Aus dem Institut für Anatomie
der Universitätsmedizin Rostock

Die intrazerebrale Applikation von Botulinum Neurotoxin-A als experimentelle antiparkinsonoide Therapie in verschiedenen Murinae

Kumulative Habilitationsschrift
zur
Erlangung des akademischen Grades
doctor rerum naturalium habilitatus (Dr. rer. nat. habil.)
der Universitätsmedizin Rostock

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Wohnhaft in Rostock

Rostock, den 19.06.2020

https://doi.org/10.18453/rosdok_id00003191

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Datum der Einreichung: 24.06.2020
Datum der Verteidigung: 31.05.2021
Datum der Probevorlesung: 02.07.2021

Inhalt

1. Zusammenfassung

Hintergrund und Rationale

In Vorarbeiten der Arbeitsgruppe Prof. Andreas Wree konnte gezeigt werden, dass motorische Defizite in einem Hemiparkinson-Modell der Ratte durch eine Injektion von Botulinumneurotoxin-A (BoNT-A) in den Caudatus-Putamen-Komplex (Striatum) gemildert werden können. In dem verwendeten Modell führt ein Dopaminmangel im Striatum zu einer Enthemmung striataler cholinerger Interneurone, verbunden mit einem striatalen Hypercholinismus. BoNT-A unterdrückt dort lokal, ohne systemische anticholinerge Nebenwirkungen, die präsynaptische Ausschüttung von Acetylcholin. Im Rahmen der vorgelegten Habilitationsarbeit wurde der mögliche therapeutische Einsatz intrazerebraler BoNT-A-Injektionen in einem Maus- und Rattenmodell vergleichend untersucht.

Zielstellung

Folgende Fragen sollten beantwortet werden:

Führt eine intrastriatale BoNT-A-Behandlung zu einem Verlust cholinerger Interneurone im Striatum? Hat eine intrastriatale BoNT-A-Behandlung über das pharmakainduzierte Rotationsverhalten hinaus einen positiven Einfluss auf die experimentell induzierten motorischen und sensomotorischen Defizite im hemiparkinsonoiden Tiermodell? Lässt sich das experimentelle Therapiekonzept der intrastriatalen Injektion von BoNT-A von der Ratte auf die Maus übertragen? Ist eine erneute Injektion von BoNT-A in das Striatum möglich, nachdem die Wirkung einer ersten Behandlung abgeklungen ist und tritt hierbei ein erneute Besserung motorischer Defizite im Hemiparkinsonmodell ein, ohne dass die wiederholte BoNT-A-Behandlung gesundheitliche Schäden bei den Versuchstieren auslöst? Lässt sich die therapeutische Zeitspanne durch eine zweite BoNT-A-Behandlung verlängern? Hat eine zweite BoNT-A-Behandlung degenerative Prozesse im Gehirn zur Folge?

Methoden

Mittels stereotaktischer Operationen wurde ein Hemiparkinsonmodell generiert und BoNT-A injiziert. Es wurden pharmakainduzierte Rotationstests angewendet, die durch Tests der forcierten als auch der spontanen Motorik und der sensomotorischen Integration der Versuchstiere ergänzt wurden. Diese Testungen wurden zu definierten Zeitpunkten bis zu einem Jahr nach den jeweils letzten Behandlungen durchgeführt.

Im Anschluss an die Verhaltenstests wurden die Gehirne der Versuchstiere histologisch, immunhistochemisch, stereologisch und volumetrisch aufgearbeitet. Die striatalen Volumina und die Anzahl der darin enthaltenen cholinergen Interneurone sowie deren Dendritenbäume beider Hemisphären wurden nach unterschiedlicher Überlebenszeit aus den Gruppen der schein- und BoNT-A-behandelten Tiere gegenübergestellt. Axonale Anschwellungen, die im Striatum nach BoNT-A-Behandlung auftreten, wurden nach demselben Aufarbeitungsprinzip einschließlich hinsichtlich der Dosisabhängigkeit des applizierten BoNT-A untersucht.

Ergebnisse

Eine ipsilaterale BoNT-A-Behandlung führt im Hemiparkinsonmodell der Ratte über drei Monate hinweg zu einer signifikanten Reduktion des apomorphininduzierten Rotationsverhaltens. Eine kontralaterale BoNT-A-Injektion führt zu einer signifikanten Aufhebung eines einseitigen Neglects der Umwelt und zu einem wieder vermehrten Gebrauch der zuvor beeinträchtigten Vorderpfote. Eine wiederholte intrastriatale Injektion von BoNT-A wird von Ratten toleriert. Die zuvor abgeklungene dämpfende BoNT-A-Wirkung auf ein pathologisches Rotationsverhalten tritt nach erneuter Behandlung ausgeprägter und langanhaltender wieder ein. Vergleichende Studien mit Mäusen und Ratten zeigten, dass BoNT-A in beiden Spezies keine neurodegenerativen Prozesse auslöst. Nach striataler BoNT-A-Behandlung wurde kein Verlust striataler cholinerger Interneurone festgestellt. Befunde histologischer und motorischen Verhaltensstudien deuten darüber hinaus auf eine antidopaminerge BoNT-A -Wirkung hin.

Schlussfolgerungen

Der Nachweis positiver Effekte von intrastriatalen BoNT-A-Injektionen im murinen Parkinsonmodell, sowie deren gute Verträglichkeit in Ratte und Maus sprechen dafür, diese experimentelle Therapieoption in weiteren Studien zu untersuchen. Es sollen Messungen extrazellulärer Transmitterkonzentrationsänderungen durch intrastriatale BoNT-A-Behandlung mittels Hochleistungsflüssigkeitschromatographie zerebraler Mikrodialysate erfolgen. Hinweise auf eine mögliche antidopaminerge Wirkung von BoNT-A machen seinen hypothetischen Einsatz bei neuropsychiatrischen Erkrankungen zumindest theoretisch interessant.

2. Abkürzungsverzeichnis

Abkürzung	Bedeutung
6-OHDA	6-Hydroxydopamin (OH = Hydroxygruppe)
ACh	Acetylcholin
AChE	Acetylcholinesterase
BiVs	Botulinum Neurotoxin induzierte Varikositäten
BoNT	Botulinum Neurotoxin
ChAT	Cholinacetyltransferase
CPu	Caudatus-Putamen-Komplex (Striatum eines Nagetiers)
GABA	γ-Aminobuttersäure
GP	Globus pallidus
Gpe	Globus pallidus externus
Gpi	Globus pallidus internus
HDB	horizontaler Schenkel des Diagonalen Bandes nach Broca
HPLC	high performance liquid chromatography
IPS	idiopathisches Parkinsonsyndrom
KG	Körpergewicht
L-Dopa	L-3,4-Dihydroxyphenylalanin
LD50	mittlere letale Dosis
LV	Lateralventrikel
MP	Morbus Parkinson
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridin
MSN	Medium spiny neuron
MVB	Mediales Vorderhirnbündel
Ncl	Nucleus
REM	rapid eye movement
SN	Substantia nigra
SNAP-25	Synaptosomal-assoziiertes Protein-25
SNARE	soluble N-ethylmaleimide-sensitive factor attachment receptor
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
SV2	Synaptisches Vesikelprotein Typ 2
TH	Tyrosinhydroxylase
U/min	Umdrehungen in der Minute
UCH-L1	Ubiquitin-Carboxy-terminale Hydrolase L1
VAMP-2	Vesikel Assoziiertes Membranprotein-2
VPL	Nucleus ventralis posterolateralis
ZNS	Zentralnervensystem

3. Verwendete Originalarbeiten

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6. **Hawlitschka A**, Berg C, Schmitt O, Holzmann C, Wree A, Antipova V. Repeated intrastriatal application of botulinum neurotoxin-A did not influence choline acetyltransferase-immunoreactive interneurons in hemiparkinsonian rat brain - A histological, stereological and correlational analysis. Brain Res. 2020; 1742:146877.

4. Einleitung

4.1 Parkinson

Die vorliegende Arbeit befasst sich mit detaillierten Untersuchungen zur Wirkung von intrastriatalen Applikationen von Botulinumneurotoxin-A (BoNT-A) zur experimentellen symptomatischen Behandlung in Tiermodellen des Morbus Parkinson (MP).

Bei MP handelt es sich um eine neurologische Erkrankung des Zentralnervensystems, die stets mit Defiziten bei motorischen Fähigkeiten einhergeht (Dauer and Przedborski, 2003; Fahn and Sulzer, 2004). Ursächlich für den Großteil der motorischen Symptome beim MP ist die fehlende Wirkung von Dopamin im Striatum (CPu). Verantwortlich hierfür ist in den meisten Fällen ein Verlust dopaminerger Neurone in der Substantia nigra parc compacta (SNpc) (**Abb.1**) oder eine Blockade von Dopaminrezeptoren im CPu durch eine systemische Behandlung mit Neuroleptika (Dauer and Przedborski, 2003; Engelhardt et al., 2018; Musco et al., 2019; Wang et al., 2005).

Im Vorfeld muss erwähnt werden, dass es verschiedene Formen von Parkinsonsyndromen gibt. Von besonders herausragender Bedeutung ist das idiopathische Parkinsonsyndrom, bei dem es sich um die zweithäufigste neurodegenerative Erkrankung handelt. Daneben gibt es Formen die familiär vererblich sind (Klein and Westenberger, 2012) sowie Parkinsonsyndrome, die durch die Exposition neurotoxischer Stoffe wie z.B. Rotenone, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP) oder Manganionen hervorgerufen werden (Cai et al., 2007; Dauer and Przedborski, 2003; Fahn and Sulzer, 2004; Kalinderi et al., 2016; Morais et al., 2009; Przedborski, 2017; Schober, 2004; Wang et al., 2005). Parkinsonsymptome können auch temporär als Nebenwirkung spezifischer medikamentöser Therapien, meist der Applikation von Neuroleptika, auftreten (Engelhardt et al., 2018; Musco et al., 2019; Sykes et al., 2017).

Leitsymptome sind Tremor, Rigor, Bradykinese bzw. Akinese und posturale Instabilität. Der Tremor bezeichnet das Zittern distaler Extremitäten mit 4 bis zu 9 Hz. Meist tritt bei Parkinsonpatienten der Tremor in Ruhe auf, kann sich aber durchaus auch als Halte- und/ oder Aktionstremor äußern. Rigor bezeichnet Muskelsteifheit – diese betrifft bei Parkinsonpatienten auch die mimische Muskulatur, wodurch die Patienten durch ein ausdrucksloses Gesicht auffallen. Unter einer Bradykinese versteht man die Verlangsamung von Bewegungen und

unter einer Akinese, die vollständige Unfähigkeit, Bewegungen initiieren zu können. Besonders diese beiden Symptome stellen für die Patienten häufig einen großen Leidensdruck dar. Die posturale Instabilität bezeichnet die Beeinträchtigung der Patienten in der Aufrechten zu verbleiben, nachdem sie im Stand einen äußeren Bewegungsimpuls, z.B. einen Stoß, erfahren haben. Dieser Aspekt der parkinsonschen Erkrankung birgt eine der größten Gefahren und Problematiken für die Patienten, da hierdurch, im Vergleich zu Gesunden, ein erhöhtes Sturzrisiko besteht. Die benannten Leitsymptome müssen nicht alle auftreten und können in ihrer Schwere variieren (Kalia and Lang, 2015; Obeso et al., 2017; Reich and Savitt, 2019).

Neben den vier Kardinalsymptomen gibt es eine Reihe weiterer Symptome unter denen Parkinson-Patienten leiden, bzw. die sie während der oder bereits vor der klinischen Manifestation herausbilden. Hierzu zählen unter anderem Riechstörungen, Depressionen, Angststörungen, Apathie, kognitive Beeinträchtigungen, Psychosen, Halluzinationen, Störungen des Farbsehens, Schmerzen, Schlafstörungen, Störungen des vegetativen Nervensystems und der Blasenfunktion (Bodis-Wollner, 2009; Herting et al., 2008; Huisman et al., 2004; Müller et al., 2002; Postuma et al., 2012; Schapira et al., 2017; Schrag et al., 2015).

4.2 Idiopathisches Parkinsonsyndrom

Die häufigste Form des MP ist das idiopathische Parkinsonsyndrom (IPS). Hier kommt es zu einem fortschreitenden Verlust diverser Neuronentypen innerhalb des ZNS, wobei der degenerative Prozess von kaudal, vom Hirnstamm ausgehend, sowie von rostral, vom Bulbus olfactorius ausgehend, im Gehirn voranschreitet (Braak et al., 2006; Braak and Del Tredici, 2017; Hawkes et al., 2009). Eine besonders vulnerable Hirnregion ist hier die SNpc, wo es im Zuge der Erkrankung zu einem fast gänzlichen Verlust dopaminerger Neurone kommt (**Abb. 1**). Sobald in der SNpc 60 – 80 % der dopaminergen Neurone untergegangen sind, treten die klinisch relevanten motorischen Symptome des MP zu Tage (Dauer and Przedborski, 2003; Marsden, 1992).

Bei den meisten Varianten des MP ist die eigentliche Ursache unbekannt, so liegen keine klar abgrenzbaren Intoxikationen, pharmakologische Interventionen, mechanische Traumata, vaskuläre Störungen oder genetische Prädispositionen als Krankheitsursache vor. Diese Formen unbekannter Ursache werden als idiopathische Parkinsonsyndrom bezeichnet. Nichts desto trotz verdichten sich in den letzten 20 Jahren in der Fachwelt die Hinweise darauf, dass

eine fehlgefaltete Version des synaptischen Proteins α-Synuclein an der Pathologie des MP beteiligt oder gar ursächlich ist. Neuropathologisch imponieren bei verstorbenen Parkinsonpatienten verschiedene Neurone in der SNpc, in der Amygdala, im Hypothalamus, im Cingulum, im Hippocampus und im frontotemporalen Kortex durch das Vorhandensein von eosinophilen Einschlusskörperchen im Zytoplasma des Somas. Diese werden als Lewy-Körperchen bezeichnet. Ähnliche Einschlüsse befinden sich auch in deren Axonen, die dann als Lewy-Neuriten bezeichnet werden (Ferrer, 2011; Ferrer et al., 2012). Die Bestandteile der Lewy-Körperchen und der Lewy-Neuriten sind fibrillär akkumuliertes α-Synuclein sowie Parkin, Ubiquitin-Carboxy-terminale Hydrolase L1 (UCH-L1), Ubiquitin, Neurofilament sowie eine Reihe anderer nitrierter Proteine.

Auf Professor Braak geht die sogenannten „Dual Hit Theorie“ zurück. Diese postuliert, dass die Pathologie des IPS zunächst in der Peripherie beginnt und zwar im Speziellen in der Nasenschleimhaut sowie dem Gastrointestinaltrakt. Anschließend soll die noch unbekannte Noxe von der Nasenschleimhaut entlang der Fila olfactoria in den Bulbus olfactorius gelangen und zur Herausbildung erster Lewy-Körperchen führen. Durch Herunterschlucken von Mucus, der das Pathogen enthält, sollen auch Plexus myentericus und Meissner Plexus infiziert werden, was sich im Vorhandensein von Lewy-Körperchen im Zytoplasma der dortigen Neurone äußert. Vom Gastrointestinaltrakt soll sich die Pathologie über den Nervus vagus bis zum dorsalen Vaguskern im Hirnstamm ausbreiten und dann in einem kaskadierten Prozess in folgender Reihenfolge voranschreiten: zunächst in den Locus coeruleus, dann in die Substantia nigra, das ventrale tegementale Areal, die Amygdala, den Mesocortex und schließlich dem Assoziationskortex. Parallel zum Progress der histologisch nachweisbaren Neuropathologie im Gehirn kann man nach Braak et al. (2003, 2006) sechs Stadien der Krankheit unterscheiden: zwei Prodromalstadien in denen Lewy-Körperchen zunächst nur im Bulbus olfactorius, dem dorsalen Vaguskern und dem Locus coeruleus nachweisbar sind, in denen es aber schon zu prämorbiden Beeinträchtigungen, vor allem Defiziten der Olfaktion (Hyposmie), Depressionen und Störungen des Schlafes in der Phase des „rapid-eye-movement“ (REM-Schlafes) kommen kann. Ab dem dritten Stadium sind Lewy-Körperchen auch in der SNpc nachweisbar und die Patienten zeigen erste motorische Beeinträchtigungen. Im Zuge der darauffolgenden Stadien breiten sich die neuropathologischen Veränderungen nach dem oben beschrieben Muster immer weiter im Gehirn aus und die Symptome verstärken sich zunehmend und werden später auf von demenziellen Beeinträchtigungen begleitet (Braak et al., 2006, 2003; Braak and Del Tredici, 2017; Hawkes et al., 2009, 2007; Lebouvier et al., 2009).

Bemerkenswert ist in diesem Zusammenhang die Erkenntnis der letzten Jahre, dass große Mengen an aggregiertem α-Synuclein im Appendix vermiformis sowohl bei Parkinsonpatienten als auch bei gesunden Personen zu finden sind und dass vollzogene Appendectomien mit einem verringerten Risiko an Parkinson zu erkranken einhergehen sollen (Killinger and Labrie, 2019; Killinger et al., 2018).

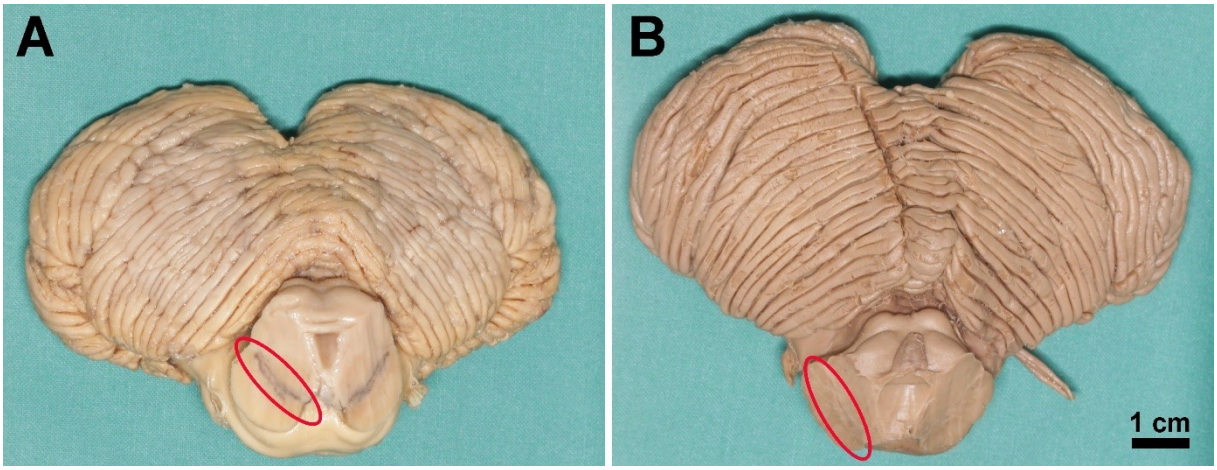

Abbildung 1.: Substantia nigra pars compacta eines Gesunden und eines Parkinsonpatienten

Teilabbildungen A und B zeigen eine Ansicht zweier Hirnstämme von kranial, die auf Höhe des Mittelhirns abgesetzt worden sind. Dorsal ist jeweils deutlich das mächtige Cerebellum zu erkennen. Ventral davon liegt das eigentliche Mittelhirn, mit dem Tectum, dem Tegmentum, beidseits der Substantia nigra und ganz ventral den Crura cerebri. Die orangen Ellipsen markieren jeweils die Substantia nigra der rechten Körperseite. Beim Gesunden imponiert die SNpc durch ihre deutliche schwärzliche Färbung aufgrund des Neuromelaningehaltes ihrer dopaminergen Neurone. Beim Parkinsonpatienten ist keine schwärzliche Färbung zu erkennen, da die dopaminergen Neurone untergegangen sind.

Die Fotos der Teilabbildungen habe ich anhand von plastinierten Gehirnpräparaten erstellt, die von Herrn Dr. rer. nat. Stefan Jean-Pierre Haas und Herrn Dipl. Biol. Marko Schulze angefertigt worden sind.

4.3 Basalganglienverschaltung

Unter dem Begriff Basalganglien subsumiert man eine Reihe an subkortikalen Kerngebieten, die in der Modulierung und Initiierung von Bewegungen, aber auch bei emotionalen und kognitiven Vorgängen involviert sind. Welche Kerngebiete zu den Basalganglien gezählt werden, ist in der Literatur nicht einheitlich festgelegt. Ich beschränke mich zunächst auf die

Beschreibung der für die vorliegenden Arbeiten relevantesten Kerngebiete: dem Striatum, der Substantia nigra, dem Pallidum, dem Nucleus subthalamicus (STN) und dem Thalamus.

4.3.1 Striatum (CPu)

Das CPu ist das größte Kerngebiet im Gehirn der Säugetiere. Beim Menschen handelt es sich hierbei um keine zusammenhängende Region, sondern es fand evolutionär durch ein Auseinanderdrängen durch die innere Kapsel eine unvollständige Trennung in den Nucleus caudatus und das Putamen statt. Daher resultiert sein weiterer Name Nucleus caudatus-putamen Komplex (CPu). Die wichtigsten Neuronentypen im CPu sind GABAerge Projektionsneurone – die Medium spiny neurons (MSNs), GABAerge Interneurone und eine kleine, aber sehr wichtige Population an großen cholinergen Interneuronen, welche exzitatorisch auf MSNs projizieren. Die MSNs können über $Dopamin_1$ (D_1)- oder $Dopamin_2$ (D_2)-Rezeptoren verfügen. Tragen sie D_1-Rezeptoren, sind sie Teil der direkten Basalganglienschleife. Verfügen sie über einen ausgeprägten D_2-Rezeptorbesatz, sind sie der indirekten Basalganglienschleife zuzurechnen.

4.3.2 Substantia nigra (SN)

Die Substantia nigra (SN) ist im ventralen Tegmentum mesencephali lokalisiert und grenzt ventral an die Crura cerebri. Sie wird in eine Pars compacta (SNpc) und eine Pars reticulata (SNpr) unterunterteilt. Die SNpc enthält überwiegend dopaminerge Neurone und fällt an Schnittpräparaten von gesunden humanen Gehirnen bereits makroskopisch durch ihre deutlich schwärzliche Färbung auf (**Abb.1**). Das Schwarz ist Resultat des Vorhandenseins an Neuromelanin in den dopaminergen Neuronen. Die SNpr enthält GABAerge spontan-aktive Neurone.

4.3.3 Globus pallidus (GP)

Der GP ist Teil des Diencephalons und grenzt dem Putamen von medial und dem Thalamus von rostro-lateral an. Er wird unterteilt in ein mediales bzw. internes Pallidumsegment (Gpi) und ein laterales bzw. externes Pallidumsegment (Gpe). Der GP enthält GABAerge Neurone, die spontan Aktionspotentiale ausbilden. Afferent ist der GP mit dem CPu, dem STN und dem Thalamus verbunden. Der GP projiziert hemmend in den Nucleus ventralis anterolateralis des Thalamus und den Nucleus subthalamicus. Weiterhin projiziert der Gpe hemmend auf den Gpi.

4.3.4 Nucleus subthalamicus (STN)

Der STN ist basal des Thalamus lokalisiert und enthält glutamaterge Neurone. Er projiziert erregend auf den Thalamus und den Gpi und wird selbst durch den Gpe gehemmt.

4.3.5 Basalganglienschleifen

Für das motorische Kardinalsymptom des MP, die Bradykinese bzw. Akinese, sind vor allem Fehlregulation in der sogenannten direkten und indirekten Basalganglienschleife (auch direkter und indirekter Weg der Basalganglienschleife(n) genannt) verantwortlich. Sehr vereinfacht lassen sich die Verschaltungen dieser Schaltkreise wie folgt erklären:

Bei der direkten Basalganglienschleife projizieren dopaminerge Neurone aus der SNpc auf D_1-Rezeptor-tragende MSNs des CPu's und werden so durch den dopaminergen Input erregt. Weiterhin projizieren hier exzitatorische Afferenzen aus dem Kortex und dem Thalamus in das CPu. Die striatalen MSNs des direkten Weges und Teile des Thalamus werden durch GABAerge Afferenzen aus der SNpr gehemmt. Die MSNs des direkten Weges projizieren hemmend auf den Gpi. Der Gpi wiederum hemmt den Nucleus ventralis anterolateralis. Dieser initiiert seinerseits durch seine Efferenzen eine erhöhte Aktivität des prämotorischen und motorischen Kortex (**Abb.2**).

Bei dem indirekten Weg der Basalganglienschleife führen dopaminerge Afferenzen aus der SNpc an einer Subpopulation von striatalen D_2-Rezeptort-ragenden MSNs und cholinergen Interneuronen zu einer Hyperpolarisation und somit zu einer Inhibition. Diese MSNs projizieren hemmend auf den Gpe, dieser hemmt mit seinen Efferenzen den Gpi und den STN. Der Gpi projiziert hemmend auf den Nucleus ventralis anterolateralis und dieser erregend auf den motorischen und prämotorischen Kortex (**Abb.2; 3**).

Auf die Darstellung des hyperdirekten Weges der Basalganglienschleife wird hier verzichtet.

