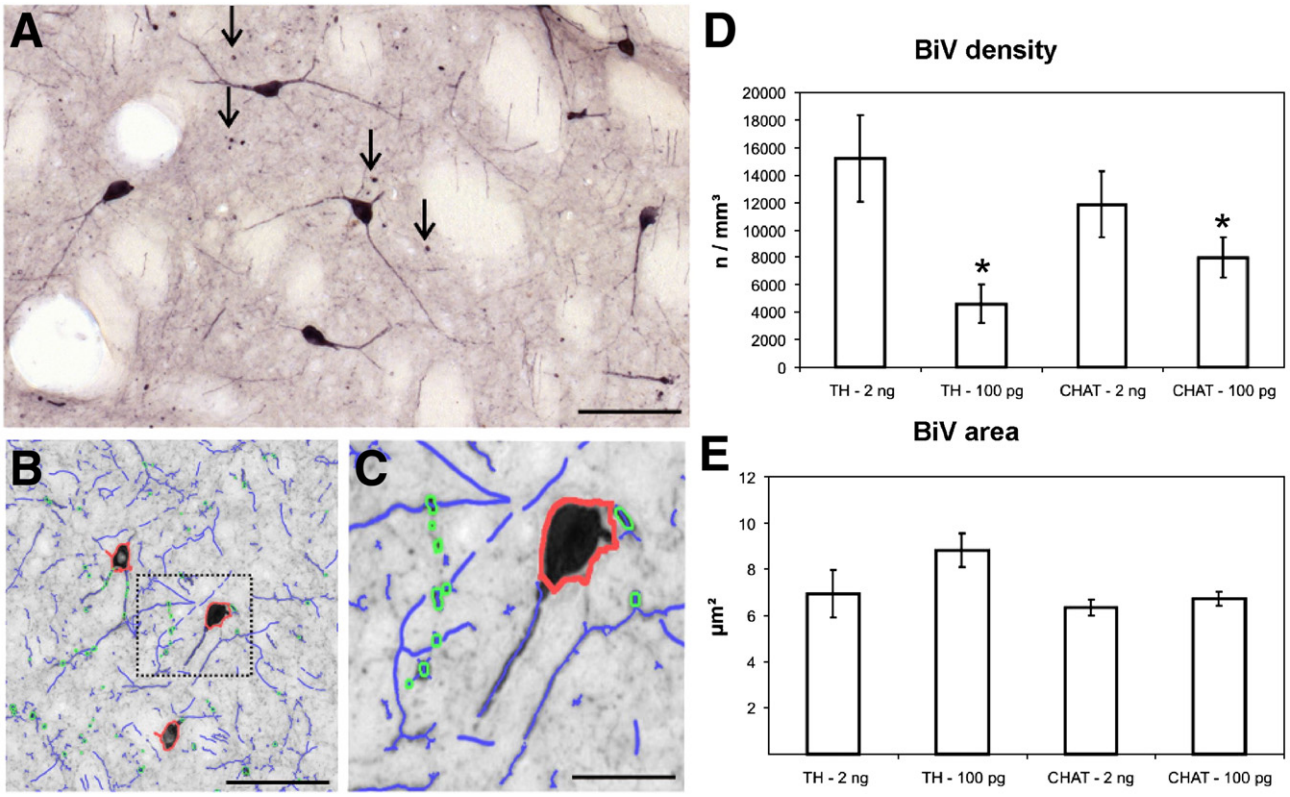

Fig. 2. Immunohistochemical staining of the rat CPu for ChAT one month after ipsilateral intrastriatal application of 2 ng BoNT-A. (A) 6 ChAT-positive neurons are visible. Arrows are pointing to ChAT-positive axonal swellings (BiVs). (B) Segmentation of nerve fibres (blue), neurons (red) and BiVs (green) with a magnification in (C). Scale bars: in A 30 µm, in B 20 µm, in C 10 µm. (D and E) Quantitative analysis of BiVs in CPu sections. (D) Density [n/mm³] of BiVs in the CPu detected in TH and ChAT immunohistochemistry after injection of 2 ng or 100 pg of BoNT-A is dose-dependently increased as confirmed by Student's t-tests (* = $p<0.05$). (E) Projection areas of BiVs were not different between the CPu treated with 2 ng and 100 pg BoNT-A. Error bars are indicating SEM.

striata and estimated their apomorphine-induced rotational behaviour. As 100 pg BoNT-A was the minimum dose revealing a tendency of asymmetric apomorphine-induced rotation and 5 ng BoNT-A was occasionally lethal to the animals, we used doses of 100 pg–2 ng BoNT-A for subsequent experiments in rats with and without prior lesioning of the substantia nigra pars compacta by 6-OHDA. Effects on motor function and on striatal neuronal morphology were measured over a time period of up to twelve months.