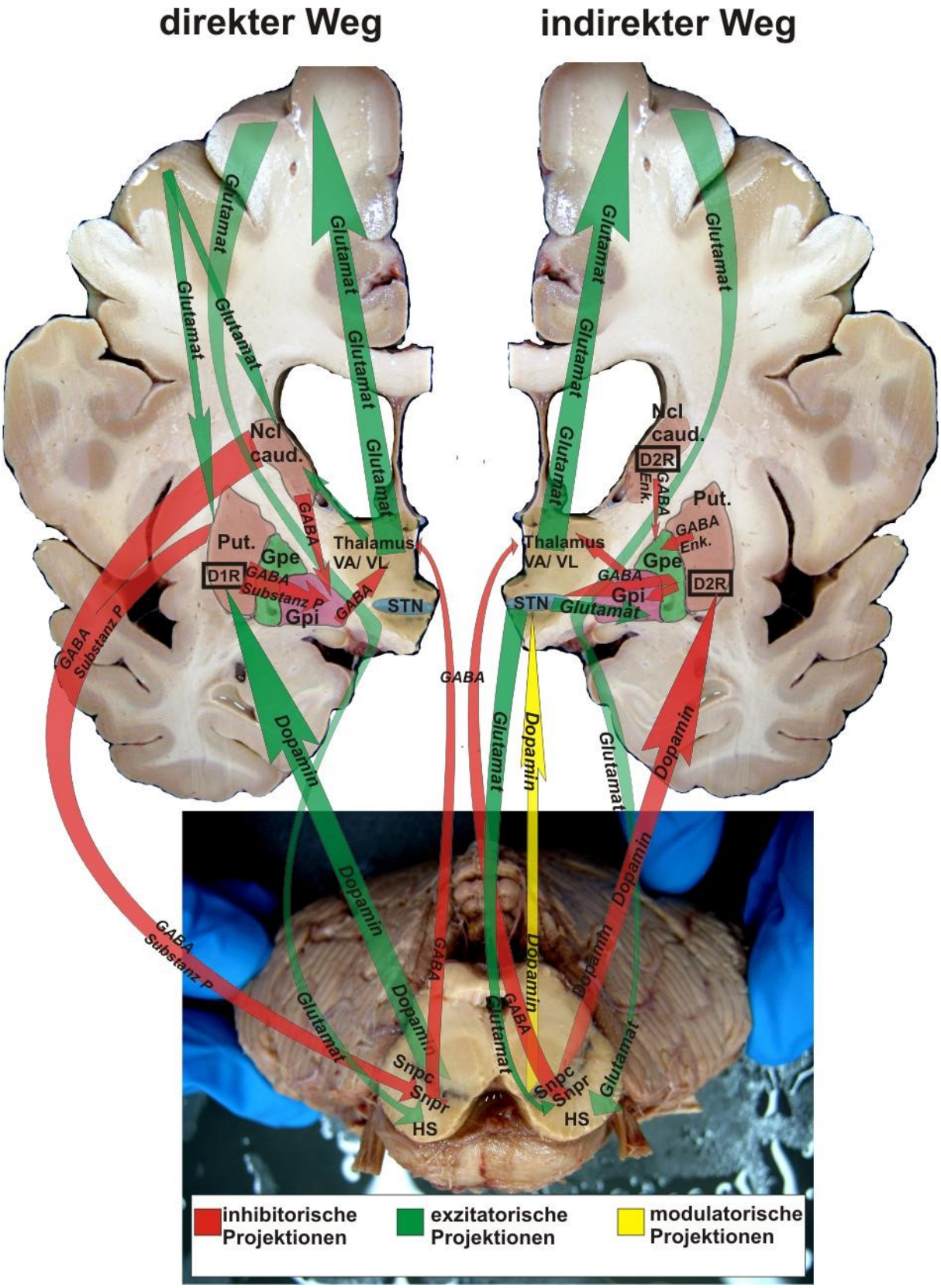

Abbildung 2.: Schematische Darstellung der direkten und indirekten motorischen Basalganglienneuronenschleifen

Rote Pfeile symbolisieren inhibitorische Bahnen. Grüne Pfeile stellen exzitatorische Faserbahnen und gelbe Pfeile modulatorische Bahnen dar. Die SNpc wirkt durch ihre dopaminergen Efferenzen sowohl inhibitorisch auf das

CPu, im Falle des indirekten Weges der motorischen Basalganglienschleife, als auch exzitatorisch, im Falle des direkten Weges. Dies ist allein durch den unterschiedlichen Besatz bestimmter Neuronentypen des CPu mit D_1- oder D_2-Rezeptoren zu erklären. Eine erhöhte Aktivität der SNpc führt immer zu einer verminderten Aktivität des Gpi, wodurch motorische Thalamuskerne vermehrt erregend auf motorische Kortexareale projizieren, die wiederum dann vermehrt erregend über die Bahnen der inneren Kapsel (Faserbahn zwischen Nucleus caudatus und Putamen sowie zwischen GP und Thalamus) und den Hirnschenkeln auf motorische Kerne des Hirnstamms und des Rückenmarks einwirken.
Die Hirnschnitte und deren Bilder, die dem Schema als Grundlage dienen, wurden im Zuge des Präparierkurses für Medizinstudenten 2011 am Institut für Anatomie Rostock durch mich angefertigt. Die obere Teilabbildung zeigt eine gespiegelte Bildmontage aus zwei Frontalschnitten des Telencephalons. Die untere Teilabbildung zeigt einen Schnitt durch das Mittelhirn, wobei die ventrale Seite nach unten und die dorsale Seite nach oben zeigt. Deutlich ist hier die schwärzliche Färbung der SNpc zu erkennen.
D_1R: Dopaminrezeptortyp 1; D_2R: Dopaminrezeptortyp 2; Gpe: Globus pallidus externus; Gpi: Globus pallidus internus; HS: Hirnschenkel; Ncl. caud.: Nucleus caudatus; Put.: Putamen; SNpc: Substantia nigra pars compacta; SNpr: Substantia nigra pars reticulata; STN: Nucleus subthalamicus
Die Abbildung wurde meiner Promotionsarbeit entnommen (Hawlitschka, 2011).

4.4 Verhältnisse beim Morbus Parkinson

Beim MP kommt es zu einem Verlust dopaminerger Neurone in der SNpc und dadurch zu einem Verlust dopaminerger Afferenzen im CPu (Bernheimer et al., 1973; Braak et al., 2006; Braak and Del Tredici, 2017; Burke and O'Malley, 2013; Cheng et al., 2010; Obeso et al., 2014). Hierdurch werden die D_1-Rezeptor-tragenden MSNs des direkten Weges weniger stark erregt und können nur noch unzureichenden den Gpi hemmen, welcher nun verstärkt den Nucleus ventralis anterolateralis hemmt. Dadurch kann dieser nur noch unzureichend den motorischen und prämotorischen Kortex erregen und die Initiierung von Bewegungen ist eingeschränkt.

Die striatalen D_2-Rezeptor-tragenden MSNs und cholinergen Interneurone des indirekten Weges der Basalganglienschleife werden durch den Dopaminmangel unzureichend gehemmt, wodurch diese überaktiv werden und vermehrt den Gpe hemmen. Der Gpe kann nun nicht oder nur unzureichend den Gpi und den STN hemmen, was wiederum zu einer überschießenden Hemmung des Nucleus ventralis anterolateralis führt, wodurch vermindert Bewegungen im motorischen und prämotorischen Kortex initiiert werden (Lanciego et al., 2012; Obeso et al., 2008b, 2008a, 2017; Pisani et al., 2007; Plotkin and Goldberg, 2018).

4.5 Die Rolle der striatalen Acetylcholinkonzentration und der cholinergen Interneurone beim Morbus Parkinson

Im CPu existiert eine Kohorte an großen cholinergen Interneuronen ohne dendritische Dornen, die ca. 1 – 2% der Gesamtpopulation der striatalen Nervenzellen ausmachen. Diese sind tonisch aktiv und projizieren auf die MSNs des CPu‘s. Ein großer Teil dieser Acetylcholin-freisetzenden Neurone trägt D_2-Rezeptoren und wird durch die dopaminergen Afferenzen aus der SNpc in der spontanen Aktivität gehemmt. Beim MP kommt es zu einem Verlust an dopaminergen Fasern im CPu und somit zu einer ausbleibenden Hemmung der tonisch aktiven cholinergen Interneurone im CPu. Diese schütten in Folge vermehrt Acetylcholin aus und erregen überschießend MSNs der indirekten Basalganglienschleife, was zu der Bradykinese bzw. Akinese beiträgt (**Abb.3**) (Antonucci et al., 2008; Bohnen and Albin, 2011; Goldberg and Reynolds, 2011; Lim et al., 2014; Obeso et al., 2008a, 2008b; Pisani et al., 2007; Quik et al., 2019; Tanimura et al., 2018; S. Ztaou et al., 2016).

4.6 Bisherige Therapieoptionen des MP

Zur Behandlung der Symptomatik des MP existieren eine Reihe von etablierten und experimentellen Behandlungsstrategien. Allen ist jedoch zu eigen, dass sie keine kausalen Therapien darstellen und der neurodegenerative Prozess im ZNS zurzeit nicht aufgehalten, verlangsamt oder im Vorfeld verhindert werden kann.

Der Goldstandard in der Parkinsontherapie stellt seit den 1960’er Jahren die Substitution des fehlenden Dopamins im CPu durch die systemische Gabe der Dopaminvorstufe L-DOPA (L-3,4-Dihydroxyphenylalanin, auch Levodopa) dar (CARLSSON et al., 1957; Connolly and Lang, 2014; Olanow and Obeso, 2011). Um den vorzeitigen Abbau von L-DOPA in der Körperperipherie zu reduzieren und damit die Menge an verfügbaren L-DOPA, das durch die Blut-Hirn-Schranke in das ZNS gelangt, zu erhöhen, werden Catechol-O-Methyltransferase-Inhibitoren wie Entacapon und Tolcapon sowie DOPA-Decarboxylaseinhibitoren wie Carbidopa und Benserazid zusätzlich verabreicht (Connolly and Lang, 2014; Fox et al., 2018). Um den Abbau von Dopamin im ZNS zu verlangsamen, erfolgt die Behandlung mit Blut-Hirn-Schranke-gängigen Monoaminoxidase-B-Hemmern wie Rasagilin und Selegilin (Fowler et al., 1996; Riederer and Müller, 2018; Youdim and Bakhle, 2006).

Tiefe Hirnstimulation

Seit Anfang der 1990er Jahre greift man für die Behandlung der Bradykinese bzw. Akinese sowie des Tremors auf die Tiefe Hirnstimulation zurück. Hier wird bilateral, mittels einer stereotaktischen Operation, eine Elektrode meistens in den STN oder auch in den Gpi eingesetzt und überaktive Neurone mittels hochfrequenter Stimulation (bis zu 200 Hz) funktionell ausgeschaltet. Diese Therapieoption ist jedoch nur Patienten vorbehalten, die noch positiv auf eine L-DOPA-Therapie reagieren und es gibt weitere Ausschlusskriterien.

Die Letalitätsrate ist zwar mit 0,4% relativ gering, jedoch gibt es bei 3,4 % der Fälle Probleme im Zusammenhang mit der intrakraniellen Operation, bei 2,4 % gibt es Probleme mit der Hardware und in 2,6 % der Fälle müssen die Elektroden ausgetauscht oder ihre intrazerebrale Position verändert werden (Engel et al., 2018). Des Weiteren kommt es aufgrund der heterogenen Funktionen des STN oft zu Beeinträchtigungen des limbischen Systems, die sich in Depressionen und/ oder Manien bis hin zu einer erhöhten Suizidrate bei Patienten äußert, die mittels Tiefer Hirnstimulation behandelt worden sind (Umemura et al., 2003; Weaver, 2009).

Anticholinerge Therapie

Vor der Etablierung der L-DOPA-Therapie war eine Behandlung mit zentral wirksamen Anticholinergika die Methode der Wahl zur Behandlung eines MP und auch heutzutage stellen Anticholinergika eine wichtige Säule in der Parkinsontherapie dar (Clarke, 2002; Duvoisin, 1967; Fox et al., 2018; Harry Kaplan, Solomon Machover, 1954; Jankovic, 2006; Quik et al., 2019). Durch die Verabreichung von BHS-gängigen Anticholinergika, soll dem striatalen Hypercholinismus beim MP entgegengewirkt werden, der zu einer vermehrten Aktivierung von MSNs führt, die in den indirekten Weg der Basalganglienschleifen eingebettet sind. Eines der bekanntesten Präparate ist Biperiden (Akineton®). Die Therapie mit Anticholinergika weist zwar gute bis sehr gute antiparkinsonoide Effekte auf, doch da die jeweiligen Präparate systemisch appliziert werden, geht ihre Anwendung mit einer Vielzahl peripherer und zentralnervöser Nebenwirkungen einher. Genannt seien hier Mydriasis, Akkomodationsprobleme, Anstieg des Augeninnendrucks, Mundtrockenheit, Entzündungen der Speicheldrüsen, trockene Augen, Muskelschmerzen, Kraftminderung, Veränderungen der Stimme, Schluckstörungen (Dysphagie), eine verminderte Peristaltik der Speiseröhre und damit verbunden Regurgitation, Verstopfung, Harnverhalt, Prostataprobleme, Tachykardie, Fieber

bei warmer/ höherer Umgebungstemperatur, Müdigkeit, Schwindel, Halluzinationen, Erinnerungsstörungen und Verwirrtheit. Insbesondere die kognitiven Störungen, die mit MP oft einhergehen, können durch eine Therapie mit Anticholinergika verstärkt werden, da es im Gegensatz zum CPu beim MP im Nucleus basalis Meynert eher zu einem Hypocholinismus kommt. Auch können sich Störungen des Gangbildes verschlechtern, da auch im Pedunculopontinen Nukleus beim MP ein ACh-Mangel auftritt (Aarsland et al., 2017; Bohnen and Albin, 2011; Calabresi et al., 2006; Clarke, 2002; French and Muthusamy, 2018; Katzenschlager et al., 2002; Lim et al., 2014; Liu et al., 2015; Ray et al., 2018; Whitney, 2007; Yarnall et al., 2011).

4.7 Botulinumneurotoxine

Botulinumneurotoxine (BoNTs) sind bakterielle Exotoxine. Chemisch handelt es sich bei ihnen um Proteinkomplexe. Sie werden durch anaerobe Bakterien der Gattung Clostridium Art *Clostridium botulinum* produziert. BoNTs besitzen eine Domäne mit Metalloproteaseaktivität, die in der Lage ist spezifische Komponenten des Transmittervesikelfusionsapparates der präsynaptischen Membran von muskulären Endplatten, aber auch von zentralnervösen Synapsen zu spalten. Durch diese Eigenschaft verursachen BoNTs eine Disruption synaptischer Signalübertragungen. Für die ausgeprägte Toxizität ist die Unterbrechung der cholinergen Signalübertragung an den muskulären Endplatten verantwortlich, die im Vergiftungsfall zu schlaffen Lähmungen der Skelettmuskulatur führt. Der Tod tritt in der Regel durch eine Lähmung der Atemmuskulatur ein. Im Allgemeinen wird die Vergiftung durch BoNTs oder die Infektion mit Clostridien, die BoNTs produzieren und so zu einer Vergiftung führen, als Botulismus bezeichnet. Botulismus kann sowohl Menschen als auch Tiere betreffen. In der Landwirtschaft in Deutschland betrifft Botulismus in der jungen Vergangenheit hauptsächlich Rinder, die sich durch Verfütterung fehlgegorener Silagen oder aus noch unbekannter Ursache mit *Clostridium botulinum* infizieren. Menschen können an Botulismus erkranken, wenn sie sich mit *Clostridium botulinum* infizieren bzw. Lebensmittel zu sich nehmen, die meist unter Luftabschluss falsch gelagert und/ oder haltbar gemacht worden sind. So besteht bei Konserven und Wurstwaren, aber auch bei eingemachtem Gemüse die Gefahr, dass sich *Clostridium botulinum* vermehrt und sich BoNTs in diesen kontaminierten Lebensmitteln ansammeln. Säuglinge sind durch die Gabe von Honig gefährdet, da im Honig Sporen von *Clostridium botulinum* enthalten sein können, die sich im unreifen Magendarmtrakt der Neugeborenen

vermehren und dann BoNTs abgeben. (Critchley, 1991; Lam et al., 2015; Montecucco and Rasotto, 2015; Pirazzini et al., 2017; Rossetto et al., 2014, 2019).

Gegenwärtig sind acht unterschiedliche BoNT-Serotypen bekannt: BoNT-A, -B, -C, -D, -E, -F, -G und –H, wobei von den meisten Serotypen noch eine Reihe weiterer Subtypen existieren (Lam et al., 2015; Peck et al., 2017; Pirazzini et al., 2017; Rossetto et al., 2014; Rummel, 2015).

4.7.1 Experimentelle Anwendungen Botulinumtoxin im ZNS

In den vergangenen 50 Jahren haben nur wenige Arbeitsgruppen an der experimentellen Anwendung von BoNTs direkt im ZNS geforscht. Die Arbeitsgruppe um Hagenah et al. hat 1977 den Einfluss von Applikationen von BoNT-A sowohl direkt in den Musculus triceps surae als auch in die Hinterwurzel und direkt in das Rückenmark auf die Aktivität von Ia inhibitorischen Interneuronen und Renshaw-Zellen des Rückenmarks beschrieben (Hagenah et al., 1977).

Die Arbeitsgruppe um Luvisetto et al. (Luvisetto et al., 2003) hat die Effekte von BoNT-A und BoNT-B nach Injektion in den Seitenventrikel von Mäusen untersucht und dabei die jeweilige LD_{50} extrapoliert sowie Veränderungen in verschiedenen kognitiven Fähigkeiten und Vitalparametern gemessen. Die LD_{50} wurde für beide Serotypen auf ca. 0,5–1,0 * 10^{-6} mg/kg KG geschätzt. Es wurde auch der Effekt der intrazerebroventrikulären Injektion von BoNT-A und BoNT-B auf kognitive und motorische Fähigkeiten untersucht (Luvisetto et al., 2004). So führte die intrazerbroventrikuläre Injektion von BoNT-A zu keinen Beeinträchtigungen des assoziativen Lernens, jedoch war die Diskriminierung neuer Objekte gestört. Weiterhin konnte diese Arbeitsgruppe einen antinozizeptiven Effekt von BoNT nach intrazerebraler als auch subkutaner Gabe nachweisen (Luvisetto et al., 2006). Chaddok et al. haben die antinozizeptive Wirkung von strukturell modifiziertem BoNT-A untersucht, bei dem das C-terminale Ende seiner schweren Kette mit Erythrina cristagalli lectin konjugiert worden ist. Dieses wurde experimentell in das Hinterhorn des Rückenmarks von Raten injiziert (Chaddock et al., 2004).

Caleo et al. haben 2007 eine Arbeit veröffentlicht, in der sie die Reifung des visuellen Cortex durch Signalinhibition der Retina mittels Injektion von BoNT-E in die Area striata untersucht haben (Caleo et al., 2007).

Antonucci et al. konnten 2008 nachweisen, dass BoNT-A im Gehirn sowohl anterograd als auch retrograd transportiert wird. Hier wurde BoNT-A unilateral in den Hippocampus injiziert. Das

Spaltprodukt von BoNT-A, gespaltenes SNAP-25, konnte beidseits nachgewiesen werden. Nach Injektion in den Colliculus superior konnte gespaltenes SNAP-25 sowohl in der kontralateralen Retina als auch im ipsilateralen visuellen Kortex nachgewiesen werden (Antonucci et al., 2008). Später konnten Antonucci et al. auch einen transsynaptischen anterograden Transport von BoNT-A nach intraokularer Injektion von BoNT-A nachweisen (Restani et al., 2011). Da bekannt ist, dass BoNTs auch dazu in der Lage sind, die Ausschüttung von Glutamat zu blockieren, wurden BoNT-E-Injektionen in den Hippocampus und BoNT-A- als auch BoNT-B-Injektionen in die Amygdala als potentielle Therapieoptionen der Epilepsie erforscht (Antonucci et al., 2009; Bozzi et al., 2006; Costantin et al., 2005; Gasior et al., 2013). Antonucci et al. untersuchten auch einen möglichen neuroprotektiven Effekt von BoNT-E im Schlaganfallmodell der Ratte (Antonucci et al., 2010).

Die Arbeitsgruppe um Ando et al. publizierte 2002 eine Studie zur Etablierung eines Tiermodels der Demenz durch Injektion von BoNT-A in den entorhinalen Kortex (Ando et al., 2002).

De Leonibus et al. (2011) zeigten, dass eine Injektion von BoNT-A in das dorsomediale CPu räumliche Lernstrategien von Mäusen beeinträchtigen kann.

4.7.2 Dosis-Findung im Vorfeld der vorliegenden Arbeiten

Bereits im Jahr 2011 wurden erstmals durch unsere Arbeitsgruppe Ergebnisse der intrastriatalen BoNT-A-Injektion zur experimentellen Behandlung des MP publiziert. Als optimale Dosis zur experimentellen Behandlung eines Parkinsonmodells bei Ratten hat sich eine Dosis von 1 ng BoNT-A je CPu erwiesen. Auch 2 ng BoNT-A je CPu wurden von den Tieren toleriert, wobei 5 ng BoNT-A je CPu zu einer erhöhten Mortalität führte. Für die Dosisfindung wurden Informationen über die LD_{50} bei Ratten und Mäusen aus Publikationen herangezogen als auch Informationen aus Veröffentlichungen, die sich ebenfalls mit der intrazerebralen Applikation von BoNTs befasst haben (Antonucci et al., 2008; Caleo et al., 2007; Lacković et al., 2009; Luvisetto et al., 2004, 2003; Williamson et al., 1996).

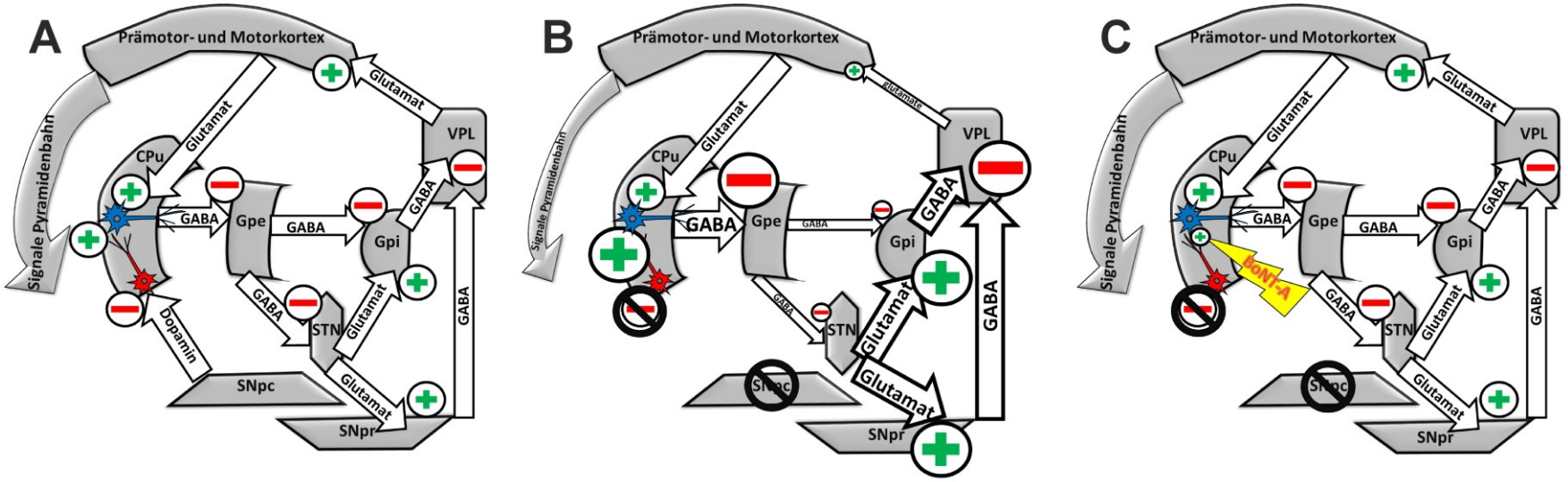

Abbildung 3.: Arbeitshypothese

Die Abbildung zeigt ein vereinfachtes Schema der indirekten Basalganglienschleife. Die Pfeile symbolisieren direkte Verbindungen. Dickere Pfeile deuten auf eine pathologisch erhöhte Aktivität, dünner Pfeile auf eine verminderte Aktivität hin. Die jeweils wichtigsten Transmitter der einzelnen Verbindungen sind in den jeweiligen Pfeilen vermerkt. Ein „+“ steht für eine Exzitation“, ein „-„ für eine Inhibition im jeweiligen Hirngebiet. Stilisierte rote Neurone im Striatum (CPu) symbolisieren cholinerge Interneurone, blaue Neurone stellen GABAerge Projektionsneurone (MSNs) dar, die auf den Gpe projizieren.

(A) Die indirekte Basalganglienschleife unter normalen Bedingungen: dopaminerge Fasern der SNpc die in das Cpu gerichtet sind, inhibieren tonisch aktive cholinerge Interneurone. Diese inhibieren ihrerseits MSNs, welche den Gpe funktionell mittels GABA hemmen. Der Gpe inhibiert den STN. Der STN erregt Gpi und SNpr, welche wiederum den VPL hemmen. Der VPL sendet aktivierende Impulse an den prämotorischen und motorischen Kortex, welche nun bewegungsfördernde Signale über die Pyramidenbahn an die α-Motorneurone der kontralateralen Seite des Rückenmarks senden.

(B) Zeigt vereinfacht die Veränderungen der Verschaltung der indirekten Basalganglienschleife beim MP und bei einem 6-OHDA-induzierten Parkinsonmodell. Hier ist der Großteil der dopaminergen Neurone der SNpc untergegangen, was zu einem Mangel an Dopamin im CPu führt. Dies hat zur Folge, dass cholinerge Interneurone überaktiv werden und vermehrt Acetylcholin ausschütten. Durch dieses werden MSNs übermäßig erregt und inhibieren verstärkt den Gpe. Dies führt zu einer verminderten Hemmung des STN und somit

zu einer verstärkten Aktivität GABAerger Projektionsneurone des Gpi und der SNpr. In der Folge, werden durch den motorischen und prämotorischen Kortex zu wenig bewegungsinitiierende Signale generiert.

(C) Arbeitshypothese der experimentellen intrastriatalen BoNT-A-Applikation beim Modell des MP: BoNT-A unterbricht die Signalübertragung zwischen cholinergen Interneuronen und MSNs im CPu. Hierdurch soll die Aktivität der MSNs, die der indirekten Basalganglienschleife zuzurechnen sind, normalisiert werden. Die nachfolgenden parkinsonbedingten Störungen der indirekten Basalganglienschleife sollten sich hierdurch ebenfalls verbessern und der motorische und prämotorische Kortex sollten wieder vermehrt Impulse für die Bewegungsinitiation generieren.

5. Fragestellung

Im Zuge des MP führt der Verlust dopaminerger Neurone in der SNpc zu einem Ausbleiben eines inhibitorischen Einflusses auf die spontane Aktivität cholinerger Interneurone im CPu. Der resultierende Hypercholinismus im Nucleus caudatus und Putamen ist ursächlich für eine Reihe von motorischen Defiziten. In der Vergangenheit konnte durch unsere Arbeitsgruppe nachgewiesen werden, dass eine Injektion von BoNT-A direkt in das dopaminerg deafferentierte CPu von hemiparkinsonoiden Ratten zu einer Verbesserung einer Reihe motorischer Symptome führt. Weiterhin wurden Aufweitungen cholinerger und katecholaminerger Nervenfasern in CPus aufgefunden, die mit BoNT-A behandelt worden sind.

Die Injektion von BoNT-A in das CPu wurde auf ihr Potential als experimentelle neue Behandlungsmöglichkeit des MP hin erforscht.

Um mögliche Risiken einer solchen neuartige Therapieform beurteilen zu können, war es wichtig zu bestimmen, inwieweit BoNT-A im Gehirn zytotoxisch wirkt.

In den folgenden Arbeiten wurde untersucht, inwiefern einmalige und mehrmalige BoNT-A-Injektionen direkt in das CPu von Ratten und Mäusen zu einer zytotoxischen Schädigung cholinerger Neurone führen. Die Entwicklung der erstmals beobachteten BoNT-A-induzierten Aufweitungen an cholinergen und katecholaminergen Nervenfasern hinsichtlich ihrer Häufigkeit und Größe sollte im temporalen Verlauf untersucht werden. Bisher durchgeführte Verhaltenstests bei BoNT-A-behandelten Tieren sollten durch weitere sensitivere Tests der spontanen Motorik als auch der sensormotorischen Integration ergänzt werden. Es sollte eruiert werden, inwiefern bereits auf der Ebene der Murinae Interspeziesunterschiede in der Wirkung von intrastriatal appliziertem BoNT-A auf histologischer als auch auf Verhaltensebene existieren.

6. Originalarbeiten

6.1 Intrastriatal injection of botulinum neurotoxin-A is not cytotoxic in rat brain - A histological and stereological analysis.

Im Zuge der Arbeiten, die der Publikation von Mehlan et al. (2016) vorausgingen, wurde untersucht, inwiefern die intrastriatale Injektion von BoNT-A in das CPu von Wistar-Ratten zu zytotoxischen Effekten auf striatale Neurone führt. Weiterhin wurde bereits im Vorfeld von unserer Arbeitsgruppe beschrieben, dass sich im BoNT-A-behandelten CPu von Ratten einen Monat nach BoNT-A-Injektion Aufweitungen von cholinergen als auch katecholaminergen Nervenfasern auffinden lassen. Diese weisen im mikroskopischen Bild eine Fläche von bis zu 10 µm² auf. Diese Aufweitungen wurden als Botulinum Neurotoxin induzierte Varikositäten, kurz BiVs, bezeichnet. Für Rattus norvegicus wurde zuvor von uns nachgewiesen, dass deren numerische Dichte als auch deren Größe dosisabhängig ist.

Fragestellung

Wirkt eine intrastriatale Injektion von BoNT-A innerhalb eines Jahrs nach Applikation zytotoxisch auf cholinerge Interneurone des CPu? Wie entwickeln sich die zuvor beobachteten BiVs in Zeitabhängigkeit hinsichtlich ihrer Größe und Menge bzw. ihrer numerischen Dichte?