Effect of instrastriatally applied BoNT-A on apomorphine-induced rotation in hemi-PD rats

6-OHDA injections into the right medial forebrain bundle (MFB) caused apomorphine-induced rotations away from the lesion site, i.e. anti-clockwise (positive values between 8 and 10 per min, 0 d in Figs. 1A–C), a hallmark of hemi-PD. When the rats six weeks after 6-OHDA application received injections of 1 ng or 2 ng BoNT-A into the ipsilateral CPu, this treatment abolished the apomorphine-induced rotations completely for about 3 months. After this time the reducing effect slowly declined (Figs. 1A and B). BoNT-A-vehicle caused no significant changes (Fig. 1C). Comparison of apomorphine-induced rotations of BoNT-A-treated 6-OHDA-lesioned rats with the corresponding vehicle-treated 6-OHDA-lesioned rats revealed that the suppressive BoNT-A effect was significant up to 6 months for 1 ng BoNT-A (p-values according to Student's t-test: BoNT-A versus vehicle at 1 month: 0.0001, at 3 months: 0.0025, at 6 months: 0.0239, at 9 months: 0.3472 and at 12 months: 0.8187). Additional control experiments were performed by sham 6-OHDA-lesioning with injection of the vehicle (0.1 M citrate buffer) into the right MFB followed by sham BoNT-A-treatment with injection of the vehicle substance PBS–BSA 0.1% into the ipsilateral CPu. This double-sham treatment did not cause significant apomorphine-induced rotations during the 12 month observation period (Fig. 1D).

If BoNT-A alone was applied to the CPu of naïve rats at doses of 1–2 ng, apomorphine induced 2–3 rotations per minute towards the injection site as soon as 2 weeks after toxin application (data not shown). However, this effect was transient and vanished after 2 months. 100 pg BoNT-A and vehicle solution had no significant effect.

In order to test the effect of intrastriatal BoNT-A application on non-drug induced sensorimotor function in hemi-PD rats we analyzed the forelimb-use asymmetry by applying the cylinder test described by Schallert and Tillerson (2000). A tendency of improvement of the asymmetric forelimb usage by BoNT-A was seen over the time period of up to 12 months with significance at the high dose of 2 ng, whereas sham lesion and sham BoNT-A application did not affect the asymmetric forelimb usage significantly (see Supplement 1).

Effect of BoNT-A on CPu morphology

Concerning the morphological consequences of intrastriatal BoNT-A-application the total number of ChAT-positive neurons was not different between BoNT-A-treated and untreated CPu, neither after injection of 100 pg BoNT-A (ipsilateral: 22,233 ± 1036; contralateral: 19,846 ± 4039; n = 3) nor after injection of 1 ng BoNT-A (ipsilateral: 20,500 ± 2076; contralateral: 20,799 ± 2979; n = 4). Preliminary counts performed 6 months after BoNT-A-application confirmed this finding (data not shown).

The most striking novel morphological finding of our study was the appearance of regularly occurring axonal swellings in the BoNT-A-infiltrated areas, which we named BoNT-A-induced varicosities (BiVs). These structures were found selectively at any injection site