Material und Methoden

Für diese Arbeit wurden initial junge gesunde adulte Wistar-Ratten mit einem Körpergewicht von 270 – 320 g verwendet. Den Ratten wurde in das rechte CPu, an zwei hintereinander gelegene Koordinaten insgesamt 1 ng BoNT-A bzw. Vehikelsubstanz injiziert. Anschließend wurden die Tiere in 7 Gruppen eingeteilt. Jede Gruppe rekrutierte sich aus 8 Ratten. Sechs Gruppen wurde 1 ng BoNT-A in das rechte CPu injiziert, der siebten Gruppe wurde lediglich die Vehikelsubstanz intrastriatal injiziert. Anschließend ließ man die Tiergruppen gestaffelt überleben. Die erste Gruppe wurde nach 2 Wochen finalisiert. Die Tiere wurden transkardial mit eiskalter 0,9%‘iger Kochsalzlösung und anschließend mit 3,7%‘iger Paraformaldehydlösung perfundiert. Die Gehirne wurden entnommen, über Nacht bei 4°C in 3,7%‘iger Paraformaldehydlösung nachfixiert und danach in 20%‘iger Succroselösung kryoprotektiert. Anschließend wurden die Gehirne in -50°C kaltem Isopentan schockgefroren

und bei -80°C bis zur weiteren Aufarbeitung gelagert. Von den Gehirnen wurden mittels eines Gefriermikrotoms (Leica) 30 µm dicke Frontalschnitte angefertigt. Es wurden sieben Schnittserien angefertigt. Jeder siebte Schnitt wurde im Gefriermikrotom direkt von der Klinge auf einen Glasobjektträger aufgenommen – diese Gehirnschnitte wurden einer Nissl-Färbung zugeführt. Die anderen Schnitte wurden alternierend in 6 verschiedene 48 Well-Platten überführt, die mit je 500 µl Kryoprotektionslösung je Well befüllt waren. Die Präparate in Kryoprotektionslösung wurden bei -20°C gelagert bis zu dem Zeitpunkt, an dem sie für immunhistochemische Färbungen genutzt worden sind. Für jede immunhistochemische Färbung wurde demnach eine Serie genutzt, die jeden siebten Anschnitt eines Gehirnes enthielt.

Neben einer Nissl-Färbung, wurden an den Gehirnen immunhistochemische Markierungen von Cholinacetyltransferase (ChAT) durchgeführt. ChAT vermittelt die Veresterung von Cholin mit einem Essigsäurerest und somit die Acetylcholinbiosynthese. Die Anfärbung von ChAT diente der Detektion cholinerger Strukturen. An einer weiteren Serie wurde eine immunhistochemische Färbung gegen Tyrosinhydroxylase (TH) durchgeführt. TH katalysiert die Bildung von L-DOPA aus Tyrosin und stellt damit das Startenzym der Katecholaminsynthese dar. Durch Markierung von TH wurden katecholaminerge Strukturen markiert.

Die gefärbten mikroskopischen Präparate der Gehirne wurden mit einem semiautomatischen Stereologiesystem untersucht. Das System bestand aus einem Mikroskop der Firma Olympus, einer digitalen Mikroskopkamera, einem Computer, einem Steuermodul für den automatischen Objektträgertisch sowie der Steuer- und Auswertesoftware Stereo Investigator 8.0.

Die gewonnen stereologischen Daten wurden mittels Einweg-Varianzanalysen (one-way ANOVA) mit den post hoc Tests nach Bonferroni und dem Student-Newman-Keuls Test auf signifikante Unterschiede hin überprüft. P-Werte von <0,05 wurden als signifikant betrachtet.

Ergebnisse

Zählung der Anzahl cholinerger Interneurone

Es wurden im Verlauf von einem Jahr nach einer Injektion von 1 ng BoNT-A in das rechte CPu von gesunden Ratten keine signifikanten Veränderungen in der Zahl cholinerger Interneurone detektiert. Dies deutet darauf hin, dass BoNT-A in der angewandten Konzentration nicht zytotoxisch für cholinerge Interneurone ist.

BiVs

Wie in den Projekten zuvor, konnten BiVs stets nur im BoNT-A behandelten CPu nachgewiesen werden und nie im kontralateralen CPu oder im Vehikel-behandelten Striatum.

Im Zuge qualitativer mikroskopischer Analysen der immunhistochemisch angefärbten Präparate fiel auf, dass BiVs oft perlschnurrartig an Nervenfasern aufgereiht waren.

Temporale Volumenentwicklung von BiVs

Im Verlauf eines Jahres hat sich das durchschnittliche Volumen eines einzelnen ChAT-immunreaktiven BiVs stätig vergrößert. So betrug das durchschnittliche Volumen eines ChAT-positiven BiVs zwei Wochen nach BoNT-A-Behandlung 18,96 ± 1,29 µm³ und nach einem Jahr 51,49 ± 2,51 µm³. Bei TH-positiven BiVs nahm das durchschnittliche Volumen eines BiVs über die Zeit ebenfalls zu, jedoch schien das maximale Volumen einzelner BiVs bereits nach 3 Monaten erreicht worden zu sein. Das durchschnittliche Volumen eines TH-positiven BiVs betrug zwei Wochen nach BoNT-A-Behandlung 15,35 ± 0,98 µm³, drei Monate nach Behandlung 36,41 ± 4,19 µm³ und nach einem Jahr 35,97 ± 4,28 µm³.

Temporale Entwicklung der numerischen Dichte von BiVs

Die numerische Dichte ChAT-positiver BiVs erreichte einen Monat nach BoNT-A-Injektion ein Maximum mit 14767 ± 2047 BiVs/mm³ und sank dann innerhalb der nächsten 8 Monate auf 8449 ± 395 BiVs/mm³ ab.

Das Maximum der numerischen Dichte TH-positiver BiVs war bereits 2 Wochen nach BoNT-A-Behandlung mit 47125 ± 2015 BiVs/mm³ erreicht und sank innerhalb eines Jahres kontinuierlich auf 21445 ± 2939 BiVs/mm³ ab.

Schlussfolgerungen

In stereologischen Analysen der Gehirne aller Versuchsgruppen wurde zu keinem Zeitpunkt nach BoNT-A-Injektion ein Verlust cholinerger Interneurone im CPu beobachtet. Auch stimmt das hier ermittelte Verhältnis aus Anzahl striataler cholinerger Interneurone und der Gesamtzahl der Nervenzellen im CPu (ermittelte Gesamtzahl aus Antipova et al., 2013

Antipova et al., 2013) mit den Angaben aus der Literatur überein, die besagen, dass es sich bei 1 – 3 % der Nervenzellen im CPu um cholinerge Interneurone handelt (Bertran-Gonzalez et al., 2012; Goldberg and Reynolds, 2011; Oorschot, 1996). Dies deutet darauf hin, dass BoNT-A nicht zytotoxisch auf cholinerge Interneurone wirkt.

Es wird festgestellt, dass unsere Arbeitsgruppe die erste ist, die Auftreibungen an Nervenfasern cholinerger und katecholaminerger Neurone im CPu nach BoNT-A-Injektion beschreibt. Ähnliche Beobachtungen wurden bis dahin nur in der Zellkultur nach BoNT-C-Exposition gemacht (Berliocchi et al., 2005). Die Zunahme des Volumens einzelner BiVs über die Zeit kann evtl. durch die langandauernde Störung des SNARE-Komplexes und damit durch die Blockade der Transmittervesikelfusion mit der präsynaptischen Membran erklärt werden. Dies kann zu einer Anhäufung von Transmittervesikeln im Axon und damit zu einer fokalen Weitung des Axons führen. Es besteht auch die Möglichkeit, dass über die Zeit eine Vielzahl kleiner BiVs zu wenigen großen BiVs fusionieren. Dies würde zum einen die wachsenden Einzelvolumina von BiVs über die Zeit erklären, zum anderen die Abnahme deren Gesamtzahl.

Die Tatsache, dass TH-positive BiVs aufgefunden worden sind lässt vermuten, dass ebenfalls katecholaminerge Axonendigungen durch BoNT-A im CPu beeinflusst wurden.

6.2 Botulinum Neurotoxin A Injected Ipsilaterally or Contralaterally into the Striatum in the Rat 6-OHDA Model of Unilateral Parkinson's Disease Differently Affects Behavior.

Das Projekt hatte zum Ziel ein tieferes Verständnis über die Mechanismen und Veränderungen der Basalganglienverschaltung zu erlangen, die durch intrastriatale Injektion von BoNT-A im Hemiparkinsonmodell der Ratte ausgelöst werden.

Erstmals wurden hier hemipakinsonoide Ratten im kontralateral zur lädierten SNpc gelegenen CPu mit 1 ng BoNT-A behandelt. Das heißt, das rechte CPu war dopaminerg deafferentiert. Hier wurde das linke und eigentlich unbeeinträchtigt dopaminerg innervierte CPu mit 1 ng BoNT-A behandelt. Durch dieses Experimentaldesign sollte eruiert werden, ob eine solche Behandlung einfach einen gegenteiligen Effekt einer ipsilateralen BoNT-A-Behandlung hervorruft. Drehen sich also hemiläsionierte Ratten im apomorphininduzierten Rotationstest nach Injektion von BoNT-A in das gesunde CPu nicht weniger häufig, sondern sogar schneller, oder werde gänzlich andere Änderungen der Motorik registriert, wie z.B. eine gleichbleibende oder sinkende Rotationsrate. Dies würde für weitaus komplexere Veränderungen in der

Verschaltung der Basalganglien nach einer BoNT-A-Behandlung sprechen, als sie ursprünglich bei der Etablierung der experimentellen intrastriatalen BoNT-A-Therapie des Parkinsonsyndroms zugrunde gelegt worden sind. Ein solcher Fall wäre ein Indiz für weitere kompensatorische Prozesse auf anderen Ebenen, z.B. dem Rezeptorbesatz verschiedener Neuronentypen des CPu's.

Fragestellung

Zeigen hemiparkinsonoide Ratten die im CPu, das kontralateral zu einer 6-OHDA-Läsion gelegen ist, mit 1 ng BoNT-A behandelt worden sind, in pharmakainduzierten Rotationstests sowie in Versuchen zur Testung der spontanen und forcierten motorischen Fähigkeiten, ein gegenteiliges motorisches Verhalten zu Tieren, die ipsilateral mit BoNT-A behandelt worden sind? Hat eine ispi- oder kontralaterale intrastriatale BoNT-A-Injektion einen Einfluss auf einen einseitigen Neglect der im Modell der Ratte durch eine unilaterale Läsion der SNpc mittels 6-OHDA ausgelöst worden ist?

Material und Methoden

Für dieses Projekt wurden Wistar-Ratten verwendet, bei denen zunächst einseitig die rechte SNpc durch Injektion von 6-OHDA in das rechte mediale Vorderhirnbündel (MVB) läsioniert worden ist. Das MVB stellt eine wichtige bemarkte Faserbahn auf- und absteigender Bahnen dar, die kortikale Regionen, Amygdala, verschiedene Basalganglien, hypothalamische Kerne, mesencephale Regionen und einige mehr miteinander verbindet. Unter anderem verbindet das MVB das CPu als auch die SNpc afferent und efferent miteinander und beinhaltet Axone dopaminerger Neurone der SNpc. Der Erfolg der Läsion wurde einen Monat nach der 6-OHDA-Injektion durch einen Apomorphin-induzierten Rotationstest verifiziert. Tiere, die eine Rotationrate von mindestens 4 Umdrehungen in der Minute kontralateral zu Läsion zeigten, wurden als erfolgreich läsioniert betrachtet.

Sechs Wochen nach der Läsion erhielt eine Gruppe dieser Tiere eine Injektion von 1 ng BoNT-A in das rechte, ipsilateral zur Läsion gelegene CPu. Der anderen Gruppe wurde 1 ng BoNT-A in das linke, kontralateral zur Läsion gelegene CPu, injiziert. Die Versuchstiere durchliefen vor Läsion, nach Läsion und nach der BoNT-A Behandlung zu definierten Zeitpunkten motorische

Verhaltenstests. Dies waren sowohl Versuche zur Ermittlung der spontanen motorischen Fähigkeiten (Zylindertest und open Field Test), forcierte motorische Tests (Stepping-Test) als auch pharmakainduzierte Tests (apomorphininduzierter und amphetamininduzierte Rotationstest). Darüber hinaus wurden die Tiere mittels des Corridor-Tasks auf einen Neglect einer Seite hin überprüft.

Ergebnisse

Apomorphininduzierter Rotationstest
Die einseitige 6-OHDA-Läsion führte bei beiden Tiergruppen zu einem deutlichen Apomorphin-induzierten Rotationsverhalten von 6 bis 10 Rotationen pro Minute. Nach ipsilateraler Injektion von BoNT-A in das CPu (rechts) kam es zu einer signifikanten Verringerung dieser apomorphininduzierten Rotationsrate. Diese Reduktion der Rotationrate war bis zu drei Monate nach BoNT-A-Injektion signifikant, danach kehrte die Rotationsrate auf ihr Ausgangsniveau zurück.

Ratten, bei denen eine Injektion von 1 ng BoNT-A in das linke CPu, das der Läsionsseite gegenüber lag, erfolgte, zeigten zwei Wochen nach dieser Behandlung eine signifikante Erhöhung der apomorphininduzierten Rotationsrate. Bei diesen Tieren verringerte sich die apomorphininduzierte Rotationsrate zwei weitere Wochen später signifikant. Die Rotationsrate vier Wochen nach der kontralateralen BoNT-A-Injektion lag sogar unter der Rate, die noch vor der BoNT-A Injektion gemessen worden ist und war auch geringer als die Rotationsrate von kontralateral Vehikel-behandelten Ratten.

Amphetamininduzierter Rotationstest
Auch beim amphetamininduzierten Rotationstest konnte zwei Wochen nach der kontralateralen BoNT-A-Behandlung ein deutlicher Trend zur Reduktion der Rotationsrate beobachtet werden, wohingegen die ipsilaterale BoNT-A-Injektion zu einer signifikanten Erhöhung der amphetamininduzierten Rotationsrate führte.

Test des spontanes Vorderpfotengebrauchs (Zylindertest)
Bemerkenswerterweise führte die Injektion von 1ng BoNT-A in das zur Läsionsseite kontralateral gelegene CPu zu einer hochsignifikanten Wiederangleichung des Links- und

Rechtspfotengebrauchs nach zuvor deutlicher Rechtspräferenz infolge der rechtsseitigen 6-OHDA-Läsion. Auch dieser Effekt verschwand in den darauffolgenden Wochen wieder. Die ipsilaterale Injektion von BoNT-A in das dopaminerg deafferentierte CPu hatte keinen Effekt auf die Händigkeit der Versuchstiere.

Test der forcierten Vorderpfotengebrauchs (Stepping Test)
Die Injektion von 1 ng BoNT-A in das linke CPu von rechtsseitig läsionierten Tieren führte zu signifikant häufigeren Auftritten (Steps) beider Vorderpfoten, im Vergleich zu Vehikel-behandelten Tieren.

Die ipsilateral zu einer 6-OHDA-Lösion durchgeführte Injektion von 1 ng BoNT-A in das CPu hatte im Gegensatz dazu keinen Effekt auf die Anzahl der Auftritte mit der linken Vorderpfote und führte bei der rechten Vorderpfote einen Monat und 6 Monate nach BoNT-A-Injektion sogar zu einer signifikanten Verringerung der Steps, verglichen mit der Vehikel-behandelten Kontrollgruppe.

Testung auf einen Neglect mittels „Corridor Task“
Die Läsion der rechten SNpc mittels Injektion von 6-OHDA in das rechte MVB führte zu einer Herausbildung eines deutlichen Neglects der linken Seite der Umwelt der jeweiligen Ratten. Dies äußerte sich dadurch, dass diese Tiere nach der 6-OHDA Läsion zu über 90 % Suchbewegungen nach Futter zu ihrer rechten Seite hin ausführten. Eine Injektion von 1 ng BoNT-A in das linke CPu hob diese Präferenz der rechten Seite hochsignifikant und fast über ein halbes Jahr hinweg wieder auf. Futter wurde nach einer solchen Behandlung wieder nahezu gleich häufig auf beiden Seiten gesucht und aufgenommen. Daraus schlossen wir, dass ein durch die Hemiläsion ausgelöster Neglect der linken Seite der Umwelt erfolgreich durch eine kontralaterale BoNT-A-Behandlung therapiert worden ist.

Eine ipsilaterale Behandlung mit BoNT-A hatte keinen Effekt auf den Neglect einer Seite der Umwelt.

Spontane Lokomotion (Open Field)
Die spontan zurück gelegte Wegstrecke in 5 min wurde weder durch eine ipsi- noch eine kontralaterale BoNT-A-Behandlung beeinflusst.

Schlussfolgerungen

Wie in den vorangegangenen Studien führte eine Injektion von 1 ng BoNT-A in das ipsilateral zu einer 6-OHDA-Läsion gelegene CPu einen Monat nach Behandlung zu einer signifikanten Reduzierung der apomorphininduzierten Rotationsrate. Diese kehrte in den darauffolgenden Monaten wieder schrittweise auf ihr Ausgangsniveau zurück. Die Ergebnisse werden mit Ergebnissen aus Rezeptorautoradiographiestudien und PET-CT-Messungen in Kontext gesetzt (T. Mann et al., 2018b; Teresa Mann et al., 2018a). Es wurde gefolgert, dass eine BoNT-A-Injektion in das CPu zu einer Reduktion der Bindungen von D_2-Rezepotorliganden (Fallypride) im CPu führte. Daraus wird geschlossen, dass BoNT-A die Dichte an D_2-Rezeptoren in der behandelten Region reduziert. Da die Wirkung von Apomorphin über D_2-Rezeptoren vermittelt wird, erklärt sich die Reduktion der apomorphininduzierten Rotationsrate nach BoNT-A-Injektion in das ipsilaterale CPu durch die Verringerung der zuvor pathologisch erhöhten D_2-Rezeptorkonzentration im dopaminerg deafferentierten CPu. Die kurzzeitige Erhöhung der apomorphininduzierten Rotationsrate nach kontralateraler BoNT-A-Injektion kann mit einer Verringerung der D_2-Rezeptorkonzentration im kontralateralen CPu und somit mit einer Erhöhung des Ungleichgewichtes des D_2-Rezeptorbestazes im linken und rechten CPu erklärt werden.

Die Erhöhung der amphetamininduzierten Rotationsrate nach ipsilateraler BoNT-A-Injektion ist womöglich auf eine Reduktion der ACh-Konzentration und des D_2-Rezeptorbesatzes im BoNT-A-behandelten CPu zurückzuführen. Auch besteht die Möglichkeit, dass BoNT-A die Konzentration an weiteren Rezeptoren im CPu beeinflusst. Die Reduktion der amphetamininduzierten Rotationsrate nach kontralateraler BoNT-A-Injektion beruht eventuell darauf, dass BoNT-A auch Präsynapsen katecholaminerger Axonendigungen blockieren kann und die Ausschüttung an Dopamin reduziert. Hierdurch würde sich die Differenz zwischen linkem (BoNT-A-behandelten) und rechtem (dopaminerg deafferentiertem) CPu hinsichtlich der Dopaminkonzentration nach Amphetamingabe verringern. Dies hätte eine etwas verminderte Initiierung von Bewegungsimpulsen im linken Kortex zur Folge, wodurch weniger Rotationen nach rechts hin ausgeführt werden.

Es ist bemerkenswert, dass eine Injektion von BoNT-A in das CPu kontralateral zu einer 6-OHDA-Läsion die Anzahl der Ausgleichsschritte im Steppingtest sowohl für die Vorderpfote

kontralateral zur Läsion als auch ipsilateral zur dopaminergen Deafferentierung des CPu's erhöht. Auch erfolgen im Zylindertest nach kontralateraler BoNT-A-Injektion wieder signifikant mehr linksseitige Wandberührungen und im Corridor Task wird eine partielle Aufhebung des linksseitigen Neglects beobachtet. Eine ipsilaterale BoNT-A-Injektion hat keinen signifikanten Einfluss im Stepping Test. Die Verbesserung der Performance der rechten Vorderpfote lässt sich dadurch erklären, dass BoNT-A in Einklang mit der Arbeitshypothese die ACh-Konzentration im CPu senkt. Dies würde zu einer verminderten Aktivierung von MSNs des indirekten Weges führen, wodurch der Gpe weniger gehemmt würde und vermehrt den Gpi und STN hemmt. Der Ncl. ventralis anterolateralis des Thalamus würde dadurch weniger gehemmt und könnte vermehrt den prämotorischen und motorischen Kortex aktivieren, in Folge wären die Bewegungsinitiierungen auf der Gegenseite und damit die Anzahl der Schritte (Steps) erhöht. Dieser theoretische Mechanismus lässt sich allerdings nicht auf den verbesserten Gebrauch der linken Vorderpfote im Stepping- und Zylindertest sowie auf die Verbesserung des Neglects der linken Umwelt nach linksseitiger BoNT-A-Injektion rechtsseitig läsionierter Tiere anwenden. Wir vermuten, dass Interhemisphärenverbindungen für dieses Phänomen verantwortlich sind.

6.3 Intrastriatally injected botulinum neurotoxin-A differently effects cholinergic and dopaminergic fibers in C57BL/6 mice.

Obwohl Ratten und Mäuse in der phylogenetischen Systematik zu der gemeinsamen Ordnung der Rodentia (Nagetiere) und sogar zur selben Familie der Muridae (Langschwanzmäuse) gehören, ist in der wissenschaftlichen Literatur umfangreich dokumentiert worden, dass sich beide Arten in vielen physiologischen, biochemischen und pharmakologischen Eigenschaften voneinander unterscheiden (Ellenbroek and Youn, 2016; Jaramillo and Zador, 2014; Overgaard et al., 2013). Aus diesem Grund war es wichtig, die experimentelle Behandlung eines Parkinsonmodells durch intrastriatale BoNT-A-Injektion auch am Mausmodell zu testen, um zu eruieren, ob bereits die Wahl eines anderen Tiermodels zu anderen Resultaten führt. Des Weiteren ist es notwendig, die Wirkung des BoNT-A auch in einem Tiermodell zu erforschen, das der Pathologie des MP näher kommt, als das 6-OHDA-Modell. Die Läsion der SNpc durch 6-OHDA vollzieht sich sehr rasch und es sind hier keine Lewy Bodies zu beobachten. Dieses Modell unterscheidet sich in seinem Verlauf von dem des IPS sehr und hat mit diesem neuropathologisch nur den Verlust dopaminerger Neurone in der SNpc und dem VTA gemein.

Viele genetische Modelle des MP, wie Mutationen und Knockouts wichtiger Gene, die bei der Pathogenese des MP eine Rolle spielen, sind nur im Mausmodell vorhanden. Die Erforschung der Wirkung einer intrastriatalen BoNT-A-Injektion auch an der Maus und nicht nur an der Ratte, stellt eine wichtige Vorarbeit dar, bevor dessen potentiell therapeutische Wirkung in einem genetischen Mausmodell des MP erforscht werden kann.

Weiterhin wurde hier der dosisabhängige Effekt von BoNT-A auf die Morphologie des CPu untersucht sowie eruiert, ob zu verschiedenen Zeitpunkten nach einer intrastriatalen BoNT-A-Behandlung Änderungen der mikroskopischen Architektur des CPu's zu Tage treten.

Fragestellung

Ist bei Mäusen ebenfalls kein Untergang cholinerger Interneurone nach Injektion von BoNT-A direkt in das CPu zu beobachten? Treten bei Mäusen ebenfalls BiVs entlang cholinerger und katecholaminerger Nervenfasern auf? Wie haben sich die Körpergewichte von Mäusen entwickelt, die mit unterschiedlichen Mengen an BoNT-A oder Sham-Substanz intrastriatal behandelt worden sind?

Material und Methoden

Für die Untersuchung der Wirkung intrastriataler BoNT-A-Applikationen bei Mäusen wurden gesunde männliche junge adulte C57BL/6-Mäuse von der Firma Charles River Wiga GmbH (Sulzfeld, Deutschland) bezogen.

Die stereotaktischen Applikationen von BoNT-A wurden, analog zu Ratten, mittels eines stereotaktischen Rahmens der Firma Kopf® (Tujunga, CA, USA) durchgeführt. An diesen Stereotakten wurde ein Mausadapter der Firma Stoelting (Wood Dale, USA) angebracht. Den Tieren wurde in das rechte CPu 1 µl einer phosphatgepufferten physiologischen Kochsalzlösung mit 0,1% bovinem Serumalbumin injiziert, die entweder 25 pg, 50 pg, 100 pg, 200 pg oder kein BoNT-A (Sham-Tiere) enthielt.

Bei Erreichen des vorgesehenen Endpunktes der jeweiligen Überlebenszeit wurden die Mäuse mit einer Überdosis Ketamin und Xylazin getötet und transkardial zunächst mit kalter 0,9%'iger Kochsalzlösung und anschließend mit 3,7%'iger Paraformaldehydlösung perfundiert. Die

Gehirne wurden entnommen, fixiert, kryoprotektiert, eingefroren und anschließend histologisch aufgearbeitet. Es wurden 30 µm-dicke Serienschnitte angefertigt. An jeweils jedem Fünften dieser Schnitte wurden Nissl-Färbungen bzw. immunhistochemische Färbungen gegen ChAT oder TH durchgeführt. Anhand dieser Färbung wurden stereologische Untersuchungen zu der Zahl striataler cholinerger Interneurone sowie eine Analyse der BiVs hinsichtlich ihrer numerischen Dichte als auch ihrer Größe durchgeführt.

Ergebnisse

Gewichte und Hirngewichte

Mäuse vertrugen eine Injektion von 25 pg – 50 pg BoNT-A in das CPu gut. Bei Applikation höherer Dosen sind einzelne Tiere verstorben. Es wurde kein Neuronenverlust in der SNpc nachgewiesen.

Es konnte ein dosisabhängiger Effekt von intrastriatal appliziertem BoNT-A auf das Körpergewicht der Tiere nachgewiesen werden. So wurde bei Mäusen die 100 pg oder 200 pg BoNT-A erhielten, ein halbes Jahr nach der Behandlung ein signifikant höheres Körpergewicht gemessen.

Mäuse denen 25 pg BoNT-A injiziert wurde, wogen 32,46 ± 0,54 g und Tiere die 50 pg BoNT- erhielten wogen 32,73 ± 1,10 g, 100 pg BoNT-A-Mäuse wogen 42,41 ± 2,11 g und 200 pg wogen BoNT-A –Mäuse 45,44 ± 0,22 g 6 Monate nach Behandlung.

Das Gehirngewicht von Mäusen, die mit 50 pg oder 100 pg BoNT-A behandelt worden sind, war signifikant kleiner als das von Mäusen, die nur mit 25 pg behandelt worden sind. So betrugen die Gehirngewichte von Mäusen, die 25 pg BoNT-A erhielten 0,478 ± 0,005 g, die von 50 pg BoNT-A-Mäusen 0,450 ± 0,006 g, die von 100 pg BoNT-A-Mäusen 0,446 ± 0,006 g und von 200 pg BoNT-A-Mäusen 0,446 ± 0,011 g.

Bei Mäusen, denen 50 pg oder 200 pg BoNT-A in das rechte CPu injiziert worden sind, war das rechte CPu signifikant kleiner als das linke, unbehandelte CPu. Bei Tieren, die mit 25 pg oder 200 pg behandelt worden sind, wurden keine signifikanten Seitenunterschiede der striatalen Volumina gemessen. Sechs Monate nach BoNT-A-Behandlung betrug das Volumen des linken CPu's von Tieren, denen 25 pg injiziert worden sind, 8,934 ± 0,224 mm^3 und des rechten, behandelten CPu's 8,499 ± 0,224 mm^3; bei Tieren, die 50 pg erhielten, betrug das Volumen des linken CPu's 10,751 mm^3 ± 0,583 mm^3 und des rechten CPu's 8,039 ± 0,583

mm³. Bei 100 pg-Tieren hatte das linke CPu ein Volumen von 8,662 ± 0,583 mm^3 und das rechte CPu von 8,023 ± 0,583 mm³ und bei 200 pg-Mäusen betrug das mittlere Volumen des linken CPu's 10,314 ± 0,923 mm³ und 6,918 ± 0,923 mm³ für das rechte CPu.

Zahl striataler cholinerger Interneurone

Im Vergleich von rechtem, behandeltem und linkem, unbehandeltem CPu, unterschied sich zu allen Zeitpunkten die Anzahl cholinerger Interneurone nicht signifikant voneinander. Auch höhere Dosen an BoNT-A führten zu keinen signifikanten Seitenunterschieden in der Anzahl striataler cholinerger Interneurone. Jedoch konnte eine prinzipiell hohe interindividuelle Variabilität der Anzahl cholinerger Interneurone unabhängig von der Art der Behandlung nachgewiesen werden.

BiVs

Bei Mäusen konnten, im Gegensatz zu Ratten, keine TH-positiven BiVs in deren Gehirnen nach BoNT-A Injektion aufgefunden werden, wohingegen ChAT-positive BiVs auch hier zahlreich nachgewiesen worden sind (Abb.4).

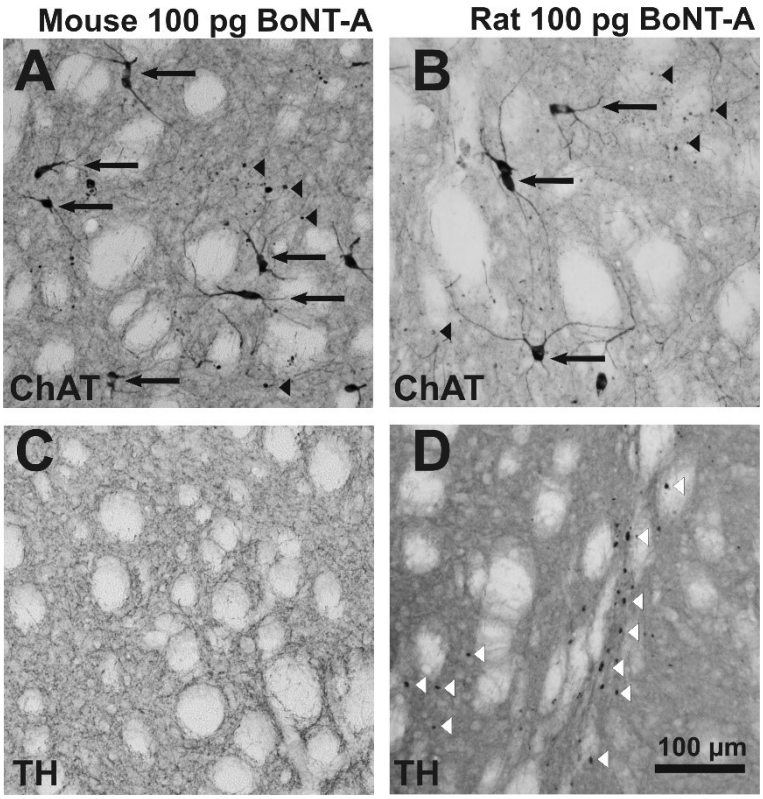

Abbildung 4.: Mikroskopische Abbildungen von BiVs im CPu von Ratte und Maus

Die Präparate bei A und C stammen von einer Maus, der 100 pg BoNT-A intrastriatale injiziert worden ist. B und D stammen von einer Ratte, der ebenfalls 100 pg BoNT-A intrastriatal injiziert worden sind. Sowohl die Maus als auch die Ratte überlebten 6 Monate nach der Operation. Die schwarzen Pfeile markieren cholinerge Interneurone. Die schwarzen Pfeilspitzen markieren ChAT-positive BiVs. Die weißen Pfeilspitzen markieren TH-positive BiVs.

Bei den Teilabbildungen A und B handelt es sich um Präparate, die gegen ChAT angefärbt worden sind, bei C und D handelt es sich um Präparate, die gegen TH angefärbt worden sind. Es ist bemerkenswert, dass im BoNT-A-behandelten CPu der Maus C, in der TH-Färbung im Gegensatz zur Ratte D keine TH-positiven BiVs aufzufinden sind.

Temporale Entwicklung der numerischen Dichte von ChAT-positiven BiVs
Die größte numerische Dichte an BiVs wurde 3 Monate nach Injektion von 25 pg in das rechte CPu gemessen. So betrug die numerische Dichte 2094 ± 367 BiVs/mm³ nach einem Monat, 3230 ± 307 BiVs/mm³ nach 3 Monaten, 1908 ± 165 BiVs/mm³ nach 6 Monaten und 749 ± 74 BiVs/mm³ nach 9 Monaten.

BoNT-A-Konzentrationsabhängigkeit der numerischen Dichte von ChAT-positiven BiVs
Die höchste numerische Dichte an ChAT-positiven BiVs im CPu wurde nach 6 Monaten Überlebenszeit bei einer Dosis von 50 pg BoNT-A je CPu gemessen. Tiere, denen 25 pg BoNT-A in das rechte CPu injiziert wurde, wiesen 1908 ± 165 BiVs/mm³ im behandelten CPu auf, Tiere die 50 pg erhielten, hatten 5066 ± 464 BiVs/mm³, Tiere die 100 pg erhielten, hatten 4056 ± 732 BiVs/mm³ und Tiere die 200 pg erhielten, hatten 3148 ± 767 BiVs/mm³.

Temporale und konzentrationsabhängige Entwicklung BiV-Volumina
Die größten Einzelvolumina wiesen BiVs nach 9 Monaten Überlebenszeit auf. Die größten Einzelvolumina nach 6 Monaten wiesen BiVs von Mäusen auf, die mit 100 pg oder 200 pg behandelt worden sind.

Zahl dopaminerger Neurone in der SNpc
Um zu eruieren, inwiefern ein möglicher zytotoxischer Effekt von BoNT-A auf dopaminerge Nervenzellen der SNpc von Mäusen ursächlich für das Fehlen von TH-positiven BiVs bei Mäusen ist, wurden exemplarisch an histologischen Schnitten des Mittelhirns von Mäusen die mit 25 pg BoNT-A intrastriatal behandelt wurden, Zählungen der TH-positiven Neurone in der SNpc durchgeführt. Hier konnten keine signifikanten Seitenunterschiede zwischen behandelter und unbehandelter Seite detektiert werden. Zusätzlich erfolgte eine solche Untersuchung an Frontalschnitten des Mesencephalons von Ratten, denen 1 ng in das rechte CPu injiziert worden ist. Auch hier wurden keine Seitenunterscheide in der Zahl TH-positiver Neurone ermittelt, so dass ein zytotoxischer Effekt der gewählten BoNT-A-Konzentrationen auf TH-positive Neurone ausgeschlossen werden konnte.

Schlussfolgerungen

Dass Mäuse, die mit hohen Dosen von 100 – 200 pg je CPu behandelt worden sind, schwerer sind als Mäuse, die mit geringeren Dosen behandelt worden sind, ist ein interessanter Befund, der sich momentan nur spekulativ erklären lässt. So könnte es sein, dass bei höherer Dosierung ein Teil des BoNT-A bis zum Hypothalamus diffundiert und dort durch die Spaltung von SNAP-25 die Exozytose von Leptin blockiert, wodurch die Mäuse ein fehlendes Sättigungsgefühl hätten und mehr fressen würden. Bei Ratten konnte eine Gewichtszunahme nach BoNT-A-Injektion nicht beobachtet werden. Es ist denkbar, dass durch das vier Mal größere Volumen eines Rattengehirns nicht ausreichende Mengen an BoNT-A in deren Hypothalamus gelangen. Des Weiteren könnten die Mäuse bezüglich ihrer hypothalamischen leptinausschüttenden Neurone sensitiver für BoNT-A sein als Ratten.

Die Gehirne von Mäusen, die mit 50 oder 200 pg BoNT-A behandelt worden sind, waren signifikant um bis zu 6 % leichter als die Gehirne von Mäusen, die mit 25 pg behandelt worden sind. Das Volumen des rechten CPu‘s dieser Tiere war ebenfalls signifikant verringert und ihr rechter Seitenventrikel erschien erweitert. Einen Neuronenverlust konnten wir in unseren bisherigen Arbeiten nicht nachweisen. Wir können jedoch nicht ausschließen, dass es eventuell einen Effekt auf Gliazellen gibt, der zu einem Verlust dieser führt und eine Erklärung für die geringfügig kleinere Masse der Mausgehirne sein kann, die mit hohen Dosen an BoNT-A behandelt worden sind. In Ratten wurde ebenfalls eine Verringerung des striatalen Volumens nachgewiesen, sofern diese innerhalb eines halben Jahres zwei Mal mit 1 ng BoNT-A behandelt worden sind (Hawlitschka et al., 2018). Die Reduktion des Gehirngewichtes bei Mäusen, schon nach einer Behandlung, kann ebenfalls auf eine höhere Sensitivität zurückzuführen sein.

In Hirnschnitten, die einer Nissl-Färbung zugeführt wurden, sind sowohl bei der Ratte als auch bei der Maus qualitativ keine Auffälligkeiten im Parenchym des CPu beobachtet worden. Diese Schnitte wurden für die Ratte auch zur Bestimmung der Gesamtneuronenzahl des CPu genutzt, wobei kein Verlust an Nervenzellen nach BoNT-A-Behandlung detektiert worden ist.

Signifikante Seitenunterschiede in der Anzahl striataler cholinerger Interneurone konnte nach einseitiger BoNT-A-Behandlung nicht festgestellt werden. Legt man die Erkenntnisse aus der Ratte zugrunde, dass 1 – 2 % der striatalen Nervenzellen cholinerge Interneurone sind (Phelps et al., 1985) und dass das CPu von C57BL/6J-Mäusen ca. 1,72 Millionen Nervenzellen besitzt,

so decken sich die von uns ermittelten Zahlen der striatalen cholinergen Interneurone mit denen bisheriger Studien.

BiVs

Bei Mäusen wurden, im Gegensatz zu Ratten, keine TH-positiven BiVs nach BoNT-A-Injektion im CPu gefunden. In diesem Zusammenhang wird deutlich, dass die Wirkung von BoNT-A auf die Gehirne von Ratte und Maus tatsächlich nicht vollkommen dieselbe ist, zumindest was das Phänomen des Auftretens TH-positiver BiVs betrifft. Der Befund, dass Mäuse keine TH-positiven BiVs ihrer dopaminergen Axonendigungen im CPu aufweisen, ist bemerkenswert, da in der Literatur beschrieben worden ist, dass BoNT-A sowohl bei der Ratte als auch bei der Maus die Freisetzung weiterer Transmitter als nur Acetylcholin aus präsynaptischen Nervenendigungen blockiert. Es ist bekannt, dass BoNT-A in genügend hohen Konzentrationen sowohl bei Ratte als auch bei Maus die Freisetzung von GABA, Dopamin, Serotonin, Glutamat, Glycin und Norepinephrin blockiert (Ashton and Dolly, 1988; Bozzi et al., 2006; Mahrhold et al., 2006; Pearce et al., 1997).

Es ist denkbar, dass Ratten und Mäuse über einen unterschiedlichen Besatz an SV2C – Rezeptoren verfügen. Der SV2C-Rezeptor an der präsynaptischen Endigung von Neuronen ist essentiell für die Wirkung von BoNT-A, da es hieran bindet und erst nach erfolgter Bindung an SV2C von dem Neuron durch endozytotische Vesikelrückgewinnung internalisiert werden kann (Dong, 2006; Kroken et al., 2017; Rummel, 2015; Stout et al., 2019). Sollten dopaminerge Axonendigungen im CPu von Mäusen einen anderen SV2-Rezeptorsubtypus tragen als Ratten, so kann dies eine Erklärung für das Fehlen von TH-positiven BiVs bei Mäusen sein. Fehlt SV2C, kann BoNT-A nicht aufgenommen werden und die leichte Kette des BoNT-A kann nicht im Zytoplasma der Nervenzelle SNAP-25 spalten, so dass nach wie vor die Transmittervesikelfusion mit der präsynaptischen Membran unbeeinträchtigt stattfinden kann. Ein hypothetischer Vesikelrückstau der zu Aufweitungen führt, wäre dann nicht gegeben.

6.4 Unilateral Botulinum Neurotoxin-A Injection into the Striatum of C57BL/6 Mice Leads to a Different Motor Behavior Compared with Rats.

Wie eingangs erwähnt gehören Ratten und Mäuse der Familie der Langschwanzmäuse an, sie unterscheiden sich aber in vielerlei Hinsicht auf biochemischer als auch Verhaltensebene.

Speziesspezifische Unterschiede einer intrazerebralen BoNT-A-Injektion müssen demnach im Vorfeld möglicher klinische Untersuchungen intrastriataler BoNT-A-Applikationen erforscht werden. Weiterhin existieren viele wichtige genetische Modelle des MP nur als Mausmodell. Aus diesem Grund wurde die Wirkung intrastriataler-BoNT-A-Injektion auch auf das Verhalten von C57BL/6-Mäusen untersucht.

In den vorangegangenen Arbeiten wurden bei Wistar-Ratten die sehr markante Beobachtung gemacht, dass ein deutlich ausgeprägtes apomorphininduziertes Rotationsverhalten im 6-OHDA-induzierten Hemiparkinsonmodell nach ipsilateraler intrastriataler BoNT-A-Injektion vollkommen aufgehoben wird, um sich innerhalb eines halben Jahres graduell wieder herauszubilden (Antipova et al., 2013, 2017; T. Mann et al., 2018b; Wedekind et al., 2018; Wree et al., 2011). Hier sollte eruiert werden, zu welch einem Verhalten eine einseitige intrastriatale BoNT-A-Injektion bei gesunden Ratten und Mäusen führt und ob sich beide Spezies in bestimmten Parametern unterscheiden.

Fragestellung

Da in histologischen Untersuchungen von Ratte und Maus speziesspezifische Unterschiede in der Wirkung von BoNT-A auf die Morphologie des ZNS aufgefunden worden sind, sollten nun folgende Fragen beantwortet werden:

Hat eine einseitige Injektion von BoNT-A in das CPu einen Einfluss auf die Gewichtsentwicklung bei Ratte oder Maus?

Bestehen speziesspezifische Unterschiede in der Wirkung einer einseitigen intrastriatalen BoNT-A-Injektion auf pharmakainduzierte Rotationstests?

Hat eine einseitige BoNT-A-Behandlung Einfluss auf forcierte und spontane motorische Fähigkeiten?

Löst eine einseitige Injektion von BoNT-A einen Seitenneglect in gesunden Mäusen aus?

Material und Methoden

Im Zuge dieser Arbeit wurden junge adulte C57BL/6-Mäuse und Wistar Ratten verwendet. Den Mäusen wurden entweder 25 pg bzw. 50 pg oder lediglich die Vehikelsubstanz in das rechte

CPu injiziert. Den Ratten wurde 1ng BoNT-A oder die Vehikelsubstanz in das rechte CPu injiziert.

Unmittelbar vor der BoNT-A-Applikation sowie innerhalb von 9 Monaten nach der Behandlung erfolgten zu mehreren Zeitpunkten Gewichtsmessungen der Tiere und motorische Verhaltenstests. Es wurden apomorphininduzierte und amphetamininduzierte Rotationstests zwei Wochen, ein, drei, sechs und neun Monate nach Behandlung durchgeführt.

Die spontane Motorik der Vorderpfoten wurde mittels des Zylindertestes untersucht. Die forcierte Motorik der Vorderpfoten wurde mittels des Steppingtestes untersucht. Weitere motorische Defizite wie z.B. Ataxien wurden mittels des Hindlimb-Clasping-Testes detektiert. Die Testung auf einen Seitenneglect hin erfolgte mittels eines Versuchs, der als „Corridor Taskes“ bezeichnet wird.

Einen Monat, drei, sechs und neun Monate nach BoNT-A-Behandlung erfolgten amphetamin- und apomorphininduzierte Rotationstests.

Ergebnisse

Körpergewicht über die Zeit
Die Körpergewichte von Mäusen, denen 25 pg oder 50 pg in das rechte CPu injiziert worden ist, waren signifikant geringer als die von Vehikel-behandelten Mäusen.

Vergleich des apomorphininduzierten und amphetamininduzierten Rotationsverhaltens von Ratten und Mäusen

Apomorphin
Bei Ratten führte die rechtsseitige Injektion von BoNT-A einen Monat nach der Behandlung zunächst zu einem apomorphininduzierten Rotationsverhalten von einer Umdrehung je Minute, das ipsilateral zur behandelten Seite ausgeführt wurde. Dieser Befund war jedoch nicht signifikant, sondern stellte einen deutlichen Trend dar. Nach drei Monaten war das gemessene apomorphininduzierte Rotationsverhalten bei Ratten ipsilateral zur Behandlung signifikant, im Vergleich zu Vehikel-behandelten Tieren.

Die Injektion von 25 pg und 50 pg BoNT-A in das rechte CPu von Mäusen führte bis zu drei Monate nach der Behandlung zu einem signifikanten apomorphininduzierten

Rotationsverhalten von ca. 2 Umdrehungen in der Minute, die kontralateral zur Injektion hin ausgeführt worden sind. Vehikel-injizierte Mäuse zeigten kein signifikantes apomorphininduziertes Rotationsverhalten.

Amphetamin
Bei gesunden Ratten führte eine einseitige intrastriatale Injektion von 1 ng BoNT-A zu keinem signifikanten amphetamininduziertem Rotationsverhalten. Im Gegensatz dazu, führte bei Mäusen eine einseitige Behandlung mit 25 pg oder 50 pg, im Vergleich zu Vehikel-behandelten Mäusen, über 9 Monate hinweg zu einem signifikanten Rotationsverhalten von 4 - 10 Umdrehungen in der Minute, die kontralateral zur BoNT-A-Injektion hin ausgeführt wurden.

Spontaner Gebrauch der Vorderpfoten/ Zylindertest
Eine einseitige Injektion von 25 oder 50 pg BoNT-A in das rechte CPu führte zu einer signifikanten Verringerung des spontanen Gebrauchs der linken Vorderpfote im Zylindertest. Diese wurde nach einer solchen Behandlung nur noch in 40% der Wandberührungen genutzt, während Vehikel-behandelte Tiere über eine Zeit von 9 Monaten beide Vorderpfoten nahezu gleich häufig verwendet haben.

Stepping Test
Bei der forcierten Testung der Vorderpfoten durch den Steppingtest, zeigten gesunde einseitig BoNT-A-behandelte Mäuse, im Vergleich zu Vehikel-behandelten Mäusen, über 9 Monate hinweg signifikante Defizite im Gebrauch der linken Vorderpfote sowie einen Monat nach Behandlung auch der rechten Vorderpfote.

Testung auf einen Seitenneglect/Corridor Task
Gesunde Mäuse suchten nach einer einseitigen intrastriatalen BoNT-A-Behandlung signifikant weniger oft auf der kontralateral zur Behandlung gelegenen Seite nach Futter. Dies spricht für die Entwicklung eines partiellen Neglects des kontralateral zur Behandlung gelegenen Teils der Umwelt der Tiere.

Hindlimb Clasping
Der Hindlimb-Clasping-Test ist dazu geeignet motorische Defizite in verschiedenen Tiermodellen neurodegenerativer Erkrankungen anzuzeigen. Die Maus wird hierzu an Schwanzbasis vorsichtig für 10 Sekunden angehoben und es wird beurteilt, ob das Tier ein oder beide Hinterpfoten an das Abdomen heranzieht und in welchem Ausmaß dies geschieht. Die Tiere wurden zu einem Testzeitpunkt drei Mal getestet und dabei gefilmt. Waren beide Hinterpfoten permanent nach außen gerichtet, so erhielt das Tier einen Wert von 0, war eine Hinterpfote während mindestens der Hälfte der Beobachtungszeit an den Bauch gezogen und nach innen gedreht, so entsprach dies einem Wert von 1, war dies für zwei Hinterpfoten der Fall, entsprach dies einem Wert von 2 (Chou et al., 2010; Guyenet et al., 2010; Lieu et al., 2013; Morris et al., 2011; Reid et al., 2013).

Es wurde bei keinem der Tiere ein positives Hindlimb-Clasping beobachtet.

Schlussfolgerungen

Geringeres Gewicht
Das, im Vergleich zu Vehikel-behandelten Mäusen, geringere Körpergewicht von Mäusen, die mit 25 pg oder 50 pg BoNT-A intrastiratal behandelt worden sind, ist bemerkenswert. In der Publikation von Hawlitschka et al. (2017) berichteten wir, dass Mäuse, denen wir 100 pg oder 200 pg BoNT-A intrastriatal injizierten, schwerer waren als Mäuse, die nur 25 pg oder 50 pg erhielten. Im Umkehrschluss wäre zu erwarten, dass diese Tiere (25 pg und 50 pg) schwerer sein sollten als Kontrolltiere, das Gegenteil war jedoch der Fall. Hier lässt sich nur spekulieren, dass 25 pg und 50 pg BoNT-A je CPu nicht ausreichend sind, die Leptinausschüttung im Hypothalamus signifikant zu beeinflussen, jedoch ausreichen um durch einen unbekannten Mechanismus eine normale Gewichtszunahme zu stören.

Verringerter Gebrauch der Vorderpfoten und Neglect
In den Arbeiten von Wedekind et al. (2017) und Mann et al. (2018) konnte gezeigt werden, dass intrastriatal injiziertes BoNT-A zu einer Verringerung der D_2-Rezeptordichte führt. D_2-Rezeptoren sind inhibitorisch und befinden sich im CPu vornehmlich auf Neuronen die dem indirekten Weg der Basalganglienschleife zuzuordnen sind. So sitzen sie auch auf MSNs die auf den Gpe projizieren und diesen hemmen. Weiterhin sitzen sie auf cholinergen Interneuronen, welche die MSNs aktivieren. Verfügen diese Neurone über weniger D_2-

Rezeptoren, werden sie weniger stark durch die dopaminerge Innervation aus der SNpc gehemmt. Die cholinergen Interneurone sollten dadurch überaktiv werden und vermehrt den Gpe hemmen, der wiederum nun weniger stark den Gpi hemmen kann und der infolge vermehrt den Thalamus hemmt, wodurch weniger Bewegungen im motorischen Kortex initialisiert werden und es aufgrund der Kreuzung der motorischen Bahnen zu motorischen Defiziten der kontralateralen Körperseite kommen könnte. Dies stellt eine mögliche Erklärung des verringerten Gebrauchs der linken Vorderpfote nach rechtsseitiger intrastriataler BoNT-A Injektion dar.

Die Störung der intrastriatalen Signalweiterleitung durch Applikation von BoNT-A in ein gesundes CPu muss ursächlich für die genannten Einschränkungen sein. Die Applikation von BoNT-A in ein gesundes CPu scheint kontraproduktiv zu sein, da noch keine Verringerung der Dopaminkonzentration vorliegt. Diese wird unter Umständen erst durch eine BoNT-A-injektion geschaffen – es ist dokumentiert, dass BoNT-A dazu in der Lage ist, die Ausschüttung an Katecholaminen zu blockieren (Ashton and Dolly, 1988; Bigalke et al., 1981, 1985; Bozzi et al., 2006; Dardou et al., 2011). Zum anderen konnten wir nachweisen, dass eine intrastriatale BoNT-A-Injektion zumindest bei Ratten zu einer Verringerung des striatalen D_2-Rezeptorbesatzes führt. Und auch die wahrscheinliche Reduktion der Acetylcholinkonzentration in einem zuvor intakten CPu nach BoNT-A-Injektion trägt zu einer Fehlregulation der Basalgangleinschleifen bei. Die Folge wären mangelnde Initiierungen von Bewegungsimpulsen im rechten Motorkortex, wodurch weniger Motorik auf der linken Seite ausgeführt wird (Li et al., 2015; Starkey et al., 2005; Welniarz et al., 2015).

Stepping Test

Die verringerte Zahl von Schrittbewegungen der linken Vorderpfoten im Steppingtest läßt sich auch durch den gestörten indirekten Weg der Basalganglienschleife aufgrund der Reduzierung von D_2-Rezeptoren im rechten CPu nach BoNT-A-Behandlung erklären. Dennoch ist es überraschend, dass kurzfristig auch auf der rechten Seite weniger Ausgleichsschritte im Steppingtest, im Vergleich zu Vehikel-behandelten Tieren, ausgeführt werden. Heuer et al. (Heuer et al., 2012) berichteten ebenfalls, dass in einem unilateralen 6-OHDA-induzierten Parkinsonmodell der Maus die Zahl der Schritte im Steppingtest auf beiden Seiten um 80% reduziert ist. Legt man die Annahme zugrunde, dass bei gesunden Mäusen BoNT-A die Dopaminausschüttung im CPu blockiert und die Konzentration an D_2-Rezeptoren herabsetzt, so kommt dies ebenfalls einer partiellen dopaminergen Läsion gleich und die Ergebnisse von

Heuer et al. würden im Einklang mit unseren Ergebnissen stehen. Dennoch existieren auch Publikationen, die nach einseitiger 6-OHDA-Läsion hauptsächlich ein einseitiges Defizit im Steppingtest messen (Boix et al., 2015; Glajch et al., 2012).

Hindlimb Clasping

Das Fehlen von Hindlimb-Claspingzeichen deutet darauf hin, dass die einseitige intrastriatale BoNT-A-Injektion in gesunde Tiere nicht auf weitere motorische Systeme, wie z.B. das cerebelläre System, übergreift und die hervorgerufenen Störungen bei gesunden Tieren nur sehr beschränkt bleiben.

Apomorphininduzierte Rotationen

Bei Mäusen führt eine unilaterale BoNT-A-Injektion in ein gesundes CPu einen und drei Monate nach der Behandlung zu einem signifikanten, apomorphininduzierten Rotationsverhalten von ein bis zwei Umdrehungen in der Minute kontralateral zur Injektionsseite hin. Bei Ratten führt eine solche Behandlung zunächst einen Monat nach Behandlung zu einem Trend, Rotationen unter Apomorphinwirkung mit ca. 1 U/min ipsilateral zur Behandlung hin auszuführen. Zwei und drei Monate nach der Behandlung, werden die Rotationen jedoch mit 1 und 1,5 U/min kontralateral zur Injektionsseite hin ausgeführt.

In vorangegangenen Arbeiten konnte mittels Rezeptorautoradiographie und PET-CT nachgewiesen werden, dass sich die Konzentration an D_2-Rezeptoren im CPu nach BoNT-A-Behandlung verringert.

Den anfänglichen Trend von Ratten, nach einseitiger intrastriataler BoNT-A-Injektion, apomorphininduzierte Rotationen ipsilateral zur Injektionsseite hin auszuführen, lässt sich mit der Verringerung an postsynaptischen D_2-Rezeptoren an cholinergen Interneuronen und GABA-ergen Medium-Spiny-Projektionsneuronen, die auf den Gpe projizieren, erklären. Denn durch das seitenspezifische Ungleichgewicht an präsynaptischen D_2-Rezeptor-Konzentrationen, kann das systemisch wirkende Apomorphin im größeren Ausmaß GABA-erge Projektionsneurone auf der unbehandelten Seite hemmen. Der Gpe wird hierdurch weniger stark gehemmt und hemmt selbst vermehrt den Gpi sowie den STN. Diese können nun weniger stark den Thalamus hemmen, der folglich im stärkeren Ausmaß auf der unbehandelten Seite den motorischen und prämotorischen Kortex aktiviert, was zu einer verstärkten Motorik der Körperhälfte führt, die kontralateral zur unbehandelten Seite bzw. ipsilateral zur BoNT-A-

behandelten Seite liegt. Dadurch drehen sich die Ratten nach Apomorphingabe ipsilateral zur BoNT-A-Injektion.

Dass zu späteren Zeitpunkten nach einseitiger BoNT-A-Injektion Ratten als auch Mäuse nach Apomorphingabe kontralateral zur Injektionsseite hin rotieren, kann man spekulativ dadurch erklären, dass hier die anticholinerge Wirkung des BoNT-A überwiegt bzw. zum Tragen kommt. Denn laut der Arbeitshypothese sollte eine intrastriatale BoNT-A-Injektion die Ausschüttung an Acetylcholin aus den cholinergen Interneuronen blockieren. Ist dies vermehrt der Fall, werden im CPu auf der behandelten Seite die GABA-ergen MSNs vermindert aktiviert und hemmen weniger stark den Gpe, der nun wiederum vermehrt den Gpi und den STN hemmt, die nun den Thalamus weniger stark hemmen können. Der Thalamus kann so vermehrt den prämotorischen und motorischen Kortex auf der BoNT-A-behandelten Seite aktivieren, wodurch auf der kontralateralen Körperhälfte vermehrt Bewegungen initiiert werden und sich das Tier kontralateral zur BoNT-A-Behandlung dreht.