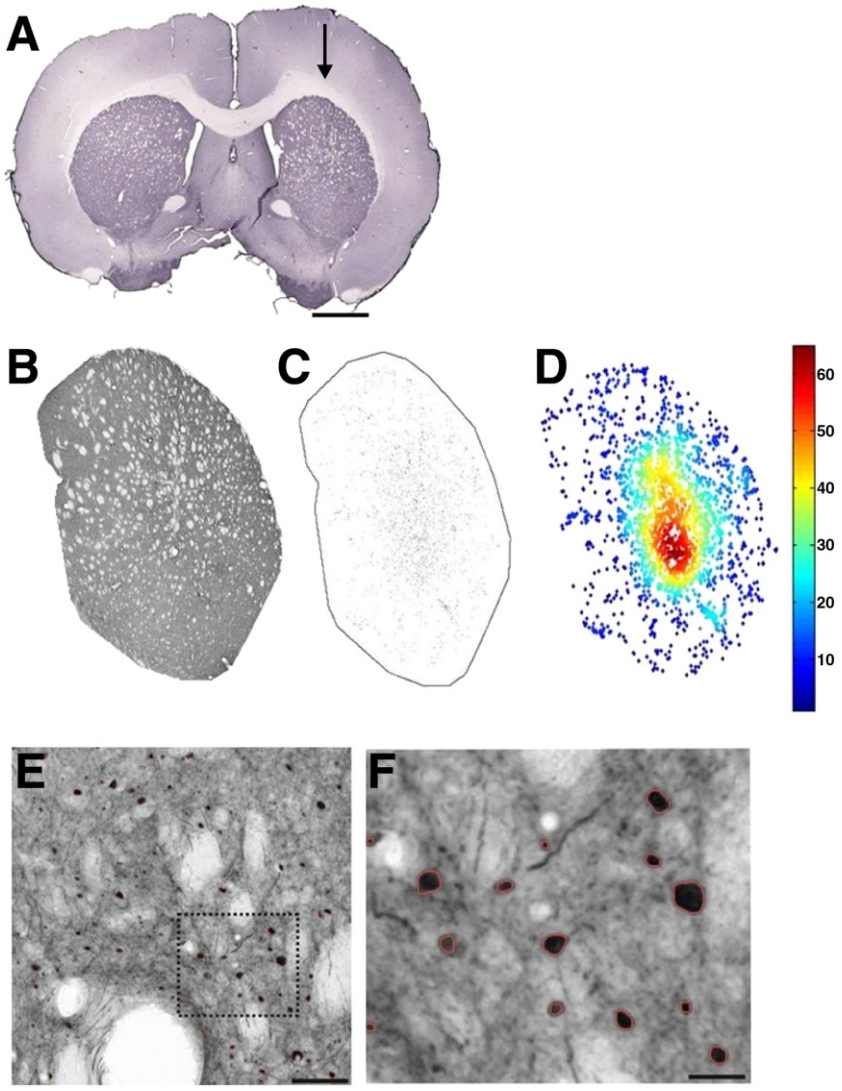

Fig. 3. Immunostaining for quantification of BiVs in the CPu one month after unilateral application of 2 ng BoNT-A. (A) TH-stained coronal brain section. The injection site is in the right CPu (arrow) and the evaluated region of interest (ROI) is shown in B. The segmented BiVs are mapped in C. In D the concentric distribution increase of BiVs is visualized. The scale displays the number of BiVs around a central BiV. (E) 1024 × 1024 large image tile of the 10,000 × 20,000 large image of the ROI (B) with segmented BiVs. (F) Magnification from E showing details of the segmentation quality. Scale bars: in A 1 mm, in E 50 μm, in F 10 μm.

of BoNT-A, i.e. in the ipsilateral, but not in the contralateral CPu of 6-OHDA-lesioned animals, as well as in the BoNT-A-treated CPu of naïve rats, but not in its opposite site. The size of the BiVs was heterogeneous ranking between 2 and 9 μm in diameter when investigating the histochemically stained CPu sections of BoNT-A treated hemi-PD or naïve rats. BiVs were reactive either for ChAT (Figs. 2A–C and 4A and B) or for TH (Figs. 3 and 4A), but never double-stained for both marker enzymes (Figs. 4A and B) and never stained for TH in the dopaminergic deafferented CPu of 6-OHDA-lesioned animals (Fig. 4B). Quantitative evaluation revealed the following (Figs. 2D and E): The density of ChAT-positive BiVs [n/mm^3] was higher in animals treated with 2 ng (11,836 ± 2404) than in animals treated with 100 pg (7989 ± 1488) BoNT-A. For TH-positive BiVs this dose-dependent difference was even higher (2 ng: 15,188 ± 3157; 100 pg: 4607 ± 1409). In contrast, the mean BiV area was not significantly different between animals treated with 2 ng and 100 pg BoNT-A, neither for ChAT-positive nor for TH-positive BiVs (ChAT-positive BiVs: 2 ng: 6.34 ± 0.34 μm^2, 100 pg: 6.73 ± 0.29 μm^2; TH-positive BiVs: 2 ng: 6.95 ± 1.02 μm^2, 100 pg: 8.82 ± 0.72 μm^2). GABAergic BiVs were not detected (data not shown).