Die Herausbildung eines kontralateralen apomorphininduzierten Rotationsverhaltens kann auch darin begründet liegen, dass bei der Verringerung der D_2-Rezeptordichte im CPu nach BoNT-A-Injektion u.U. nicht nur postsynaptische D_2-Rezeptoren betroffen sind, sondern, womöglich zeitversetzt, sich auch die Konzentration an präsynaptischen D_2-Rezeptoren an dopaminergen Nervenendigungen verringert. Da D_2-Rezeptoren einen inhibitorischen Charakter haben, hätte eine Verringerung der präsynaptischen D_2-Rezeptorkonzentration an dopaminergen Präsynapsen eine stärkere Ausschüttung an Dopamin zur Folge als im kontralateralen CPu. Hierdurch würde sowohl der direkte als auch der indirekte Weg der Basalganglienschleife vermehrt durch Dopamin stimuliert, was ebenfalls eine vermehrte Aktivierung des ipsilateralen prämotorischen und motorischen Kortex zur Folge hätte. Die Tiere würden dann aufgrund einer gesteigerten Bewegungsinitiation auf der kontralateralen Körperhälfte kontralateral zur Behandlung hin rotieren.

Warum Mäuse im Gegensatz zu Ratten keine anfängliche Phase eines ipsilateralen Rotationsverhaltens unter Apomorphinwirkung zeigen, lässt sich nur spekulativ beantworten. Zunächst ist festzuhalten, dass das anfängliche ipsilaterale Rotationsverhalten der Ratten, im Vergleich zu Vehikel-behandelten Kontrolltieren, nur einen Trendcharakter hat. Weiterhin kann es sein, dass bei Mäusen die anticholinerge Wirkung und/ oder die Wirkung von BoNT-A auf die präsynaptischen D_2-Rezeptoren überwiegt. Es ist jedoch auch möglich, dass der Zeitpunkt des ersten apomorphininduzierten Rotationstests mit zwei Wochen nach der

Behandlung, zu spät gewählt worden ist und während einer möglichen Messung zuvor evtl. auch ein solches Verhalten erfasst worden wäre. Aufgrund der Notwenigkeit, den Mäusen nach der stereotaktischen Injektion eine ausreichend lange Zeit zur Erholung zu gewähren, waren jedoch vorherige Messungen nicht möglich.

Amphetamininduzierte Rotationen

Gesunde Mäuse und Ratten, die einseitig intrastriatal mit BoNT-A behandelt worden sind, zeigen ein unterschiedliches amphetaminindiziertes Rotationsverhalten. Während bei Ratten kein signifikantes Rotationsverhalten zu messen ist, rotieren Mäuse deutlich und signifikant kontralateral zur injizierten Seite hin. Diese speziesspezifischen Unterschiede in der BoNT-A-Wirkung lassen sich am ehesten durch unterschiedlich starke Wirkungen auf die verschiedenen präsynaptischen Endigungen der verschiedenen Transmittersysteme im CPu bei Ratte und Maus erklären. Es ist wahrscheinlich, dass es zwischen Ratte und Maus Unterschiede in der Konzentration an SV2C-Rezeptoren im CPu gibt. Diese sind an Axonendigungen diverser Neuronentypen lokalisiert, die in das CPu projizieren. SV2C-Rezeptoren stellen die Bindungsstelle für BoNT-A dar und sind eine essentielle Komponente, die die Endozytose des BoNT-A und somit seine Wirkung vermittelt. Es ist bereits in einigen Publikationen gut dargelegt, dass SV2C je nach Spezies und je nach Hirnregion sowie je nach Neuronentyp unterschiedlich stark exprimiert wird (Crèvecœur et al., 2013; Davies et al., 2018; Dunn et al., 2018; Janz et al., 1999; Lam et al., 2015; Mahrhold et al., 2006; Rummel, 2015). Offensichtlich überwiegt bei Mäusen die anticholinerge Komponente der BoNT-A-Wirkung. D.h. BoNT-A muss im CPu von Mäusen eher die Exozytose von Acetylcholin blockieren als die von Dopamin, da die dopaminergen Axonendigungen der Maus im Gegensatz zur Ratte u.U. weniger oder gar keine SV2C-Rezeptoren tragen und BoNT-A dadurch nicht an dopaminergen Präsynapsen des Maus-CPu‘s binden kann. Hierfür spricht, dass wir bei Mäusen keine TH-positiven BiVs beobachten konnten. Dadurch geht die bewegungsfördernde Komponente des Dopamins für den indirekten und direkten Weg der Basalgangleinschleifen nicht in dem Maße verloren, wie die bewegungshemmende Komponente des Acetylcholins für den indirekten Weg der Basalgangleinschleifen. Amphetamin wirkt hauptsächlich als Freisetzer von Katecholaminen aus der Präsynapse (Herrera-Marschitz et al., 2010; Kahlig et al., 2005; Lam et al., 2011). Nach systemischer Applikation von Amphetamin in einseitig BoNT-A-behandelten Mäusen wird anscheinend sowohl im BoNT-A-behandelten CPu als auch im kontralateralen unbehandelten CPu überschießend Dopamin freigesetzt – ein

Rotationsverhalten ist dann zunächst nicht zu erwarten. Vorausgesetzt, dass im Einklang mit der Arbeitshypothese BoNT-A die Acetylcholinfreisetzung im behandelten CPu verringert hat, wird die bewegungshemmende Wirkung des behandelten CPu durch dessen nun verringerten inhibitorischen Einfluss auf den Gpe verkleinert und auf der kontralateralen Körperhälfte muss es zu einer vermehrten Initiierung von Bewegungen und somit zu einem Rotationsverhalten kontralateral zur Behandlung kommen.

Bei Ratten ist kein signifikantes Rotationsverhalten nach einseitiger intrastriataler Behandlung mit 1 ng BoNT-A unter Amphetamineinwirkung zu beobachten. Ursächlich hierfür kann sein, dass der Besatz an SV2C-Rezeptoren an den Axonendigungen der unterschiedlichen Neuronentypen, die im CPu projizieren, in Ratten anders als in Mäusen verteilt ist. So ist es möglich, dass in der Ratte nicht nur die Acetylcholinausschüttung durch BoNT-A stark verringert ist, sondern durch einen erhöhten Besatz an SV2C-Rezeptoren an dopaminergen Axonendigungen auch die Dopaminausschüttung blockiert ist. Dadurch würde nach Amphetamingabe die bewegungsfördernde Komponente der ACh-Konzentrationsverringerung im BoNT-A-behandelten CPu durch eine, im Vergleich zum unbehandelten CPu, verminderte Dopaminausschüttung kompensiert werden. Folglich würden beide Striata wieder einen annähernd gleichen Einfluss auf die Bewegungsinitierung durch den direkten und indirekten Weg der Basalganglienschleifen vermitteln – ein vermehrtes Initiieren von Bewegungen auf einer der beiden Seiten wäre dann nicht zu erwarten.

Zusätzlich kann auch die Verringerung an D_2-Rezeptoren im BoNT-A behandelten CPu der Ratte ursächlich für das Ausbleiben eines Amphetamininduzierten Rotationsverhaltens sein, da hierdurch die bewegungsfördernde Wirkung des Dopamins, die durch den indirekten Weg der Basalganglienschleife vermittelt wird, verringert wird.

6.5 Repeated Intrastriatal Botulinum Neurotoxin-A Injection in Hemiparkinsonian Rats Increased the Beneficial Effect on Rotational Behavior.

Vorarbeiten ergaben, dass der Effekt einer intrastriatalen BoNT-A-Behandlung auf das motorische Verhalten von Nagetieren temporärer Natur und in der Regel nach 3 bis 6 Monaten wieder abgeklungen ist. Hier wurde eruiert, inwiefern eine zweite intrastriatale BoNT-A-Behandlung in Ratten sechs Monate nach einer ersten BoNT-A-Behandlung, also nach dem

Abklingen der Wirkung der ersten Behandlung, möglich ist und welche Besonderheiten auf Verhaltensebene nach einer solchen Zweitbehandlung zu beobachten sind.

Darüber hinaus sollten Hinweise darauf gewonnen werden, ob durch die intrastriatale BoNT-A-Injektion in der Tat, wie in der Arbeitshypothese dargestellt, die extrazelluläre Konzentration an ACh im CPu reduziert wird. Hierzu sollten die Effekte, die in pharmakainduzierten motorischen Verhaltenstests nach BoNT-A-Behandlung auftreten, bei gleichzeitiger Gabe eines Blut-Hirn-Schranke-gängigen Acetylcholinesteraseinhibitoren untersucht werden.

Fragestellung

Ist es möglich, hemiparkinsonoide Ratten, die bereits einmal mit 1 ng BoNT-A im dopaminerg deafferentierten CPu behandelt worden sind, ein halbes Jahr nachdem die Wirkung des BoNT-A auf den apomorphininduzierten Rotationstest abgeklungen ist, ein weiteres Mal dieser Behandlung zu unterziehen? Führt diese zweite BoNT-A-Behandlung zu einer gesundheitlichen Beeinträchtigung der Tiere? Ist eine zweite intrastriatale BoNT-A-Behandlung dazu in der Lage, die apomorphininduzierte Rotationsrate, die nach einer ersten BoNT-A-Behandlung zunächst signifikant gesenkt worden ist und im Verlauf von 6 Monaten wieder auf ihren Ursprungswert gestiegen ist, ein weiteres Mal signifikant zu senken? Wie stark fällt eine eventuelle zweite Senkung der apomorphininduzierten Rotationsrate aus? Sind durch eine zweite ipsilaterale BoNT-A-Behandlung innerhalb eines halben Jahres andere Effekte auf einen 6-OHDA induzierten Seitenneglect oder auf forciertes motorisches Verhalten zu beobachten, als bei einmaliger BoNT-A-Injektion? Lässt sich durch die systemische Applikation des Acetylcholinesteraseinhibitors Donepezil, der dazu in der Lage ist die Blut-Hirn-Schranke zu überwinden, die apomorphininduzierte Rotationsrate wieder steigern und somit der Nachweis erbringen, dass die Reduktion der apomorphininduzierten Rotationsrate bei hemiparkinsonoiden Ratten nach einseitiger BoNT-A-Injektion abhängig ist von einer Reduktion der striatalen Acetylcholinkonzentration?

Material und Methoden

Junge männliche Wistar-Ratten wurden zunächst dem Corridor Task und dem Stepping Test unterzogen. Anschließend wurde mittels sterotaktischer Injektion von 6-OHDA in das rechte

MVB eine rechtsseitige Läsion der SNpc ausgelöst. Einen Monat später erfolgten Stepping Test, Corridor Task und apomorphininduzierter Rotationstest. Die Tiere wurden nach diesem Schema einen, drei und sechs Monate nach der ersten BoNT-A-Behandlung getestet. Sechs Monate nach der ersten BoNT-A-Behandlung erfolgte eine zweite Injektion von 1 ng BoNT-A in das rechte, dopaminerg deafferentierte CPu. Einen Monat, drei, sechs, neun und zwölf Monate nach der zweiten BoNT-A-Behandlung durchliefen die Tiere die oben genannten Batterien an Verhaltenstests.

Zu Untersuchung der Acetylcholinkonzentrationsabhängigkeit der Reduktion der apomorphininduzierten Rotationsraten hemiparkinsonoider Ratten nach intrastriataler BoNT-A-Injektion, wurden im Rahmen dieser Arbeit apomorphininduzierte Rotationstests nach dem Muster der vorherigen Publikationen durchgeführt, wobei zusätzlich zu den jeweiligen Untersuchungszeitpunkten Rotationstests unter dem Einfluss von Donepezil durchgeführt worden sind. Donepezil ist Blut-Hirn-Schranke-gängig und blockiert die Acetylcholinesterase (AChE). Es lag die Annahme zugrunde, dass falls der mindernde Effekt auf die apomorphininduzierte Rotationsrate nach BoNT-A-Gabe auf einer Reduzierung der ACh-Konzentration zurückzuführen ist, die Erhöhung der extrazellulären ACh-Konzentration diesen Effekt wieder rückgängig machen muss. Donepezil sollte die AChE im CPu blockieren. Hierdurch sollte die extrazelluläre Konzentration an ACh im CPu kurzzeitig erhöht werden. Diese temporäre Erhöhung der striatalen ACh-Konzentration durch Donepezilgabe sollte zum jeweils zweiten apomorphininduzierten Rotationstest eines Untersuchungszeitpunktes erfolgen. Anschließend sollten die jeweiligen Rotationsraten, einmal ohne Donepezileinfluss und einmal unter Donepezileinfluss, miteinander verglichen werden. Dass überhaupt noch funktionsfähige cholinerge Präsynapsen im Ratten-CPu, das mit 1 ng BoNT-A behandelt worden ist, vorhanden waren, schlossen wir daraus, dass die Effekte auf die verschiedenen motorischen Tests nach Injektion von 2 ng BoNT-A in das CPu stärker waren als die nach Injektion von 1 ng (Antipova et al., 2013; Wree et al., 2011).

Aufgrund unterschiedlicher Angaben über die Halbwertszeit von Donepezil (Barnes et al., 2000; Geerts et al., 2005; Goh et al., 2011; Mumenthaler et al., 2003; Nagy et al., 2004; Nirogi et al., 2012; Ohnishi et al., 1993; R and Davis, 2012; Snape et al., 1999; Tiseo et al., 2002), musste dieses Experiment in verschiedenen Varianten erfolgen. Diese unterschieden sich in ihrem zeitlichen Muster. Zu jedem Untersuchungszeitpunkt lagen der erste und der zweite Rotationstest 72 h auseinander. Im ersten Schema erfolgte die Donepezil- bzw.

Scheindonepezilgabe 24 h vor dem zweiten Rotationstest und bei dem zweiten Schema erfolgte die Donepezilgabe 1 h vor dem zweiten Rotationstest.

Ergebnisse

Körpergewichte
Sowohl die erste als auch die zweite BoNT-A-Injektion (6 Monate nach der ersten) wurde von den Tieren gut vertragen. Es wurden zu keinem Zeitpunkt signifikante Unterschiede zwischen BoNT-A-behandelten und Vehikel-behandelten Tieren bezüglich des Körpergewichtes beobachtet.

Apomorphininduzierte Rotationen
Einen Monat nach einer rechtsseitigen 6-OHDA-Injektion konnte ein durchschnittliches apomorphininduziertes Rotationsverhalten von 6 U/min kontralateral zur Läsion gemessen werden. Nachdem ein erstes Mal 1 ng BoNT-A in das rechte CPu injiziert wurde, ist die Rotationsrate signifikant auf 2 U/min gesunken. In den folgenden 5 Monaten stieg die Rotationsrate kontinuierlich auf den Ursprungswert an. Daraufhin folgte die zweite Injektion von 1 ng BoNT-A an dieselben Koordinaten wie zuvor. Die apomorphininduzierte Rotationsrate sank daraufhin noch stärker auf ca. 0 U/ min ab. Auch in den folgenden Monaten nach der zweiten BoNT-A-Behandlung stieg die apomorphininduzierte Rotationsrate wieder schrittweise an. Jedoch vollzog sich der Wiederanstieg langsamer, als nach der ersten BoNT-A-Behandlung.

Stepping Test
Zur Detektion einer möglichen Akinesie der Vorderpfoten wurde auch hier der Steppingtest angewendet. Die rechtsseitige 6-OHDA-Läsion führte auf der linken Seite zu einer deutlichen Reduktion der Zahl der Ausgleichsschritte („Adjusting-Steps“) von ca. 12 auf ca. 4 – 6. Eine BoNT-A-Behandlung führte zu keinen signifikanten Veränderungen der Anzahl der Adjusting-Steps, im Vergleich zu Vehikel-behandelten Tieren.

Corridor-Task

Die Versuchstiere wurden mittels des Corridor-Tasks auf einen Neglect einer Seite ihrer Umwelt hin getestet. Rechtsseitig 6-OHDA-läsionierte Ratten suchten im Schnitt nur noch zu 5 % Futter im Bereich ihres linken Gesichtsfeldes, obwohl ihnen im Korridor des Corridor-Taskes alle 15 cm Futter auf beiden Seiten angeboten worden ist. Dieser kontralaterale Neglect konnte durch eine Injektion von BoNT-A in das rechte CPu nicht gebessert werden. Auch die zweite BoNT-A-Injektion hatte keinen Effekt auf die Seitendifferenz bei den Futtersuchen.

Donepezil-Effekte

Hier wurden die Werte von je zwei Rotationstests nach Apomorphingabe für jeden Untersuchungszeitpunkt miteinander verglichen. Die beiden Rotationstests lagen stets je 72 h auseinander. Je nach Studiendesign erhielten die Ratten 24 h oder 1 h vor dem jeweils zweiten Rotationstest 2 mg/ kg KG Donepezil als intraperitoneale Injektion.

Die ersten Donepezilwirkungen konnten bei den Tieren nach 8 - 10 min beobachtet werden. So führten die Tiere vermehrt Kaubewegungen über einen Zeitraum von 2,5 h aus und es konnte eine Reduktion der Körpertemperatur von 1°C gemessen werden.

Bei hemiparkinsoiden Ratten, die anfänglich eine hohe apomorphininduzierte Rotationsrate aufwiesen, führte eine BoNT-A-Injektion einen Monat nach der Behandlung zu einer deutlichen Reduktion der Rotationsrate. Wurden diese Tiere 72 h später einem zweiten Rotationstest unterzogen, nachdem diese 24 h zuvor mit Donepezil behandelt worden sind, so war die Rotationsrate zum Zeitpunkt des zweiten Tests, im Vergleich zu der Rotationsrate des ersten Tests, signifikant erhöht. Ein ähnlicher Effekt wurde auch in Schein-BoNT-A-behandelten Ratten gemessen. Sowohl bei BoNT-A behandelten als auch Vehikel-behandelten hemiläsionierten Ratten war die Rotationsrate des zweiten Rotationstestes (24 h nach Donepezilgabe) zu den Untersuchungszeitpunkten einen Monat und zwei Monate nach BoNT-A-Behandlung signifikant gegenüber dem Test vor Donepezilgabe erhöht. Drei und vier Monate nach BoNT-A-Behandlung bestand ein deutlicher Trend dahingehend, dass die Rotationsrate des jeweiligen Testes nach Donepezilgabe gegenüber dem ersten Test erhöht war.

Dieses Testdesign wurde ein weiteres Mal durchgeführt, jedoch erhielten die Ratten 24 h vor dem zweiten Rotationstest lediglich die Vehikelsubstanz (0,9%‘ige NaCl-Lösung) des Donepezil intraperitoneal injiziert. Auch hier zeigten sowohl BoNT-A-behandelte Ratten als auch sham-behandelte Ratten einen Monat nach BoNT-A-Behandlung signifikant erhöhte

Rotationsraten im Zuge des zweiten Rotationstest, der 72 h nach einem ersten Rotationstest erfolgte.

Aufgrund stark abweichender Informationen über die Halbwertszeit von Donepezil in der Literatur wurde eine dritte Versuchsreihe durchgeführt. Hierzu wurden ebenfalls hemiläsionierte Ratten untersucht, die entweder BoNT-A- oder Vehikel-behandelt wurden. Folgende drei Versuchsgruppen wurden getestet: BoNT-A-behandelte Hemiparkinsonratten, die zu jedem Testzeitpunkt einen ersten apomorphininduzierten Rotationstest durchliefen und 72 h danach ein zweites Mal getestet wurden, jedoch 1 h vor dem zweiten Rotationstest 2 mg/ kg KG Donepezil erhielten; BoNT-A-behandelte Hemiparkinsonratten die 1 h vor dem zweiten Rotationstest die Vehikelsubstanz des Donepezil erhielten und Vehikel-behandelte Hemiparkinsonratten, die 1 h vor dem zweiten Rotationstest ebenfalls 2 mg/ kg KG Donepezil erhielten.

Bei BoNT-A-behandelten Hemiparkinsonratten hatte die Donepezilgabe 1 h vor dem zweiten Rotationstest zu den meisten Untersuchungspunkten keinen signifikanten Effekt auf die Rotationsrate, lediglich 9 Monate nach der BoNT-A-Behandlung war die Rotationsrate während des zweiten Rotationstests unter Donepezileinwirkung um 2 U/min signifikant erniedrigt. Bei schein-BoNT-A-behandelten Hemiparkinsonratten war fast zu jedem Untersuchungszeitpunkt die Rotationsrate unter Donepezileinwirkung signifikant um 3 bis 4 U/min erniedrigt. Eine Scheindonepezilbehandlung hatte keinen signifikanten Effekt auf die apomorphininduzierte Rotationsrate.

Schlussfolgerungen

Das, im Vergleich zu Vehikel-behandelten Tieren, unveränderte Körpergewicht von den mit BoNT-A injizierten Ratten und der nicht zu unterscheidende Gesamteindruck der beiden Tiergruppen bis zu ein Jahr nach einer zweiten BoNT-A-Behandlung und 18 Monate nach einer ersten Behandlung mit 1 ng BoNT-A, spricht für eine gute Verträglichkeit auch einer zweiten intrazerebralen BoNT-A-Applikation innerhalb von 6 Monaten.

apomorphininduzierte Rotationen
Der Effekt der zweiten BoNT-A-Behandlung auf die apomorphininduzierte Rotationsrate war stärker ausgeprägt als der Effekt der ersten BoNT-A-Injektion. So betrug die

apomorphininduzierte Rotationsrate einen Monat nach der ersten BoNT-A-Behandlung 2,27 ± 0,86 U/min und 0,17 ± 0,58 U/min. Und auch der Wiederanstieg der Rotationsrate war nach der zweiten Behandlung weniger steil, als nach der ersten Behandlung. Die Rotationsrate 3 Monate nach der ersten BoNT-A-Injektion betrug 4,44 ± 0,86 U/min und 1,32 ± 1,06 U/min 3 Monate nach der zweiten Injektion sowie 6,85 ± 1,05 U/min sechs Monate nach der ersten BoNT-A-Injektion und 2,20 ± 0,91 U/min sechs Monate nach der zweiten BoNT-A-Injektion.

Die Ergebnisse legen die Vermutung nahe, dass trotz der Tatsache, dass sechs Monate nach der ersten BoNT-A-Injektion die Rotationsrate wieder einen Wert erreicht hat, der dem Wert vor der BoNT-A-Behandlung entsprach, dennoch eine Restwirkung der ersten BoNT-A-Behandlung vorgelegen haben muss. Unsere Befunde entsprechen auch den Erkenntnissen anderer Arbeitsgruppen über die BoNT-A-Wirkung in der Körperperipherie, die besagen, dass nach wiederholter intramuskulärer Injektion, die BoNT-A-Wirkung jeweils länger andauert. So konnten Rogozhin et al. (Rogozhin et al., 2008) zeigen, dass sich nach wiederholter Behandlung des Musculus epitrochleoanconeus mit BoNT-A bei Mäusen, die neuromuskuläre Signalweiterleitung langsamer normalisiert als nach einer einmaligen BoNT-A-Behandlung. Und auch aus der klinischen Anwendung beim Menschen ist gut belegt, dass mit der Anzahl der BoNT-A-Behandlungen einer Körperregion deren Wirkstärke und Wirkdauer steigt (Brashear et al., 2005; Colosimo et al., 2012; Flynn, 2010; Şen and Arpaci, 2015).

Dass das stärkere Absinken der apomorphininduzierten Rotationsrate und deren langsamerer Wiederanstieg nach der zweiten BoNT-A-Behandlung auf die wiederholte BoNT-A-Injektion und nicht auf das fortgeschrittene Alter der Versuchstiere zurückzuführen ist, schließen wir daraus, dass auch in vorangegangenen Experimenten und bei schein-BoNT-A-behandelten Ratten dieses Experimentes die apomorphininduzierte Rotationsrate 6-OHDA-hemiläsionierter Ratten nicht abhängig war von der Zeit, die zwischen dem jeweiligen Rotationstest und der zurückliegenden 6-OHDA-Läsion lag.

Stepping test und Corridor task

Wie in den vorangegangenen Studien (Antipova et al., 2018, 2017) wurde nach einmaliger ipsilateraler Injektion von 1 ng BoNT-A in das rechte, dopaminerg deafferentierte CPu keine Erhöhung der Schrittzahl der kontralateralen Vorderpfote beobachtet. Auch im Corridor Task wurde keine Aufhebung des linksseitigen Neglects beobachtet. Eine zweite Injektion von BoNT-A hatte ebenfalls keinen signifikanten Effekt in diesen Tests.

Die dopaminerge Deafferentierung des rechten CPu‘s führt zu einer Enthemmung von MSNs, die auf den Gpe projizieren, woraufhin der Gpi und STN enthemmt werden, was zu einer vermehrten Hemmung des Thalamus und einer geringeren Aktivierung des motorischen und prämotorischen Kortex führt. Auch werden MSN’s, die direkt auf den Gpi projizieren, nicht mehr durch Dopamin aktiviert und können den Gpi weniger stark hemmen, so dass dies zu seiner Überaktivität und so zu einer verringerten Aktivierung des motorischen und prämotorischen Kortex beiträgt. Diesen Umstand konnte auch die zweimalige ipsilaterale Injektion von 1 ng BoNT-A anscheinend nicht aufheben, auch wenn man annehmen musste, dass BoNT-A die intrastriatale ACh-Konzentration herabsetzen sollte und damit die übermäßige Aktivierung von MSNs verringern sollte, die auf den Gpe projizieren.

Donepezil bei apomorphininduzierten Rotationstest

Da in der Literatur sehr unterschiedliche Angaben über die Halbwertszeit von Donepezil gemacht worden sind, wurde Donepezil bzw. die Vehikelsubstanz entweder 1 h oder 24 h vor einem zweiten apomorphininduzierten Rotationstest appliziert.

Hemiläsionierte Ratten, die 24 h vor einem zweiten apomorphininduzierten Rotationstest mit 2 mg/ kg KG Donepezil behandelt worden sind, zeigten eine signifikant erhöhte Rotationsgeschwindigkeit, im Vergleich zu einem 72 h zuvor ausgeführten Rotationstest. Hierdurch schien sich zunächst unsere Arbeitshypothese zu bestätigen, dass durch die systemische Gabe des Acetylcholinesterasehemmers Donepezil die ACh-Konzentration im BoNT-A-behandelten CPu wieder erhöhen lässt und eine durch BoNT-A erniedrigte Rotationsrate wieder steigt, vorausgesetzt dass die apomorphinduzierte Rotationsrate eine Acetylcholinabhängigkeit aufweist. Auch schein-BoNT-A-behandelte Hemiparkinsonratten, wiesen 24 h nach Donepezilgabe einen solchen Sprung der apomorphiniduzierten Rotationsrate auf. Jedoch zeigten auch hemiparkinsonoide BoNT-A-behandelte Ratten einen gleichartigen signifikanten Sprung der Rotationsrate, die 24 h vor dem zweiten Rotationstest lediglich mit der Vehikelsubstanz des Donepezils behandelt worden sind. Daraus muss geschlossen werden, dass der beobachtete plötzliche und signifikante Wiederanstieg nicht auf die Wirkung des Donepezils zurückzuführen ist, sondern auf eine mögliche Sensitivierung des extrapyramidalmotorischen Systems nach einer ersten Gabe von Apomorphin im Rahmen eines ersten Rotationstestes. Solche Sensitivierungen nach mehrfachen Apomorphinapplikationen wurden bereits beschrieben (Castro et al., 1985; Gancher et al., 1995).

Entgegen der Arbeitshypothese konnte bei schein-BoNT-A-behandelten hemiläsionierten Tieren die Beobachtung gemacht werden, dass eine Stunde nach einer Donepezilgabe, die apomorphininduzierte Rotationsrate, im Vergleich zu einem Test der 72 h zuvor durchgeführt worden ist, sogar herabgesetzt war. Hier muss spekuliert werden, dass die systemische Wirkung des Donepezils eine prinzipielle Beeinträchtigung der Motorik und damit eine Verlangsamung der Rotationsgeschwindigkeit zur Folge hat. In Einklang hiermit wurde in der Literatur berichtet, dass es bei Patienten, die mit Donepezil behandelt werden, zu einer Verschlechterung der motorischen Fähigkeiten kommen kann (Fabbrini et al., 2002). Aus den Ergebnissen ist auch zu schließen, dass die Halbwertszeit von Donepezil den Publikationen entspricht, die diese mit einigen Stunden angeben (Geerts et al., 2005; Goh et al., 2011; Liang and Tang, 2006; Naik et al., 2009; Nirogi et al., 2012). So konnten wir einen Effekt von Donepezil auf die Rotationsrate nur beobachten, wenn dieses 1 h vor dem Rotationstest appliziert worden ist. Wurde Donepezil 24 h vor einem zweiten Rotationstest gegeben, so unterschied sich das Verhalten der jeweiligen Ratten nicht von Tieren, denen lediglich die Vehikelsubstanz von Donepezil 24 h vor einem zweiten Rotationstest injiziert worden ist.

Aus den Resultaten zogen wir den Schluss, dass die systemische Gabe von Donepezil vor apomorphininduzierten Rotationstests nicht geeignet war, eine Acetylcholinanbhängigkeit der Wirkung von intrastriatal appliziertem BoNT-A auf die apomorphininduzierte Rotationsrate nachzuweisen.

6.6 Repeated Intrastriatal Application of Botulinum Neurotoxin-A did not Influence Choline Acetyltransferase Immunoreactive Interneurons in Hemiparkinsonian Rat Brain - A Histological, Stereological and Correlational Analysis

In dieser Arbeit wurde der Effekt einer wiederholten BoNT-A-Behandlung hemiparkinsonoider Ratten auf die Histologie des CPu's hin untersucht. Im Kern sollten Hinweise darauf gewonnen werden, ob bei einer mehrfachen intrazerebralen BoNT-A-Behandlung das Risiko zytotoxischer Effekte oder anderer degenerativer Prozesse im Gehirn besteht. Diese Studie war notwendig, da bei einem potentiellen Einsatz der intrazerebralen BoNT-A-Applikation als Therapieoption wiederholte Anwendungen notwendig wären, da die Wirkung einer einmaligen Anwendung im Verlauf weniger Monate wieder nachlässt. Aus dem klinischen Einsatz von BoNT-A in der Peripherie ist dieser Umstand bekannt (Şen and Arpaci, 2015).

Fragestellung

Hat eine zweimalige Injektion von je 1 ng BoNT-A in das rechte CPu von Ratten einen zytotoxischen Einfluss auf dessen cholinergen Interneurone? Wird deren Anzahl oder deren Dendritenlänge durch die Behandlung beeinflusst? Wirkt BoNT-A nach Injektion in das CPu auch auf benachbarte cholinerge Regionen und beeinflusst die Dendritenlängen der dortigen cholinergen Nervenzellen? Hat die zweite BoNT-A-Behandlung innerhalb eines halben Jahres Einfluss auf die Größe und numerische Dichte der zuvor beobachteten Aufweitungen an cholinergen Nervenfasern?

Material und Methoden

Es wurden Ratten untersucht, bei denen die rechte SNpc läsioniert wurde und denen sechs Wochen sowie sieben Monate nach 6-OHDA-Läsion jeweils 1 ng BoNT-A oder die Vehikelsubstanz in das rechte, dopaminerg deafferentierte CPu injiziert wurde.

Die Gehirne der Ratten wurden 12 Monate nach der letzten BoNT-A-Injektion entnommen und gewogen. Anschließend wurden sie fixiert, kryoprotektiert, eingefroren und es wurden 30 µm dicke Serienschnitte am Gefriermikrotom angefertigt. Jeder siebte Schnitt wurde entweder Nissl-gefärbt oder immunhistochemisch gegen ChAT gefärbt. Anhand der gegen ChAT gefärbten Schnitte wurde mittels stereologischer Zählungen mithilfe des Programms Stereo Investigator 8.0 (MicroBrightFieldBioscience, Vermont, USA) die Zahl striataler cholinerger Interneurone in den Gehirnen und das Volumen der Striata bestimmt. Dies geschah sowohl für die behandelte als auch für die unbehandelte Hemisphäre. Weiterhin wurden Sholl-Analysen durchgeführt, um die Dendritenlängen der cholinergen Neurone des CPu zu bestimmen. Untersucht wurde das behandelte (rechte) und das unbehandelte (linke) CPu von 3 Ratten, die 12 Monate nach einer einseitigen BoNT-A-Injektion getötet worden sind und von 3 Ratten, die 12 Monate nach einer zweiten BoNT-A-Injektion und 3 Ratten die 12 Monate nach einer zweiten Vehikel-Injektion finalisiert worden sind. Für die Analyse wurden, virtuell vom Zentrum des Somas cholinerger Neurone ausgehend, konzentrische Kreise im Abstand von 25 µm gelegt. Die Anzahl der Kreuzungen von den Dendriten mit den jeweiligen Kreisen wurde ermittelt und daraus die totale Dendritenlänge berechnet.

Um beurteilen zu können, ob BoNT-A über das CPu hinausgehend wirkt, wurden anhand der gegen ChAT-angefärbten Hirnschnitte auch die Dendritenlängen der cholinergen Neurone des horizontalen Schenkels des Diagonalen Bandes nach Broca (HDB) bestimmt. Dies geschah insbesondere deshalb, um Hinweise auf potentiell unerwünschte Effekte des intrastriatal applizierten BoNT-A auf andere Hirnregionen zu erlangen, in den ebenfalls eine ausgeprägte cholinerge Signalweiterleitung erfolgt und die eine zentrale Rolle bei kognitiven Prozessen spielen. Der HDB wurde gewählt, da er im Gegensatz zum Nucleus basalis Meynert bei der Ratte besser abgrenzbar ist und sich auf denselben Schnitten befunden hat, die auch für Sholl-Analysen im CPu genutzt worden sind (Liu et al., 2015).

Weiterhin wurde untersucht, wie sich, im Vergleich zu einer einmaligen BoNT-A-Injektion, bei gesunden Mäusen die numerische Dichte und Größe ChAT-positiver BiVs nach einer zweimaligen BoNT-A-Injektion in 6-OHDA-hemilesionierten Ratten entwickelt. Hierzu wurden anhand mikroskopischer Aufnahmen der entsprechenden CPus, die BiVs digital segmentiert und hinsichtlich ihrer Fläche und Anzahl analysiert.

Ergebnisse

Körpergewichte und Hirngewichte

Am Ende des Untersuchungszeitraumes 12 Monate nach der letzten Behandlung wogen die Tiere, die zweimal mit BoNT-A behandelt worden sind 500 ± 23,8 g (MW ± SEM) und Ratten, die lediglich mit der Vehikelsubstanz injiziert worden sind 594,0 ± 61,4 g. Die Unterscheide waren nicht signifikant.

Auch die Hirngewichte und die Quotienten aus Hirngewicht und Körpergewicht unterschieden sich nicht signifikant voneinander.

Striatales Volumen

Das Volumen des behandelten rechten CPu‘s von Tieren, denen zweimal BoNT-A injiziert worden ist, betrug 29,45 ± 1,22 mm³ und war damit signifikant kleiner als das linke, unbehandelte CPu mit 36,48 ± 1,29 mm³. Bei Vehikel-behandelten Tieren unterschieden sich die Volumina des rechten (33,43 ± 1,74 mm^3) und linken (37,07 ± 1,44 mm^3) CPu’s nicht signifikant voneinander.

Zahl und numerische Dichte cholinerger striataler Interneurone

Weder doppelt-BoNT-A-behandelte Ratten noch Vehikel-behandelte Ratten wiesen signifikante Unterscheide in der Anzahl cholinerger Interneurone des rechten (20546,3 ± 866,6 (Doppel-BoNT-A), 20257,2 ± 655,23 (Doppel-Sham)) und linken (21573,1 ± 788,2 (Doppel-BoNT-A), 20496,4 ± 488,4 (Doppel-Sham)) CPu's auf. Auch zwischen den verschiedenen Experimentalgruppen existierten keine signifikanten Unterschiede bezüglich der Zahl striataler cholinerger Interneurone.

Bei doppelt-BoNT-A-behandelten Ratten war die numerische Dichte der cholinergen Interneurone des rechten CPu's (703,6 ± 33,2 ChAT-positive Neurone/mm^3) jedoch signifikant höher als die des linken, unbehandelten CPu's (594,8 ± 26,3 ChAT-positive Neurone/mm^3). Bei Sham-behandelten Ratten gab es keine signifikanten Unterschiede zwischen den Hemisphären bezüglich der numerischen Dichte cholinerger Neurone (rechts: 614,5 ± 55,2 ChAT-positive Neurone/mm^3, links: 556,9 ± 34,3 ChAT-positive Neurone/mm^3).

Sholl-Analyse der Dendritenlängen striataler cholinerger Interneurone

Die Gesamtdendritenlänge der cholinergen Neurone des rechten CPu's (132,83 ± 10,87 µm) und des linken CPu's (117,52 ± 6,15 µm) bei doppel-BoNT-A-behandelten Ratten unterschied sich nicht signifikant untereinander. Ebenso wurden keine signifikanten Unterschiede zwischen den beiden Hemisphären von Vehikel-behandelten Ratten (rechts: 89,67 ± 17,31 µm, links: 95,22 ± 10,96 µm) gemessen. Auch unterschieden sich die Dendritenlängen striataler cholinerger Interneurone der Vehikel-behandelten Tiere von der doppel-BoNT-A-behandelter Tiere nicht signifikant voneinander.

Allerdings waren bei gesunden Ratten, die nur einmal mit BoNT-A behandelt worden sind und ein Jahr überlebt haben, die Dendriten des linken (unbehandelten) CPu's (206,94 ± 17,64 µm) signifikant länger als die des rechten (BoNT-A-behandelten) CPu's (155,08 ± 20,14 µm). Auch waren deren striatale ChAT-positive Dendriten signifikant länger, als die der anderen beiden Tiergruppen.

Sholl-Analyse der Dendritenlängen cholinerger Neurone des horizontalen Schenkels des Diagonalen Bandes nach Broca (HDB)

Die Dendritenlängen ChAT-positiver Neurone des HDB zeigten zwischen linker und rechter Hemisphäre keine signifikanten Unterschiede. Auch waren keine signifikanten Unterschiede zwischen hemiläsionierten Ratten, die 12 Monate nach einer zweit-BoNT-A-Injektion überlebt haben und Tieren, denen nach Läsion lediglich zwei Mal die Vehikelsubstanz injiziert worden ist, messbar. Die Dendritenlängen von gesunden Tieren, die nur einmal einseitig mit BoNT-A behandelt worden sind und die nach einem Jahr finalisiert worden sind, unterschieden sich zwischen linkem und rechtem HDB ebenfalls nicht signifikant voneinander. Allerdings waren auch hier die Dendriten signifikant länger, als die von hemiparkinsonoiden Tieren, die zweimal mit BoNT-A oder der Vehikel-Substanz behandelt worden sind, die also nach einer 6-OHDA-Läsion noch 19 Monate lebten.

Die Dendritenlängen der Doppel-BoNT-A-Tiere betrugen für das rechte HDB: 86,90 ± 8,64 µm und für das linke HDB: 83,33 ± 8,55 µm. Bei zweimal Sham-behandelten Tieren waren die Dendriten im rechten HDB: 111,18 ± 0,42 µm und im linken HDB: 112,53 ± 6,86 µm lang und bei gesunden einmal BoNT-A-behandelten Tiere betrugen die Dendritenlängen für das rechte HDB: 141,44 ± 12,21 µm und für das linke HDB: 135,11 ± 8,51 µm.

Numerische Dichte der striatalen ChAT-positiven BiVs

Bei hemiparkinsonoiden Ratten, denen zweimal 1 ng BoNT-A in das dopaminerg deafferentierte CPu (rechts) injiziert worden ist, wurde für cholinerge BiVs im rechten CPu eine numerische Dichte von 4717,4 ± 562,2 Varikositäten je mm^2 ermittelt. Für gesunde Tiere, die ein Jahr nach einer einmaligen Injektion von 1 ng BoNT-A überlebt haben, betrug dieser Wert 1963,8 ± 369,1 Varikositäten je mm^2 und war damit nur halb so groß.

Größe und Größenverteilung striataler ChAT-positiver BiVs

Die mittlere Größe der BiVs, gemessen als Fläche auf einem digitalen Bild, war bei zweifach BoNT-A-behandelten hemiläsionierten Ratten (3,401 ± 0,089 $µm^2$) und gesunden Ratten, die einmal BoNT-A-behandelt worden sind (3,270 ± 0,220 $µm^2$), annähernd gleich.

Die Analyse der Größenverteilung ergab, dass doppel-BoNT-A-behandelte Ratten signifikant mehr BiVs kleiner Größenkategorien bis 3,84 µm² aufwiesen als einmal behandelte Tiere.

Schlussfolgerungen

Hirn-und Körpergewichte
Ein negativer Einfluss der zweimaligen BoNT-A-Injektion auf die Gesundheit der Versuchsratten konnte nicht beobachtet werden. Dafür sprachen unter anderem, dass, im Vergleich zu doppel-Sham-behandelten Tieren, keine signifikanten Unterschiede im Körpergewicht, im Hirngewicht und im Quotienten von Hirngewicht und Körpergewicht gemessen worden sind.

CPu-Volumina, Anzahl und numerische Dichte cholinerger Interneurone im CPu
Bei hemiläsionierten Ratten, die zweimal mit BoNT-A behandelt worden sind, waren die Volumina der behandelten Striata signifikant kleiner als die der kontralateralen Striata. Dies war bei Vehikel-behandelten Ratten und bei gesunden Ratten, die nur einmal mit BoNT-A behandelt worden sind, nicht der Fall. Es konnte noch nicht bestimmt werden, welche Anteile des striatalen Neuropils bei doppel-BoNT-A-behandelten Tieren verringert sind.

Es wurden keine signifikanten Unterschiede in der Anzahl cholinerger Interneurone 12 Monate nach der zweiten BoNT-A-Injektion gemessen, jedoch war die numerische Dichte der cholinergen Interneurone im BoNT-A-behandelten CPu signifikant erhöht. Dies ist mit der Tatsache zu begründen, dass bei gleichbleibender Zellzahl, das striatale Volumen verringert war. Dass kein Verlust striataler ChAT-positiver Nervenzellen gemessen worden ist, steht im Einklang mit den Ergebnissen, die zuvor auch in Ratten und Mäusen nach einmaliger BoNT-A-Injektion ermittelt worden sind (Antipova et al., 2013; Mehlan et al., 2016).

Sholl Analysen
Da es bekannt ist, dass verschiedene neurodegenerative Erkrankungen nicht ausschließlich auf einen Untergang ganzer Neurone beruhen, sondern oftmals zuvor mit einer Störung der dendritischen Architektur einhergehen (Cochran et al., 2014; Connolly and Lang, 2014; Forman et al., 2004; Polo, 2015), wurden Sholl-Analysen sowohl in den BoNT-A-behandelten und Vehikel-behandelten CPus als auch in den jeweils kontralateralen CPus ausgeführt (Binley et al., 2014; SHOLL, 1953).

Zur Detektion möglicher über das CPu hinausreichender Wirkungen von BoNT-A auf cholinerge Strukturen, wurde ebenfalls am horizontalen Schenkel des HDB eine Sholl-Analyse ausgeführt.

Das HDB wurde zur Analyse des Einflusses von striatal appliziertem BoNT-A auf die Arborisierung nichtstriataler cholinerger Neurone gewählt, da er in der Nachbarschaft des CPu's liegt und zwar in einer Region, die es erlaubt, an ein und denselben Objektträgern sowohl die Sholl-Analyse am CPu als auch am HDB durchzuführen und weil sich dieses Kerngebiet sehr gut abgrenzen lässt (Woolf, 1997).

Weder bei hemiparkinsonoiden Tieren, die zweimal mit BoNT-A behandelt worden sind, noch bei hemi-PD-Ratten, die mit Vehikelsubstanz behandelt worden sind, konnten signifikante Unterschiede hinsichtlich der Gesamtdendritenlänge des linken und des rechten CPu's nachgewiesen werden. Dies spricht dafür, dass intrazerebral appliziertes BoNT-A keinen zytotoxischen Effekt auf cholinerge Nervenzellen hatte. Es ist jedoch festzuhalten, dass bei gesunden Ratten, die nur einmal mit 1 ng BoNT-A behandelt worden sind, die Dendritenbäume der cholinergen Neurone im behandelten CPu signifikant kürzer waren, als im unbehandelten CPu. Warum dieser Effekt bei nativen Tieren auftritt, die nur einmal mit BoNT-A behandelt worden sind, lässt sich anhand der vorliegenden Datenlage nicht erklären. Ein Unterschied in der Wirksamkeit der BoNT-Charge scheint unwahrscheinlich, da die anderen Tiergruppen ebenfalls einen ausgeprägten rotationsratenmindernden Effekt der BoNT-A-Behandlung im apomorphininduzierten Rotationstest aufwiesen und zahlreiche BiVs in den Striata nachgewiesen werden konnten. Beim HDB konnte für keine der Tiergruppen ein signifikanter Unterschied in der totalen Dendritenlänge zwischen linkem und rechtem CPu registriert werden.

Bemerkenswert ist, dass die Gesamtdendritenlänge sowohl für das CPu als auch für das HDB bei nativen Tieren, die einmal BoNT-A-behandelt worden sind, signifikant länger war, als bei hemiläsionierten Tieren, die entweder zweimal mit BoNT-A oder zweimal mit der Vehikelsubstanz behandelt worden sind. Dieses Phänomen kann zum einen in der Tatsache begründet liegen, dass die nativen Tiere nicht mittels 6-OHDA-läsioniert worden sind. Viel weitreichender scheint jedoch der Alterseffekt. So waren die nativen, einmal behandelten Tiere zum Zeitpunkt der Finalisierung 15 Monate alt und die beiden anderen Tiergruppen hatten ein Alter von 24 Monaten. In der Literatur ist beschrieben, dass die Komplexität der Arborisierung

zentraler Neurone mit zunehmendem Alter abnimmt (Dickstein et al., 2007; Matamales et al., 2016; Umegaki et al., 2008)

Analyse BiVs

Die numerische Dichte der BiVs hat sich bei Tieren, die zweimal mit 1 ng BoNT-A behandelt worden sind und 12 Monate überlebt haben, im Vergleich zu Tieren, die nur einmal mit BoNT-A behandelt worden sind, nahezu verdoppelt, wobei sich die Größenverteilung kaum verändert hat. Dies spricht für einen kumulativen Effekt einer mehrfachen BoNT-A-Injektion.

7. Diskussion

7.1 Morbus Parkinson und das unilaterale 6-OHDA-induziertes Parkinsonmodell

Einer der wichtigsten Punkte, der bei der Beurteilung des BoNT-A-Effektes im 6-OHDA induzierten Parkinsonmodell zu beachten ist, besteht darin, dass im Rahmen der bisher beschriebenen Experimente lediglich ein unilaterales Parkinson-Modell genutzt worden ist, um den therapeutischen Effekt einer intrastriatalen Injektion zu eruieren. Der MP bzw. das idiopathische Parkinsonsyndrom ist jedoch eine Erkrankung, die beide Seiten des Gehirns betrifft. Lediglich zu Beginn der Erkrankung ist die Symptomatik oft auf einer Körperhälfte deutlicher ausgeprägt (Noh et al., 2015; Riederer et al., 2018).

Die Wahl eines unilateralen Parkinson-Modells erfolgte, da es hier möglich ist, die gesunde Seite als interne Kontrolle je Individuum bzw. als Referenz anzusehen. Einzelne Tests sind auch erst möglich, sobald ein Ungleichgewicht der Basalganglienschleifen beider Seiten besteht. So ist der apomorphininduzierte Rotationstest nur möglich, da es durch die einseitige dopaminerge Deafferentierung des rechten CPu's dort zu einer kompensatorischen Induktion des D_2- und D_3-Rezeptorbesatzes kommt, woraufhin die systemische Gabe des D_2-Agonisten Apomorphin zu einer vermehrten Initiierung von Bewegungen auf der kontralateralen Körperseite führt. Die spontane Initiierung von Bewegungen auf der ipsilateralen Körperseite wird jedoch nicht in dem überschießenden Ausmaß gesteigert. Durch dieses Ungleichgewicht kommt es zur Herausbildung eines Rotationsverhaltens nach Apomorphingabe.

Der amphetamininduzierte Rotationstest ist ebenfalls nur durch ein Ungleichgewicht bei den Aktivitäten der linken und rechten Basalganglienschleife möglich. Amphetamin vermittelt die Ausschüttung von Katecholaminen wie Dopamin aus der präsynaptischen Endigung und hemmt deren Wiederaufnahme (Cadet and Shu Ming Zhu, 1992; Cannazza et al., 2012; Herman et al., 1993; Herrera-Marschitz et al., 2010; Lam et al., 2011; Sulzer et al., 2005). Einen solchen Effekt kann systemisch appliziertes Amphetamin hauptsächlich im unläsionierten CPu vermitteln, jedoch nicht in einem 6-OHDA-läsionierten CPu, da dort über 90 % der dopaminergen Axonendigungen untergegangen sind. In Folge führt die Applikation von Amphetamin in 6-OHDA-hemiläsionierte Ratten zu einer vermehrten Aktivierung des Motorkortex auf der unläsionierten Seite und zu einer verstärkten Bewegungsinitiierung auf der Körperseite, die ipsilateral zur Läsion gelegen ist. Unter Amphetamineinfluss fangen 6-OHDA-hemiläsionierte Ratten an, sich in Richtung zu der Läsion zu drehen, während sie unter Apomorphin kontralateral zur Läsion rotieren.

Auch die Testung der Präferenz des spontanen Vorderpfotengebrauchs (Händigkeit/ Zylindertest) und die Untersuchung der Versuchstiere auf einen Seitenneglect hin, ist nur in einem unilateralen Krankheitsmodell möglich.

Durch die Injektion von 6-OHDA in das mediale Vorderhirnbündel werden spezifisch die ipsilaterale SNpc sowie das ipsilaterale ventrale tegmentale Areal geschädigt.

Das idiopathische Parkinsonsyndrom ist eine neurodegenerative Erkrankung, die sich über Jahrzehnte hinzieht. Es gibt Hinweise darauf, dass der neurodegenerative Prozess zunächst im peripheren Nervensystem und hier vor allem im vagal innervierten Bereich der Verdauungsorgane beginnt. Ebenfalls sehr früh ist das olfaktorische System betroffen. Im Verlauf weiterer Jahre scheinen sowohl Nervus vagus und schließlich die Vagus-Kerne im Hirnstamm als auch der Bulbus olfactorius nach und nach umgebende Hirnareale „anzustecken“. Der degenerative Prozess mit der Ausbildung von intrazytoplasmatischen Ubiquitin- und α-Synuclein-reichen Einschlüssen in Somata und Axonen von Nervenzellen breitet sich so immer weiter im Gehirn aus. Relativ früh und vergleichsweise stark sind, im Vergleich zu anderen Hirnarealen, die dopaminergen Zellen der SNpc und des Locus coeruleus betroffen. Im weiteren Verlauf der Erkrankung weitet sich dieser pathologische Effekt auf alle weiteren Hirnareale aus und greift auf nicht-katecholaminerge Neurone über. Dieser Prozess nimmt, wie eingangs erwähnt, Jahrzehnte in Anspruch (Braak et al., 2006, 2003; Braak and Del Tredici, 2017; Hawkes et al., 2009, 2007; Lebouvier et al., 2009). Die Applikation von 6-OHDA in das mediale Vorderhirnbündel führt jedoch innerhalb eines Tages zum retrograden Transport von 6-OHDA in die SNpc und dort innerhalb weniger Stunden zu einem Tod der dopaminerge Neurone.

Diese Unterschiede von sehr schnellem und einseitigem Verlauf der Neurodegeneration nach 6-OHDA-Injektion sowie der Spezifität von 6-OHDA für katecholaminerge Neurone einerseits und des langsamen, über Jahrzehnte andauernden, progredienten Verlaufs, der eine Vielzahl von Neuronentypen betrifft, bei dem IPS andererseits, spielen bei der Einordnung des potentiell therapeutischen Effektes der intrastriatalen BoNT-A-Injektion eine wichtige Rolle.

Bei der Bewertung des möglichen therapeutischen Nutzens der intrastriatalen BoNT-A-Injektion ist zu berücksichtigen, dass auch dieses experimentelle Konzept keine ursächliche Behandlung der degenerativen Prozesse im ZNS darstellt, sondern lediglich die pathologisch erhöhte Konzentration an Acetylcholin im CPu senken soll.

7.2 Bewertung des experimentellen Ansatzes einer zentralnervösen lokal begrenzten anticholinergen Therapie

Die Tatsache, dass keine Seitenunterscheide hinsichtlich der Anzahl von Neuronen gemessen wurden, spricht dafür, dass das intrazerebral applizierte BoNT-A sehr wohl eine starke neuromodulatorische Wirkung entfaltet, jedoch ohne dabei zytotoxisch auf die Neurone der dazugehörigen beeinflussten Axonendigungen zu wirken.

Ziel der BoNT-A-Injektion in das CPu bei MP ist es, den gestörten Regelkreis der Steuerung motorischer Aktivität durch die Basalganglienschleifen zu regulieren. Hierbei sollte auf folgendes Verschaltungsmuster Einfluss genommen werden: dopaminerge Neurone der SNpc senden Efferenzen in das CPu und hemmen dort unter Anderem tonisch aktive cholinerge Interneurone. Beim MP kommt es zu einem Untergang der dopaminergen Neurone der SNpc. Der Verlust an Dopamin im CPu beim MP führt im CPu zu einer vermehrten Ausschüttung an ACh durch die dortigen Interneurone, die aufgrund der nun mangelnden Hemmung durch Dopamin überaktiv werden. Die cholinergen Neurone wirken nun vermehrt exzitatorisch auf MSNs ein, die nun vermehrt den Gpe hemmen. Dieser wirkt in Folge nur noch insuffizient hemmend auf den Gpi und den STN ein. Sowohl Gpi als auch STN werden überaktiv, wobei der STN selbst exzitatorisch auf den den Gpi einwirkt. Als Konsequenz hemmt der Gpi überschießend den Nucleus ventralis anterolateralis des Thalamus, der nun nur noch unzureichend den prämotorischen und motorischen Kortex aktivieren kann, dadurch generiert der Kortex weniger Bewegungsimpulse für die kontralaterale Körperhälfte, als dies im gesunden Zustand der Fall wäre. BoNT-A sollte im CPu die Freisetzung an ACh durch cholinerge Interneurone blockieren und so die Aktivitäten aller nachgeschalteten Kerne der indirekten Basalganglienschleife normalisieren.

Dass es möglich ist durch gezielte anticholinerge Intervention im CPu beim Parkinsonmodell der Maus motorische und sensormotorische Defizite zu verbessen, konnte unter anderem durch die Arbeitsgruppe um Ztaou et al. (2016) (Samira Ztaou et al., 2016) gezeigt werden. Hier wurde bei Mäusen durch 6-OHDA einseitig die SNpc läsioniert, anschließend wurden entweder durch optogenetische Methoden die cholinergen Interneurone im CPu blockiert oder es wurden Hemmer der Muscarinrezeptoren verabreicht. Beide Interventionen verbesserten die parkinsonoide Symptomatik.

7.3 Intrastriatalen Behandlung mit BoNT-A – Anticholinerge vs. Antidopaminerge Wirkung

Bei der experimentellen Injektion von BoNT-A in das CPu sollte die Ausschüttung von ACh durch cholinerge Interneurone inhibiert werden. Diese sind beim MP überaktiv, was einen Großteil der motorischen Symptomatik des MP mitverursacht. Im Zuge der vergangenen Arbeiten konnte gezeigt werden, dass BoNT-A ebenfalls katecholaminerge Afferenzen im CPu, sowohl auf präsynaptischer als auch auf postsynaptischer Ebene, beeinflusst. Dies äußerte sich bei Ratten durch die Ausbildung von TH-positiven BiVs an dopaminergen Axonen. Zum anderen führt BoNT-A zu einer Verringerung der D_2/D_3-Rezeptordichte im CPu. In der Literatur ist bereits seit Jahrzehnten beschrieben, dass BoNT-A in der Lage ist, die Ausschüttung an Dopamin zu blockieren (Ashton and Dolly, 1988; Bigalke et al., 1985, 1981; Bozzi et al., 2006; Dardou et al., 2011).