TH-positive BiVs in naïve rats injected with BoNT-A were further investigated by electron microscopy. Ultrastructurally, these structures appear as large axonal dilations filled with TH-immunoreactive vesicles and some scattered mitochondria (Figs. 4D and E). Synapses, however, were not positively identified so far.

Discussion

For detection of functional effects of intrastriatal BoNT-A in the rat hemi-PD model we chose the apomorphine-induced rotation test. This test provides a sensitive and rapid behavioural correlate of striatal damage and has been used to evaluate the potential efficacy of therapeutic trials with tissue and/or cell grafts in different conditions of striatal degeneration (Emerich et al., 1996; Nikkhah et al., 1994; Norman et al., 1988). As a first step, the effective and tolerated BoNT-A dose was determined. In the literature an effective dose range of BoNT-A between 4 ng and 150 ng was reported for injections into skeletal muscles in humans (Borodic et al., 1996) and between 135 pg and 1875 pg for injections into whisker pad muscles (135 pg), corpus vitreous (1.875 ng), superior colliculus (225 pg) and hippocampus (300 pg), respectively, in Sprague Dawley rats (Antonucci et al., 2008). We found effective doses of 1 ng–2 ng BoNT-A for the intrastriatal application, whereas higher doses turned out to be toxic. Therefore, the dose most suitable for therapeutic intervention in