Auch Verhaltenstests an unilateral lediglich mit BoNT-A behandelten Mäusen (Antipova, et al. 2018) erbrachten Hinweise darauf, dass die Applikation von BoNT-A in das CPu zumindest bei Mäusen eine Komponente aufweisen muss, die auf eine Bewegungsinitiierung hemmend wirken kann. Insbesondere die Ergebnisse des Zylindertestes wiesen darauf hin, dass gesunde Mäuse nach einer einseitigen BoNT-A-Behandlung motorische Defizite auf der kontralateralen Körperhälfte aufwiesen. Hierfür können antidopaminerge Wirkungen des BoNT-A verantwortlich sein. Diese können auf eine Blockade der Dopaminausschüttung beruhen, wodurch der direkte als auch der indirekte Weg der Basalganglienschleife gestört wird. Oder eine BoNT-A-Behandlung bewirkt auch in Mäusen eine Verringerung der striatalen D_2-Rezeptordichte. In diesem Fall wäre der indirekte Weg der Basalganglienschleife gestört.

Eine Vielzahl an Neuroleptika stellen Antagonisten des Dopamins am D_2-Rezeptor dar, was in der Psychopharmakologie ein ernstes Problem darstellt. Diese Neuroleptika sollen in Teilen des limbischen Systems einem Dopaminüberschuss bzw. einer erhöhten Sensitivität gegenüber Dopamin entgegenwirken, indem sie Dopaminrezeptoren blockieren. Durch deren systemische Anwendung, geschieht dies jedoch auch zu einem mehr oder weniger starken Ausmaß in motorischen Anteilen des CPu‘s, wodurch die Patienten bei suboptimaler Dosierung unter parkinsonähnlichen Symptomen wie Bradykinese und Tremor leiden (Farde et al., 1992; Leucht et al., 2009; Miyamoto et al., 2012). Zwar wirkt BoNT-A nicht als Antagonist an Dopaminrezeptoren, es blockiert jedoch sehr wahrscheinlich auch die Freisetzung an Dopamin

und vermindert die numerische Dichte an D_2-Rezeptoren. Dies könnte zu denselben Effekten führen, wie die Anwendung von Dopaminrezeptorantagonisten, die als Neuroleptika eingesetzt werden. Die theoretische Gefahr der Verschlechterung einer Parkinsonsymptomatik besteht also gerade zu Beginn des klinisch auffälligen Teils der Erkrankung, bei dem noch ca. 30 % der dopaminergen Afferenzen erhalten sind.

Für die Bewertung des therapeutischen Potentials der intrastriatalen BoNT-A-Injektion für die Parkinsonbehandlung ist es darum essentiell zu eruieren, welche der beiden Effekte, die anticholinerge Wirkung oder die antidopaminerge Wirkung, im CPu überwiegt. Während eine anticholinerge Wirkung im CPu gewünscht ist, würde hier eine antidopaminerge Wirkung die motorische Symptomatik des MP unter Umständen sogar verschlimmern.

Vorzugsweise ist daran zu denken, BoNT-A in einer Spätphase des MP anzuwenden, bei der bereits ein Großteil der dopaminergen Neurone der SNpc untergegangen sind und eine mögliche antidopaminerge Komponente der intrastriatalen BoNT-A-Anwendung nicht mehr zum Tragen kommen kann.

7.4 Ergebnisse anderer Arbeitsgruppen zur intrazerebralen BoNT-A-Injektion als mögliche Therapieoption des Morbus Parkinson

Im Verlauf der letzten 5 Jahre wurden Arbeiten anderer Arbeitsgruppen publiziert, die ebenfalls die Option der intrazerebralen Injektion von BoNT-A zur Parkinsontherapie untersucht haben.

Hierbei hat sich die Gruppe um Itakura et al.(Itakura et al., 2014a, 2014b) an den zuvor durch uns veröffentlichten Ergebnissen orientiert. Es wurden intrastriatale Applikationen von 0,1 ng, 0,5 ng und 1 ng vom BoNT-A Subtyp 1 und vom BoNT-A Subtyp 2 auf Unterschiede in ihrer Wirksamkeit in motorischen Verhaltenstests, ihrer Wirksamkeit auf die Substrukturen des Gehirns und auf den Allgemeinzustand der Tiere untersucht. Itakura et al. konstatierten, dass die Wirkung von BoNT-A des Subtypes 2 einen stärkeren Effekt auf die Rotationsrate von Hemiparkinsonratten hat und dass es in der Lage ist, SNAP-25 effizienter zu spalten als BoNT-A des Subtyps 1. Die Wirkung von BoNT-A des Subtypes 2 soll auf die Region um die Injektionsstelle herum beschränkt bleiben. Dies würde bei einer künftigen therapeutischen Anwendung für eine höhere Sicherheit des Subtyps 2 sprechen. Es gab teilweise abweichende Befunde dieser Arbeitsgruppe. So sank bei Itakura et al. die amphetamininduzierte Rotationsrate nach BoNT-A-Behandlung, während wir festgestellt haben, dass die

amphetamininduzierte Rotationsrate nach BoNT-Behandlung zunimmt. Jedoch muss man feststellen, dass die Studien von Itakura et al. nur bedingt dazu geeignet sind, mit denen unserer Arbeitsgruppe verglichen zu werden. So wurde durch Itakura et al. das 6-OHDA zur Generierung eines Hemiparkinsonmodels nicht, wie bei uns, in das MVB injiziert, sondern in das CPu. Es ist bekannt, dass Applikationen von 6-OHDA in das CPu zu einem weit weniger vollständigen Untergang dopaminerger Neurone in der SNpc führen als Injektionen in das MVB (Deumens et al., 2002). Auch wurde von dieser Arbeitsgruppe ein anderer Rattenstamm verwendet als von uns. So verwendeten Itakura et al. Sprague Dawley Ratten, während wir an Wistar Ratten geforscht haben. Bei Itakura et al. wurde der erste Rotationstest bereits 7 Tage nach der 6-OHDA-Läsion durchgeführt, während in unserer Arbeitsgruppe der erste Rotationstest einen Monat nach Läsion erfolgte. Itakura et al. injizierten BoNT-A bereits 2 Wochen nach der 6-OHDA-Läsion, während diese Behandlung in unserer Arbeitsgruppe frühestens einem Monat nach der Läsion erfolgte. Die Applikation der Gesamtmenge des BoNT-A erfolgte bei Itakura et al. nur an einer Injektionsstelle im CPu, bei uns jedoch an zwei hintereinander gelegene Koordinaten, um dem großen Volumen dieses Kerngebietes Rechnung zu tragen.

Eine weitere Arbeitsgruppe, die erst kürzlich ihre Ergebnisse zur intrazerebralen BoNT-A-Injektion veröffentlichte, war die um Tsang et al. Hier bestand der experimentelle Ansatz darin, BoNT-A bei hemiparkinsonoiden Ratten in den Entopedunculären Nucleus der Ratte zu injizieren und so eine Verbesserung der Symptomatik zu erzielen (Tsang et al., 2019a, 2019b). Der Entopedunculäre Nucleus (EPN) ist bei der Ratte das Äquivalent des Gpi des Menschen. Hier sollte nicht unsere Arbeitshypothese verfolgt werden und durch BoNT-A die striatale Acetylcholinkonzentration gesenkt werden, um die motorischen Fähigkeiten zu verbessern. Tsang et al. wollten die Glutamat-Ausschüttung von Afferenzen aus dem STN im EPN blockieren. Dadurch sollte einer pathologischen Übererregung des EPN durch den ebenfalls bei Parkinson überaktiven STN entgegengewirkt werden. Man wollte sich den Umstand zu Nutze machen, dass BoNT-A neben Acetylcholin auch die Ausschüttung von Glutamat aus der Präsynapse blockieren kann (Bigalke et al., 1981; Mcmahonss et al., 1992; SANCHEZ-PRIETO et al., 1987). Auch diese Behandlungen wurden von den Versuchsratten gut vertragen. Nach Injektion von BoNT-A in den EPN bei 6-OHDA-hemiläsionierten Tieren wurde ebenfalls eine massive Reduktion einer nach 6-OHDA-Läsion hohen apomorphininduzierten Rotationsrate beobachtet. Des Weiteren hat sich die Schrittweite, die Geschwindigkeit einzelner Schrittbewegungen, die Bewegungsgeschwindigkeit und die Kadenz der Schrittfolge

hemiparkinsonoider Ratten im Verlauf eines Monats gesteigert. Nach drei Monaten kehrten die in motorischen Verhaltenstests gemessenen Parameter wieder auf das Ausgangsniveau vor der BoNT-A-Behandlung zurück. Im BoNT-A-behandelten EPN nahm die Immunreaktivität gegenüber Synaptophysin, einem präsynaptischen Protein und dem Vesikulären Glutamattransporter 2, der auf glutamatergen Präsynapsen verortet ist, ab. Die Immunreaktivität gegenüber Glutamatdecarboxylase 67, einem wichtigen Enzym GABA-erger Neurone, nahm nicht ab. Daraus schlossen die Autoren, dass die GABAergen Präsynapsen nicht beeinträchtigt wurden.

Die Autoren diskutieren richtig, dass die Abnahme der apomorphininduzierten Rotationsrate nicht zu erwarten war, da Apomorphin ein D_2-Rezeptoragonist ist. Der D_2-Rezeptorbesatz ist im dopaminerg deafferentierten CPu erhöht, wodurch Apomorphin hier stärker wirkt und unter Apomorphineinfluss das läsionierte CPu den Gpe weniger hemmt. Der Gpe hemmt seinerseits den STN und den Gpi, was zu einer Enthemmung des Thalamus und einer vermehrten Erregung des motorischen und prämotorischen Kortex und damit zu einem Rotationsverhalten in Richtung der kontralateralen Seite führt. Eine weitere Hemmung des Gpi bzw. des EPN durch BoNT-A müsste zu einer noch stärkeren Enthemmung des Thalamus und zur Generierung weiterer Bewegungsimpulse im motorischen Kortex und zu einer höheren apomorphininduzierten Rotationsrate führen. Das Gegenteil war allerdings auch hier der Fall. Die Autoren spekulieren, dass BoNT-A evtl. retrograd in den Motorkortex transportiert worden ist und hier zu einer Hemmung führt oder dass sich die Basalganglienverschaltung unter BoNT-A-Einfluss auf nicht näher benannte Art reorganisiert hat (Tsang et al., 2019a, 2019b).

7.5 Rezeptordichtenänderung nach BoNT-A-injektion

In den vergangenen Jahren konnten durch unsere Arbeitsgruppe Ergebnisse über die Änderungen von Neurotransmitterrezeptordichten mittels Rezeptorautoradiographie und Positronenemissionstomographie publiziert werden. Hervorzuheben ist, dass eine Injektion von 1 ng BoNT-A in das CPu von gesunden Ratten zu einer Reduktion der Bindung eines D_2-Rezeptorliganden im CPu führt, was einen Hinweis auf eine Reduktion der D_2-Rezeptordichte im behandelten CPu darstellt (T. Mann et al., 2018b). Weiterhin führte eine einseitige 6-OHDA-Läsion, wie erwartet, zu einer erhöhten D_2-Rezeptorligandenbindung, was auf eine kompensatorisch erhöhte D_2-Rezeptorexpression striataler Neurone nach dopaminerger

Deafferentierung zurückzuführen ist. Eine Behandlung dieser hemiparkinsonoiden Ratten mit 1 ng BoNT-A im dopaminerg deafferentierten CPu führte hier zu einer signifikanten Reduktion der pathologisch erhöhten D_2-Rezeptordichte und somit zu einer Wiederangleichung des D_2-Rezeptorbesatzes der Neurone im linken und rechten CPu (T. Mann et al., 2018b, 2018a; Wedekind et al., 2018). Diese Ergebnisse sind bemerkenswert, da sie zum ersten Mal eine hinreichende Antwort auf die Frage geben, warum hemiparkinsonoide Ratten nach zuvor hoher apomorphininduzierter Rotationsrate, in Folge einer intrastriatalen BoNT-A-Behandlung, ein deutlich reduziertes oder gar kein apomorphininduziertes Rotationsverhalten mehr zeigen. Das Wesen des apomorphininduzierten Rotationstestes beruht auf der Imbalance der D_2-Rezeptordichte zwischen dem CPu auf der unläsionierten Seite und dem CPu auf der dopaminerg deafferentierten Seite. Im CPu der 6-OHDA-läsionierten Seite vollzieht sich aufgrund der massiv verringerten Dopaminkonzentration eine kompensatorische Hochregulation der Biosynthese von D_2-Rezeptoren. Aufgrund der nun erhöhten numerischen Dichte von D_2-Rezeptoren auf der Oberfläche von Neuronen des läsionierten CPu's, wirkt Apomorphin nach systemischer Applikation hier stärker. D_2-Rezeptoren sind hauptsächlich auf cholinergen Interneuronen und MSN's des indirekten Weges der Basalganglienschleife lokalisiert. Eine systemische Apomorphingabe führt also zu einer stärkeren Hemmung der MSNs des indirekten Weges der Basalgangleinschleife auf der läsionierten Seite als auf der unläsionierten Seite, so dass der Gpe auf der Seite der 6-OHDA-Läsion weniger stark gehemmt wird und selbst wiederum stärker den Gpi und den STN hemmen kann. Der Gpi hemmt nun weniger stark den Nucleus ventralis anterolateralis, der wiederrum nun verstärkt exzitatorische Signale an den motorischen und prämotorischen Kortex sendet, woraufhin vermehrt Bewegungen auf der kontralateralen Körperhälfte initiiert werden. Eine vermehrte Lokomotion auf der gegenüberliegenden Körperhälfte äußert sich dann in einer stetigen Drehbewegung der Ratte kontralateral zur 6-OHDA-Injektion hin. Im Umkehrschluss heißt dies, dass nach einer Reduktion der pathologisch erhöhten D_2-Rezeptordichte im dopaminerg deafferentierten CPu durch eine BoNT-A-Injektion auf einen Wert, der sich der D_2-Rezeptordichte der kontralateralen Seite annähert, nach systemischer Gabe von Apomorphin, dieses nicht mehr verstärkt in einem der beiden CPus wirken kann. Es kommt nun nicht mehr oder nur noch in abgeschwächter Form zu einer ungleichmäßigen Aktivierung des indirekten Weges der Basalgangleinschleife durch Apomorphin, wodurch die apomorphininduzierte Rotationsrate sinkt.

7.6 Ratten vs. Mäuse - BiVs und SV2-Rezeptoren

Der Charakter, der von uns aufgefunden BiVs im CPu nach BoNT-A-Behandlung, konnte nicht abschließend geklärt werden. Bis auf unsere Arbeitsgruppe, konnten bisher nur in der Arbeit von Berliocchi et al. axonale Aufweitungen nach Kontakt mit einem BoNT beschrieben werden. Allerdings wurde hier BoNT-C zu Nervenzellkulturen der Maus gegeben, woraufhin axonale Schwellungen auftraten, die man als „blebs“ bezeichnet hat (Berliocchi et al., 2005). Auch Schädelhirntraumata induzieren an Axonen lange persistierende Aufweitungen, die als Retraction bulbs oder Retraction balls bezeichnet werden. Verschiedene Arbeitsgruppen (Ertürk et al., 2007; Gennarelli et al., 1982; Marmarou et al., 2005; Vowles et al., 1987) beschrieben solche Phänomene, nach mechanischen Traumata des Gehirns.

Es steht aus, das Wesen der von uns aufgefunden BiVs in der Zellkultur zu untersuchen und elektrophysiologisch zu messen, ob Axone die BiVs ausgebildet haben, noch in der Lage sind, Aktionspotentiale auszubilden und an ihren Endigungen Transmitter auszuschütten.

Momentan gehen wir davon aus, dass es sich bei den BiVs um eine Folge einer Retention von intrazytoplasmatischen Transmittervesikeln handelt, die zwar durch einen anterograden Transport in Richtung Axonendigungen transportiert werden, dort jedoch durch den gestörten Vesikelfusionsapparat nicht entleert werden können, wodurch es zu einem Rückstau kommt. Eine Störung des Vesikelumsatzes durch eine BoNT-A-Behandlung katecholaminerger Neurone konnte die Arbeitsgruppe um Zhou et al. 2018 beschreiben. Eine experimentelle Behandlung mit BoNT-A verringerte eine arterielle Vasokonstriktion. Zhou et al. folgern, dass dies wahrscheinlich auf einer Hemmung der Noradrenalinausschüttung aus sympathischen Nervenfasern zurückzuführen ist. Hinweise auf eine solche Retention von Noradrenalin in präsynaptischen Endigungen konnten Zhou et al. in BoNT-A-behandelten kultivierten sympathischen Ganglienzellen finden, deren Vesikelumsatz nach BoNT-A-Exposition gestört war. Tatsächlich konnte die Arbeitsgruppe im Medium kultivierter sympathischer Ganglienzellen eine signifikant verringerte Noradrenalinkonzentration messen. Immunhistochemisch konnte nachgewiesen werden, dass diese Zellen SV2C-positiv waren (Zhou et al., 2018). SV2C ist ein Protein auf der präsynaptischen Membran, das die vornehmliche Bindungsstelle für BoNT-A darstellt.

BoNT-A bindet nicht ausschließlich an SV2C, sondern auch im geringeren Maße an die Subtypen SV2A und SV2B (Lam et al., 2015; Rummel, 2015). Gerade SV2C ist im Gehirn

unterschiedlicher Spezies auch unterschiedlich auf den Axonen der verschiedenen Neuronentypen verteilt (Crèvecœur et al., 2013; Davies et al., 2018; Dunn et al., 2018; Janz et al., 1999; Mahrhold et al., 2006). Dieser speziesspezifische, kerngebietsspezifische und Neuronentypus-spezifische unterschiedliche Besatz mit SV2C, aber auch SV2A und SV2B kann eine Erklärung für die Unterschiede in der Ausbildung von TH-positiven BiVs nach BoNT-A-Injektion in das CPu sein. So ist es möglich, dass SV2C in den Neuronen der SNpc von Ratte und Maus unterschiedlich stark exprimiert wird (Dunn et al., 2018, 2017; Hu et al., 2017; Stout et al., 2019). Fehlt SV2C auf der präsynaptischen Membran, kann BoNT-A in das entsprechende Axon nicht internalisiert werden und die Fusion der Transmittervesikel mit der präsynaptischen Membran nicht stören.

Die Arbeitsgruppe um Dunn et al. (Dunn et al., 2017) konnte immunhistochemisch nachweisen, dass die Verteilung von SV2C sowohl in den Gehirnen von MP-Patienten als auch von genetisch veränderten Mäusen die α-Synuclein überexprimieren verändert ist und SV2C im CPu nicht mehr gleichmäßig verteilt, sondern punktuell aggregiert ist. Eine solche Alteration der α-Synuclein-Expression kann unter Umständen eine potentielle Therapie des IPS mittels BoNT-A beeinträchtigen, da SV2C die Hauptbindungsstelle des BoNT-A an der präsynaptischen Membran ist.

7.7 BoNT-A-Dosis

Es ist bemerkenswert, dass die genutzten Dosen an BoNT-A in diesen Arbeiten die LD50 von BoNT-A für Ratten und Mäuse überschritt. So wird die LD50 für Mäuse mit 0,25 bis 1,15 ng/ kg KG angegeben (Pirazzini et al., 2017). Da die Mäuse in unseren Arbeiten mit einem KG von ca. 20 g mit BoNT-A injiziert worden sind, ergibt sich für diese Tiere eine theoretische LD50 von 5 pg bis zu 23 pg. Die Ratten wogen zum Zeitpunkt der stereotaktischen BoNT-A-Applikation 300 g. Extrapoliert man die LD50 der Mäuse auf das Gewicht von Ratten, ergibt das eine LD50 von 75 pg bis zu 345 pg. Die Mäuse in unseren Arbeiten wurden allerdings mit Dosen von 20 pg bis zu 200 pg behandelt und die Ratten dieser Arbeiten erhielten Dosen von 1 ng. In Arbeiten zuvor wurden sogar 2 ng je CPu und 5 ng je CPu injiziert, wobei lediglich die Dosis von 5 ng je CPu zu einer erhöhten Mortalität führte (Antipova et al., 2013; Wree et al., 2011).

Hier muss bedacht werden, dass für Toxizitätsstudien und Mausbioassays die jeweiligen BoNT-Lösungen intraperitoneal injiziert werden. Durch die intraperitoneale Gabe erfolgt eine sehr rasche Aufnahme des BoNTs in den Blutkreislauf durch das Omentum majus und das Peritoneum. Durch die intraperitoneale Injektion wirkt BoNT-A also systemisch und führt so zu einer Lähmung der Skelettmuskulatur. Insbesondere die Lähmung der Atemmuskulatur führt dann zum Tod.

Da in unseren Arbeiten das BoNT-A jedoch in die Tiefe des Hirnparenchyms verabreicht worden ist, ist es wahrscheinlich, dass es dort nicht hinausdiffundieren konnte. Insbesondere die Blut-Hirn-Schranke hat womöglich einen Übertritt von BoNT-A vom Gehirn in das Blut erschwert.

7.8 Perspektivische Ansätze zur Untersuchung der intrazerebralen BoNT-A-Wirkung und mögliche weitere therapeutische Anwendungen der intrazerebralen BoNT-Injektionen

Die Verwendung von Donepezil als theoretischen Antagonisten einer intrazerbralen BoNT-A-Wirkung zum einfachen Nachweis eines anticholinergen Effektes der intrastriatalen BoNT-A-Injektion hat sich als nicht zielführend erwiesen. Zu bedenken ist hierbei, dass Donepezil, als AChE-Hemmer der Wirkungsweise der meisten Nervenkampfstoffe (VX, Tabun, Soman, Sarin…) und einiger Pestizide (Parathion, Malathion, Disulfoton) homolog ist. Zwar überwiegen beim Menschen innerhalb der therapeutischen Dosis die zentralnervösen Wirkungen des Donepezils, allerdings kann nicht ausgeschlossen werden, dass bei der Ratte aufgrund von Interspeziesunterschieden, das Verhältnis von zentralnervöser AChE-Hemmung und peripherer AChE-Hemmung, die dem Großteil der Nebenwirkungen bzw. der Toxizität zugrunde liegt, anders gelagert ist, als beim Menschen.

Prinzipiell lässt sich in Zukunft die Frage welche Transmittersysteme durch BoNT-A beeinflusst werden, nur mittels Mikrodialyse im CPu und nachgeschalteter Analytik der Transmitterkonzentrationen der Dialysate durch eine Hochleistungsflüssigkeitschromatographie (HPLC) beantworten. Weiterhin müssen elektrophysiologische Messungen in den einzelnen Kerngebieten durchgeführt werden und dies möglichst noch auf neuronenspezifischer Ebene (Patch Clamp an Nervenzell- und/ oder Lebendhirnschnittkulturen der einzelnen Kerngebiete), um Aussagen treffen zu können, ob die

Aktivität der einzelnen Kerngebiete als auch die jeweils inhärenten Nervenzelltypen die Aktivitätsänderungen zeigen, die durch unsere Arbeitshypothese proklamiert worden sind.

Weiterhin sind Studien mittels immunhistochemischen Markierungen und molekularbiologischen Methoden zur Untersuchung der Expression von SV2C, aber auch von SV2A und SV2B als Bindungsstellen von BoNT-A für Ratte und Maus in den verschiedenen Stationen der Basalganglienschleifen notwendig, um Aussagen über die teils divergierenden Wirkungen von BoNT-A bei Ratte und Maus tätigen zu können. Aus denselben Gründen wären langfristig die Wiederholungen der rezeptorautoradiographischen Studien, die bei der Ratte getätigt worden sind, auch für die Maus sinnvoll (T. Mann et al., 2018; Teresa Mann et al., 2018; Wedekind et al., 2017).

In der Vergangenheit wurde der Nachweis durch mehrere Arbeitsgruppen erbracht, dass BoNTs dazu in der Lage sind, mittel- bis langfristig nicht nur die Ausschüttung an ACh zu unterbrechen, sondern auch die Freisetzung an Dopamin und Glutamat zu blockieren. Einer erhöhten Konzentration dieser Transmitter bzw. einer gesteigerten Empfindlichkeit gegenüber Dopamin und Glutamat schreibt man in der Fachwelt eine ursächliche Rolle bei der Schizophrenie zu (Leucht et al., 2009; Miyamoto et al., 2012). Vor diesem Hintergrund ist die experimentelle Anwendung eines BoNTs z.B. im Ncl. Accumbens, im ventralen CPu oder direkt im ventralen tegmentalen Areal im Tiermodel der Schizophrenie in Erwägung zu ziehen. Eine Verringerung der Konzentration an erregenden Transmittern in beteiligten Strukturen des limbischen Systems ist unter Umständen dazu in der Lage, die Symptomatik des Wahns über eine längere Zeit zu verringern, ohne dass regelmäßige Einnahmen von Neuroleptika nötig wären. Auf diese Weise würden auch die systemischen Nebenwirkungen dieser Medikamente im besten Fall umgangen, da hier die Transmitterausschüttung nur lokal unterbrochen wird.

8. Literatur

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9. Anlagen

9.1 Originalarbeiten

BRAIN RESEARCH 1630 (2016) 18–24

Available online at www.sciencedirect.com

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Research Report

Intrastriatal injection of botulinum neurotoxin-A is not cytotoxic in rat brain – A histological and stereological analysis

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ARTICLE INFO

Article history:
Accepted 31 October 2015
Available online 10 November 2015

Keywords:
Botulinum neurotoxin A
Striatum
Basal ganglia
Unbiased stereology
Rat brain

ABSTRACT

Parkinson's disease (PD) is caused by progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta, resulting in a deficiency of dopamine in the striatum and an increased release of acetylcholine by tonically active interneurons. Botulinum neurotoxin-A (BoNT-A) is well known for blocking transmitter release by cholinergic presynaptic terminals.

Treating striatal hypercholinism by local application of BoNT-A could be a possible new local therapy option of PD. In previous studies of our group, we analyzed the effect of BoNT-A injection into the CPu of 6-OHDA lesioned hemiparkinsonian rats. Our studies showed that BoNT-A application in hemiparkinson rat model is capable of abolishing apomorphine induced rotations for approximately 3 months. Regularly occurring axonal swellings in the BoNT-A infiltrated striata were also discovered, which we named BoNT-A induced varicosities (BiVs).

Résumé: Here we investigated the long-term effect of the injection of 1 ng BoNT-A into the right CPu of naive Wistar rats on the number of ChAT-ir interneurons as well as on the numeric density and the volumetric size of the BiVs in the CPu. Significant differences in the number of ChAT-ir neurons between the right BoNT-A treated CPu and the left untreated CPu were not detected up to 12 month post BoNT-A injection. The numeric density of BiVs in the treated CPu reached a maximum 3 months after BoNT-A treatment and decreased afterwards, whereas the volume of single BiVs increased steadily throughout the whole time course of the experiment.

Abbreviations: Ach, acetylcholine; BiVs, BoNT-A-induced varicosities; BoNT-A, botulinum neurotoxin-A; ChAT, choline acetyltransferase; CPu, caudatus putamen complex; ir, Immunoreactive; L-DOPA, L-3,4-Dihydroxyphenylalanine; PD, Parkinson's disease; SNAP-25, synaptosomal-associated protein-25; SNARE, N-ethylmaleimide-sensitive-factor attachment receptor; SNC, substantia nigra pars compacta; TH, tyrosine hydroxylase.

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http://dx.doi.org/10.1016/j.brainres.2015.10.056
0006-8993/

1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative diseases (de Lau and Breteler, 2006). Parkinsonism is caused by progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNC) which results in a deficiency of dopamine mainly in the striatum. This leads to a cascade of functional changes in basal ganglia circuitry and an increased release of acetylcholine (ACh) by tonically active interneurons in the caudate putamen complex (CPu) (Coffield and Yan 2009; Pisani et al., 2007).