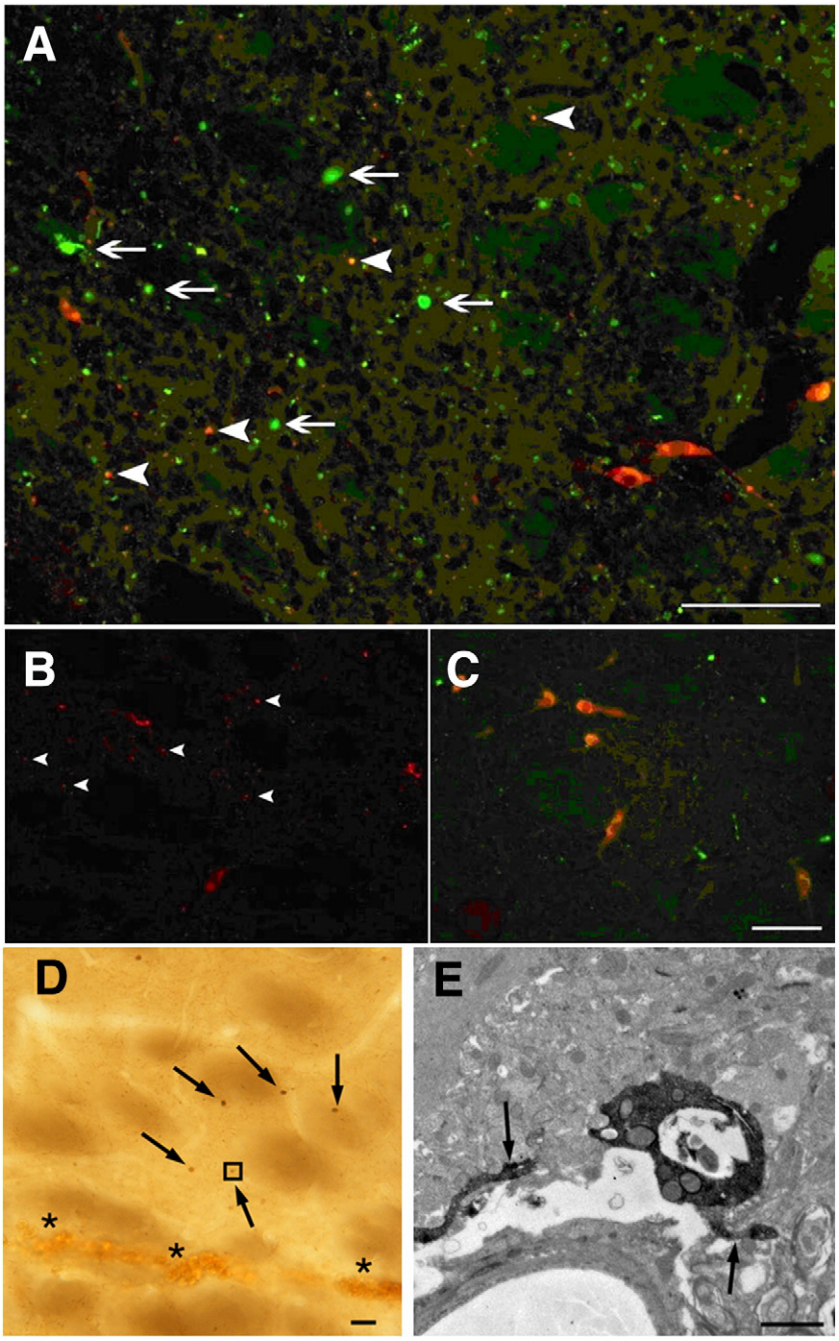

Fig. 4. Immunofluorescence of the rat CPu for ChAT (red) and TH (green) visualized by confocal laser scanning microscopy. (A) Section of the CPu of a naïve rat one month after application of 2 ng BoNT-A. ChAT-positive cell bodies and projections are clearly separated from TH-positive projections. Both types of projections display several axonal swellings (BiVs) of heterogeneous size (arrows: TH-positive BiVs, arrowheads: ChAT-positive BiVs). Double-positive BiVs are not detected. (B) CPu of a rat with 6-OHDA-lesion and subsequent ipsilateral injection of 1 ng BoNT-A. Double staining for ChAT and TH shows ChAT-positive cell bodies and BiVs (arrowheads), but no TH-positive projections. (C) A corresponding section contralateral to the side shown in B displays no BiVs, but ChAT-positive cell bodies and projections and TH-positive projections of similar density as at the ipsilateral side of rats treated with BoNT-A only (shown in A). (D–E) Electron microscopy of TH-positive BiVs. (D) A 50 μm thick TH-positive section of a naïve BoNT-A treated rat, embedded in Epon, exhibiting BiVs (arrows) approx. 5 μm in diameter. Asterisks mark the injection channel, filled with macrophages. The insert marked by a rectangle shows a BiV similar to that enlarged in (E). A dilated axonal projection of a TH-positive neuron is filled with vesicles (approx. 30 nm) and mitochondria. The axon partly wraps other, non-TH-reactive axonal processes. Two TH-positive projections of this BiV are arrow-marked. Scale bars: 100 μm in A–C, 20 μm in D and 1 μm in E.

the rat PD model appears to be around 1 ng and this dose is also recommended to start with in other conditions of local cholinergic hyperactivity in the CNS.

Functional effect of intrastriatally applied BoNT-A

In the 6-OHDA-lesioned hemi-PD rats the dopaminergic input into the right CPu is destroyed leading to a right side DA receptor supersensitivity mainly involving DRD2 receptors (Kaasinen et al., 2000). Thereby apomorphine acts in the right CPu as an enhancer of the inhibitory action with the consequence of anti-clockwise rotation. In PD the well-known hypercholinergic state interferes negatively with the basal ganglia loops (2), the hyperactive tonic cholinergic interneurons targeting the GABAergic medium spiny projection neurons of the CPu. Local application of BoNT-A into the CPu abolishes ACh release, thus reducing the apomorphine-induced contralateral rotations. It cannot be excluded that striatal damage, which would also yield ipsilateral rotations, may antagonize the contralateral

circling. However, striatal neuron loss by BoNT-A application is unlikely, since the number of cholinergic neurons is not reduced in the CPu after BoNT-A treatment. Analysis of the asymmetric forelimb usage argues for a beneficial effect of intrastriatal BoNT-A application on non-drug induced motor function, too, although the effect was slight and extended studies with different non-drug induced tests are needed in order to evaluate its therapeutic potential.

Surprisingly, the rotation-reducing effect of BoNT-A in the hemi-PD rats lasts for at least half a year. This is interesting, since thereby the compensatory BoNT-A effect in the CPu lasts longer than the conventional inhibitory BoNT-A effect on peripheral muscle activity, which is usually terminated to about 3 months due to intermittent axonal sprouting and reformation of affected transport proteins (Benecke and Dressler, 2007; de Paiva et al., 1999). Of course, the regeneration time in the periphery depends also on the injection site and size of affected structures. It is somewhat longer than 3 months, when small muscles or perspiratory and salivary glands are injected (Heckmann et al., 2005; Reid et al., 2008). However, there may also be more differences between the mechanisms of peripheral and central BoNT effects. In this context, the novel morphological finding of axonal swellings that we intentionally called BiVs could be of relevance.

Effect of BoNT-A on striatal morphology

In any BoNT-A-infiltrated areas, whether they contained only cholinergic terminals, i.e. in 6-OHDA-lesioned rats, or whether they contained both cholinergic and dopaminergic terminals, i.e. in naïve rats, BiVs appeared along the neuronal axons. At first glance, they resemble classical boutons as previously described, e.g., for cortical neurons (de Paola et al., 2006). Concerning the mechanism of their formation, BoNT-A binds via its heavy chain component with low affinity to gangliosides and with high affinity to the synaptic vesicle protein SV2 (Dong et al., 2006; Mahrhold et al., 2006) of presynaptic membranes. As a next step receptor-mediated endocytosis occurs followed by release of the light chain of BoNT-A from the acidified endosome into the cytoplasm due to reduction of a disulfide bond. At the vesicular membrane the light chain due to its Zn^{2+}-dependent metalloprotease activity cleaves the SNARE target SNAP25. This prevents the fusion of neurotransmitter containing vesicles with the presynaptic plasma membrane, the prerequisite of neurotransmitter secretion into the synaptic cleft, and may lead to accumulation of several vesicles, which subsequently become confluent and form the varicosity-like structures. Of interest, at the motor endplate ACh is predominantly released via full vesicle fusion, whereas in the CNS a transient “kiss-and-run” mechanism may prevail under physiological conditions (Brunger et al., 2009), which are impaired in the presence of BoNTs. Details of the differences of the mechanisms of action of BoNT-A in the CNS and at the motor endplate are currently not fully understood. Their disclosure remains a task of future investigations.

The selectivity of BoNT binding to nerve cell terminals determines the selectivity of neurotransmitter blockade by BoNTs with preference of cholinergic terminals. However, it is well known that depending on their concentration BoNTs can inhibit the evoked release of a variety of neurotransmitters including DA, norepinephrine, serotonin, glutamate, GABA and glycine (Ashton and Dolly, 1988; Bigalke et al., 1981; Pearce et al., 1997). In the femtomolar range BoNT-A blocks the neuromuscular junction, whereas in the picomolar range it affects almost all transmitter systems (Bigalke et al., 1985). Therefore, it is not surprising that we found in addition to BiVs containing the marker enzyme of cholinergic neurons ChAT also BiVs containing the marker enzyme of dopaminergic neurons TH. The exact mechanism by which the BiVs are generated is unknown. Sequential electron micrographs will be required in order to get deeper insight in the early events of BiV formation after BoNT-A-application.

Since intrastriatal BoNT-A-application did not substantially affect the number of ChAT-positive neurons in the BoNT-A-injected CPu, there is no morphological indication for a non-specific toxic effect of BoNT-A on this cell population of the BoNT-A-treated CPu. However, testing of their functional integrity awaits further histochemical and electrophysiological investigations.

In summary, intrastriatal application of BoNT-A leads to a well-tolerated long-lasting localized reduction of cholinergic activity within the CPu. In the 6-OHDA rat model of PD, pharmacologically induced pathological motor activity is attenuated and spontaneous motor activity slightly improved. Extended functional test in the rodent and primate models of PD will show, whether these findings can contribute to new therapeutic strategies for treatment of neurodegenerative diseases with localized imbalanced hypercholinergic activity such as PD. Since detection of TH-positive BiVs argues for an inhibition of residual intrastriatal DA release by BoNT-A, a comprehensive new therapeutic concept for PD should combine local application of BoNT-A by a gentle procedure (Rogawski, 2009) with conventional systemic application of L-DOPA or DA agonists (Whitney, 2007). Respective trials in the animal model are under way. In addition, our findings may enable the development of improved animal models of hypo-cholinergic disorders such as Huntington's disease and progressive supranuclear palsy for pathophysiological investigations and pharmacological manipulation (Pisani et al., 2007).

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