Down to the present day a couple of therapy options of PD symptoms were developed. The substitution of the absent dopamine occurs by administration of L-3,4-Dihydroxyphenylalanine (L-DOPA) (Carlsson et al., 1957; Playfer, 1997; Reichman, 2015). This method is initially tolerated well and shows a high efficacy, but in the course of several years L-DOPA treated patients exhibit increasingly side effects like dyskinesias and dystonias. In addition there exist hinds for a neurotoxic effect of L-DOPA itself (Olanow and Obeso, 2011; Stansley and Yamamoto, 2013).

Treatments with catechol-O-methyltransferase inhibitors and monomanine oxidase inhibitors, which are mostly combined with L-DOPA-treatment, should enhance the available amount of dopamine in the brain by inhibition of catecholamine degradation. Because of their systemic action catechol-O-methyltransferase inhibitors and monoamine oxidase inhibitors show several side effects like hypertension, dizziness, vertigo, nausea, hepatotoxicity hallucination and headache (Connolly and Lang, 2014).

Bradykinesia, akinesia and tremor in PD can be treated neurosurgically by lesion of the medial pallidum or the specific regions of the thalamus. These procedures are irreversible and may include side effects like cognitive impairments, paresthesia, head pain and nausea (Tarazi et al., 2014). Since two decades good results in PD treatment were achieved with the deep brain stimulation, nevertheless also here severe side effects and complications like seizures, anxiety, depressions, manias or changes of the personality occur. Furthermore there are a number of exclusion cirteria for the treatment with deep brain stimulation (Reichman, 2015; Tarazi et al., 2014; Umemura et al., 2003; Weaver et al., 2009).

Transplantations of catecholaminergic cells in PD patients were performed experimentally since 20 years. In case of transplantation of human fetal mesencephalic cells there are ethical problems of acquisition and dispensation of potential transplants. Remarkably it is shown in post mortem analyses that fetal implants in PD patients brains developed also PD characteristic neurodegenerative phenomena (Angot and Brundin, 2009; Angot et al., 2010; Frost and Diamond, 2010; Olanow and Prusiner, 2009; Tarazi et al., 2014).

Beside the application of L-DOPA, one of the first pharmacological interventions in the history of PD therapy was the administration of central acting anticholinergic substances like Akineton®. This form of treatment shall counteract to a striatal hypercholinism caused by overactive cholinergic interneurons in the CPu in PD. The anticholinergic therapy shows good results in the reduction of rigidity, bradykinesia and akinesia. But due to the fact that these substances act systemically, there are a lot of central and peripheral side effects, like mydriasis, accomodation problems, mouth dryness and inflammations of the salivary glands, dry eyes, muscle pain, loss of strength, dysphagia, regurgitation, urinary retention, tachycardia, fever, memory disorders (Clarke, 2002; Whitney, 2007). Moreover systemic anticholinergic therapy increases PD-related hypocholinism in the Nucleus basalis of Meynert and the Pedunculopontine nucleus which worsens symptoms like dementia and gait disturbances (Bohnen and Albin, 2011; Emre et al., 2004).

Our intention was to avoid these side effects of the classical anticholinergic therapy by direct and local disruption of the transmitter release of overactive cholinergic interneurons in dopminergic deafferentiated CPu's by injection of Botulinum neurotoxin-A (BoNT-A) directly into the CPu. Thereby we wanted to circumvent unwanted anticholinergic actions in the periphery and other brain areas except the CPu. BoNT-A binds on presynaptic terminals and blocks the release of ACh by enzymatic cleavage of synaptosomal-associated protein-25 (SNAP-25) (Coffield et al., 1994; Restani et al., 2012; Rossetto et al., 2015). Due to the fact that it is well known that BoNT-A action is limited to several month this method would not be irreversible like neurosurgical lesions and does not prohibit the switch to other therapy strategies.

Here we wanted to decrease the pathological increased ACh level in the CPu (Coffield and Yan 2009) of hemiparkinsonian rats by intrastriatal application of BoNT-A (Pisani et al., 2007; Wree et al., 2011) in order to investigate the therapeutic potential of intrastriatal BoNT-A injection regarding the treatment of motor symptoms. In earlier experiments we could show that intrastriatally applied BoNT-A at doses of 1 ng in the 6-OHDA rat hemiparkinson model (Ungerstedt, 1968) reverses the rotation behavior in the apomorphine rotation test, for a period of at least 3 months (Antipova et al., 2013; Wree et al., 2011). Furthermore the intrastriatal application of BoNT-A leads to axonal swellings which we named botulinum toxin-induced varicosities (BiVs). Those BiVs are immunoreactive (ir) either for choline acetyltransferase (ChAT) or for tyrosine hydroxylase (TH) (Wree et al., 2011).

We here investigate whether the intrastriatal application of BoNT-A has a long term cytotoxic effect on cholinergic interneurons within the CPu to clarify the therapeutic potential of intrastriatal BoNT-A application. In addition we quantified the previously described BiVs (Wree et al., 2011) with respect to number and size in a time dependent manner up to 12 month following BoNT-A injection (Fig. 1 A-L). For that purpose naïve rats were treated with 1 ng BoNT-A, which previously turned out to be an effective dose (Wree et al., 2011).

2. Results

2.1. *Number of ChAT-ir neurons*

We measured the number of ChAT-ir neurons (Fig. 1 A-F; Fig. 2 A) at 6 time points (14 days, 1, 3, 6, 9 and 12 months)

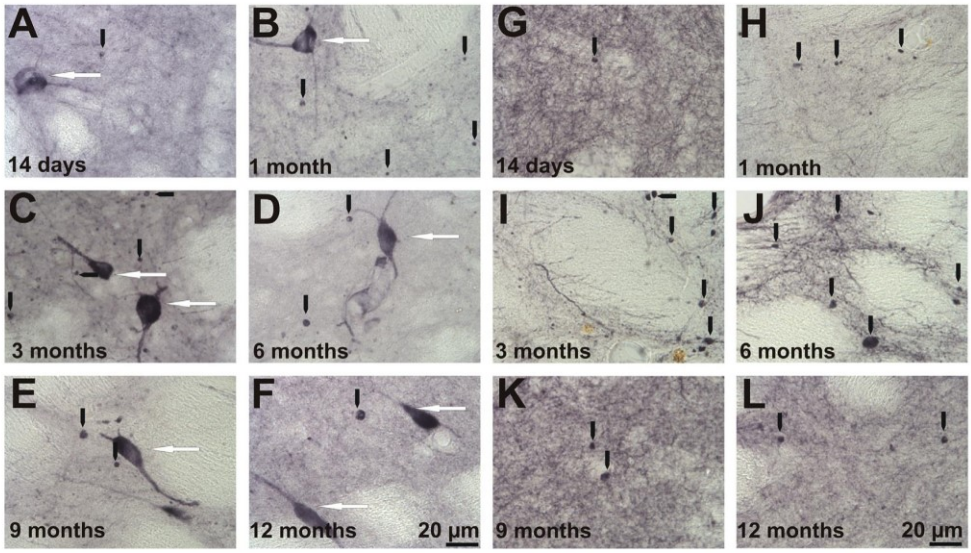

Fig. 1 – ChAT and TH-ir BiVs in temporal progress. (A–F) Immunohistochemical stainings for ChAT: cholinergic interneurons are marked by white arrows and ChAT-ir varicosities by black peaks. (G–L) Immunohistochemical stainings for TH: TH-ir varicosities in the BoNT-A treated CPu are clearly visible and marked by black peaks.

after BoNT-A application. For each animal we counted the ChAT-ir neurons of the right and left CPu. Approximately 30,000 cholinergic neurons were found per rat CPu. No significant differences in the number of cholinergic interneurons could be detected between the right (BoNT-A injected) and left (contralateral and untreated) CPu over the whole time span of the experiment (Fig. 2 A).

2.2. *Botulinum neurotoxin-A induced varicosities (BiVs)*

2.2.1. *Numeric density of ChAT-ir BiVs decreases over time (Fig. 2B)*

Two weeks after intrastriatal injection of BoNT-A we counted 11,582±387 ChAT-ir varicosities per mm^3 (mean±SE). One year after BoNT-injection, the numeric density decreased significantly to 9085±611 varicosities per mm^3 (Pearson product-moment correlation coefficient $r=-0.778$) (Fig. 2 B).

2.2.2. *Volume of ChAT-ir BiVs increases over time (Figs. 1A–F, 2C and 3A)*

The mean volume of single ChAT-ir BiVs increased during the course of one year significantly, from 18.96±1.29 μm^3 two weeks after the injection of BoNT-A into the striatum up to averaging 51.49±2.51 μm^3 one year after BoNT-A application (Figs. 1A–F and 2C). In the histogram of Fig. 3A it is clearly recognizable, that the rate of single BiVs with small volumes up to 20 μm^3 decreases whereas BiVs with large volumes (25 μm^3 and more) increases over time.

2.2.3. *Numeric density of TH-ir BiVs decreases over time (Figs. 1G–L; 2 D)*

Also the numeric density of TH-ir BiVs decreased steadily from 47,125±2015 per mm^3 14 days after injection of BoNT-A to 21,445±2939 per mm^3 12 month after injection. The measured changes were significant (Fig. 2 D).

2.2.4. *Volume of TH-ir BiVs increases over time (Figs. 1G–L; 2 E; 3 B)*

The volume of single TH-ir BiVs also increased significantly during one year after intrastriatal BoNT-A application (Pearson product-moment correlation coefficient $r=0.718$). We measured a mean initial volume of 15.35±0.98 μm^3 of TH-ir BiVs 14 days after intrastriatal injection of BoNT-A, and 12 month after injection the mean volume of single TH-ir BiVs was 35.97±4.28 μm^3 (Figs. 1 G-L; 2 E; 3 B).

3. Discussion

3.1. *Number of ChAT-ir neurons*

Our analysis showed that there is no loss of cholinergic interneurons in the CPu even one year after intrastriatal BoNT-A application. These findings extend previous results where the total number of all neurons in the CPu of rats (about 3,000,000 per hemisphere) after BoNT-A application was determined, and where overall no neuronal loss was observed (Antipova et al., 2013). Previous publications pointed out that cholinergic interneurons have a stake of 1–3% of the total number of neurons (Bertran-Gonzalez et al., 2012; Goldberg and Reynolds, 2011). Actually we found the same ratio of cholinergic neurons referring to the total number of rat striatal neurons known from the literature (Antipova et al., 2013; Oorschot, 1996). Both the stable numbers of all striatal neurons and especially the cholinergic interneurons speak in favor of a lack of cytotoxic effects of the injection of

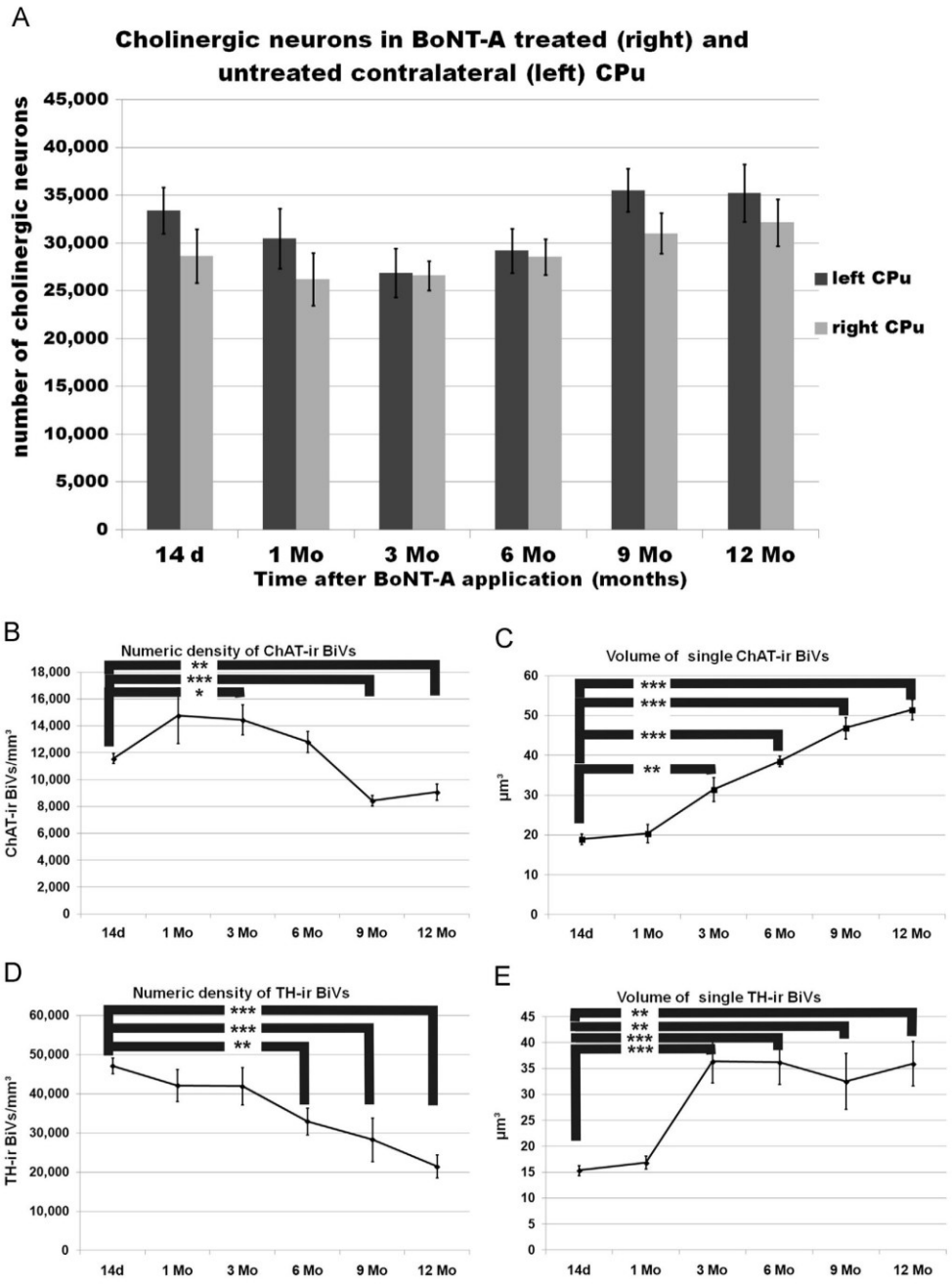

Fig. 2 – Analysis of cholinergic interneurons and numeric density of BiVs as well as single BiV volume. (A) Number of ChAT-ir neurons. Significant differences in the number of ChAT-ir neurons between the right BoNT-A treated CPu and the left untreated CPu were not detected at each time point of examination. (B) and (C) Analysis of TH-ir BiVs: The numeric density of TH-ir BiVs decreases continuously over the period of observation (A), whereas the mean volume of each single BiV increases over time significantly (B). (D) and (E) Analysis of ChAT-ir BiVs: Also the number of ChAT-ir BiVs decreases during one year after BoNT-A application, whereas the volume of single BiVs increases significantly. Asterisks of significance always refer to a comparison the data measured 14 d after operation.

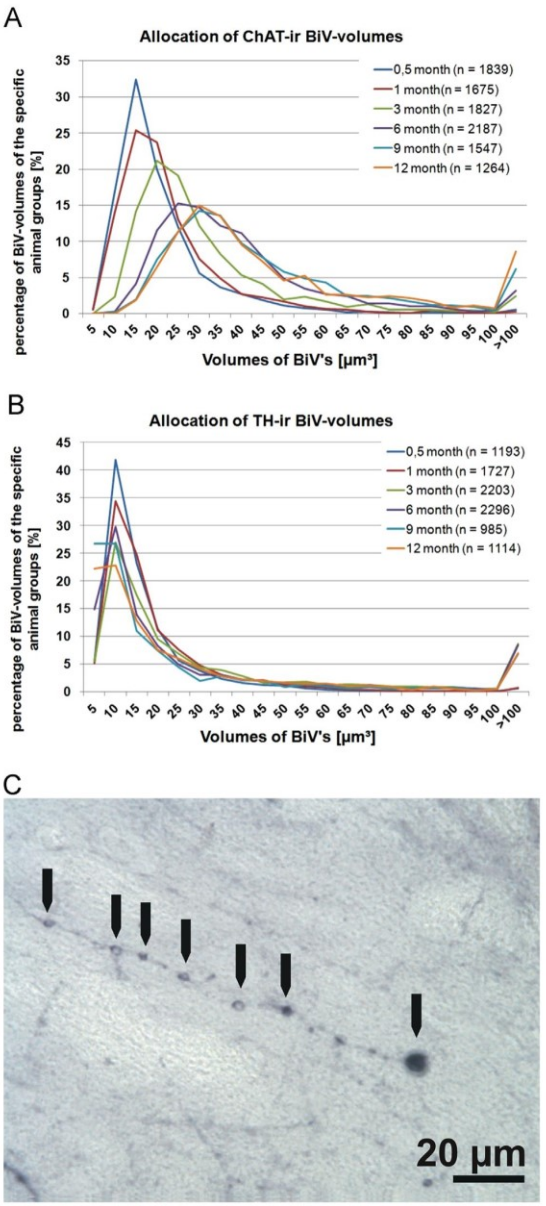

Fig. 3 – Histograms of BiV-volume distribution.
(A) Histogram of ChAT positive BiV-volumes. Percentage of distinct single BiV-volumes of the entirety (*n*) of measured BiVs per time group. For acquisition of the allocation volume categories in 5 µm³ steps were created. (B) Histogram of TH-ir BiV-volumes. Percentage of distinct single BiV volumes of the entirety (*n*) of measured BiVs per time group. For acquisition of the allocation volume categories in 5 µm³ steps were created. (C) Bead chain shaped stringing of ChAT-positive BiVs. The arrows mark ChAT positive BiVs.

1 ng BoNT-A. Thus intrastriatal BoNT-A injection could theoretically be eligible for therapeutically use in PD.

3.2. *Numeric density and volume of the BiVs*

TH-ir and ChAT-ir BiVs in BoNT-A treated striata of rats were described by our group for the first time (Wree et al., 2011). Heretofore of all publications which are concerned with the injection of BoNT into the CNS (Antonucci et al., 2008, 2009, 2010; Costantin et al., 2005; Gasior et al., 2013; Hagenah et al., 1977; Itakura et al., 2014a, 2014b; Lacković et al., 2009; Luvisetto et al., 2003) our work group is the only one which described swellings on cholinergic and catecholaminergic nerve fibers. Only Berliocchi et al. (2005) described the appearance of axonal varicosities 12 h after exposure of cultured cerebellar granule cells to BoNT-C.

In BoNT-A injected striata, BiVs were constantly found along neuronal branches. Sometimes BiVs were organized as a stringed bead chain (Fig. 3C). BiVs were found exclusively in BoNT-A treated striata and never in the respective contralateral hemispheres, nor in control animals injected with vehicle substance. BoNT-A binds at its target cell via its heavy chain, utilizing both, specific gangliosides and the synaptic vesicle glycoprotein 2 as receptors on presynaptic membranes (Mahrhold et al., 2006). Once attached, receptor-mediated endocytosis carries the toxin molecule into the cell. During this phase, acidification of the endocytotic vesicles leads to a cleavage of the light chain from the heavy chain by eliminating electrostatic interactions, partially unfolding the light chain and reduction of the interconnecting disulfide bond, leading to a release of the light chain into the cytosol. The light chain acts as a zinc dependent endopeptidase, cleaving the SNARE-protein SNAP-25 (Brunger and Rummel, 2009). Cleaving of SNAP25 inhibits the exocytosis of neurotransmitter-containing synaptic vesicles. We suppose that after BoNT-A administration transmitter vesicles accumulate presynaptically and eventually merge to bigger inclusions resulting in the formation of BiVs. Following our observations, this process could progress over time and may lead to increasing volumes of ChAT-ir as well as TH-ir BiVs. A possible explanation for the decreasing numeric density of both types of BiVs over a period of 12 months might be, that it is more probable for vesicles, i.e. BiVs to merge as their volume increases due to geometric parameters such as distance, curvature and surface tension (Zimmerberg and Chernomordik, 1999). This could lead to the observed decreasing numeric densities and the increasing volumes of TH-ir and ChAT-ir BiVs over months.

Overall a long-lasting and well-tolerated effect of the application of BoNT-A can be seen, which in the case of the 6-OHDA rat model leads to a reduction of apomorphine induced rotation behavior (Hawlitschka et al., 2013; Holzmann et al., 2012; Wree et al., 2011). Interestingly, as seen from the presence of ChAT-ir and TH-ir BiVs in naive rats, which were treated intrastriatally with BoNT-A, BoNT-A does not only effect the cholinergic presynaptic terminals but also influences the dopaminergic

axons. To what extend this effect is concentration dependent and may affect or even counteract a potential positive action on parkinson's symptoms is currently investigated by further experiments.

4. Experimental procedure

4.1. Animals

Young (3 months old), male Wistar rats, were obtained from Charles River WIGA (Sulzfeld, Germany). Animals were housed temperature-controlled (22±2 °C) under a 12-h light/12-h dark cycle. The rats had free access to food and water and were treated according to legal obligations for animal welfare at any time.

4.2. Intrastriatal injection

Animals weighted 270–320 g at the time point of surgery. The rats were anesthetized with a mixture of ketamine (50 mg/kg body weight) and xylazine (4 mg/kg body weight) and received two injections of 1 μl each with a total dose of 1 ng BoNT-A (lot No. 13028A1A; List, Campbell, CA; purchased via Quadratech, Surrey, UK) solved in phosphate-buffered saline (PBS) sublimented with 0.1% bovine serum albumin. The solution was injected into the right striatum into two injection sides at the following coordinates in relation to Bregma ap +1.3 mm and –0.4 mm, l −2.6 mm and –3.6 mm, v –-5.5 mm and –5.5 mm.

4.3. Histochemistry

4.3.1. Fixation

For the acquisition of the rat brains, animals were killed with an overdose of ketamine and xylazine and perfused with 3.7% paraformaldehyd solved in phosphate-puffered saline (pH 7.4). Brains were removed out of the skull, postfixed in 3.7% paraformaldehyd, cryprotected and frozen at −80 °C. Frontal 30 μm thick brain slices were made by serial cryo-cutting.

4.3.2. Stainings

In order to evaluate the BiVs as well as the ChAT-ir neurons of the striatum, 30 μm thick brain sections were stained for TH or ChAT. Staining of cholinergic structures occurred by incubation of a polyclonal biotinylated goat anti-ChAT affinity purified antibody (Millipore, Schalbach, Germany, 1:200), followed by incubation of rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA, 1:67). Staining of catecholaminergic structures occurred by incubation of a monoclonal mouse anti-TH antibody (clone TH2, Sigma-Aldrich, St. Louis, MO, USA) followed by incubation of biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, 1:67). The methods of staining have previously been described by Wree et al. (2011).

4.4. Stereological analysis

The immunohistochemically stained brain slices were examined stereologically using the program Stereo Investigator 8.0. The cholinergic cells and the numeric density of BiVs where calculated by unbiased counting by means of the optical fractionator method.

4.5. Statistical analysis

For stereology, a one-way ANOVA was used to analyze the morphometric quantities with the post hoc Bonferroni and Student–Newman–Keuls tests. $P<0.05$ was considered statistically significant. The mean coefficient of error of optical fractionator estimates was calculated according to the method of Gundersen and Jensen (1987) and was <0.05 in all analyses.

Acknowledgments

We gratefully acknowledge Mrs. Frauke Winzer for her excellent technical assistance.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 21 June 2017
doi: 10.3389/fnbeh.2017.00119

Botulinum Neurotoxin A Injected Ipsilaterally or Contralaterally into the Striatum in the Rat 6-OHDA Model of Unilateral Parkinson's Disease Differently Affects Behavior

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Parkinson's disease (PD) is one of the most frequent neurodegenerative disorders. The loss of dopaminergic neurons in the substantia nigra leads to a disinhibition of cholinergic interneurons in the striatum. Pharmacotherapeutical strategies of PD-related hypercholinism have numerous adverse side effects. We previously showed that ipsilateral intrastriatal injections of 1 ng in unilaterally 6-hydroxydopamine (6-OHDA)-lesioned rats inhibit apomorphine-induced rotation behavior significantly up to 6 months. In this study, we extended the behavioral testing of ipsilateral botulinum neurotoxin A (BoNT-A)-injection and additionally investigated the impact of intrastriatal BoNT-A-injections contralateral to the 6-OHDA-lesioned hemisphere on the basal ganglia circuity and motor functions. We hypothesized that the interhemispheric differences of acetylcholine (ACh) concentration seen in unilateral hemi-PD should be differentially and temporally influenced by the ipsilateral or contralateral injection of BoNT-A. Hemi-PD rats were injected with 1 ng BoNT-A or vehicle substance into either the ipsilateral or contralateral striatum 6 weeks after 6-OHDA-lesion and various behaviors were tested. In hemi-PD rats intrastriatal ipsilateral BoNT-A-injections significantly reduced apomorphine-induced rotations and increased amphetamine-induced rotations, but showed no significant improvement of forelimb usage and akinesia, lateralized sensorimotor integration and also no effect on spontaneous locomotor activity. However, intrastriatal BoNT-A-injections contralateral to the lesion led to a significant increase of the apomorphine-induced turning rate only 2 weeks after the treatment. The apomorphine-induced rotation rate decreases thereafter to a value below the initial rotation rate. Amphetamine-induced rotations were not significantly changed after BoNT-A-application in comparison to sham-treated animals. Forelimb usage was temporally improved by contralateral BoNT-A-injection at 2 weeks after BoNT-A. Akinesia and lateralized sensorimotor integration were also improved, but contralateral BoNT-A-injection had no significant effect on spontaneous locomotor activity. These long-ranging and different effects suggest that intrastriatally applied BoNT-A acts not only as an inhibitor of ACh release but also has long-lasting impact on transmitter expression

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Edited by:
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Received: *03 February 2017*
Accepted: *06 June 2017*
Published: *21 June 2017*

Citation:
Antipova VA, Holzmann C, Schmitt O, Wree A and Hawlitschka A (2017) Botulinum Neurotoxin A Injected Ipsilaterally or Contralaterally into the Striatum in the Rat 6-OHDA Model of Unilateral Parkinson's Disease Differently Affects Behavior. Front. Behav. Neurosci. 11:119. doi: 10.3389/fnbeh.2017.00119

and thereby on the basal ganglia circuitry. Evaluation of changes of transmitter receptors is subject of ongoing studies of our group.

Keywords: botulinum toxins, hemiparkinsonism, behavior, Wistar rats, striatum

INTRODUCTION

Parkinson's disease (PD) is one of the most prevalent debilitating chronic progressive neurodegenerative movement disorders and mainly caused by degeneration of dopaminergic neurons especially in the substantia nigra pars compacta (SNC; Hornykiewicz and Kish, 1987; Braak et al., 2004). This results in a deficit of striatal dopamine (DA) that leads to the impairment in cortico-striatal-thalamo-cortical or nigrostriatal pathways (Bagga et al., 2016; Inan et al., 2016; Kim et al., 2016) and is responsible for the major motor symptoms of PD, including muscular rigidity, resting tremor, bradykinesia and postural instability (Dauer and Przedborski, 2003; Kortekaas et al., 2005; Ren et al., 2016), and non-motor disturbances (Bargiotas and Konitsiotis, 2013).

In the striatum the decrease of DA is followed by an increase in the concentration of acetylcholine (ACh) released from disinhibited tonically active cholinergic striatal interneurons (Gerfen, 1994; Day et al., 2006; Pisani et al., 2007; Obeso et al., 2008b). Therefore, one possible therapeutic approach in PD is using anti-cholinergic drugs (Klockgether, 2003; Horstink et al., 2006). However, systemic application of anti-cholinergics has some peripheral and central side effects (Clarke, 2002; Fernandez, 2012; Connolly and Lang, 2014). To avoid these undesirable effects connected with systematic administration of anti-cholinergic drugs, we tested a local anti-cholinergic treatment by injecting botulinum neurotoxin A (BoNT-A) directly into the caudate putamen (CPu; Wree et al., 2011; Holzmann et al., 2012; Antipova et al., 2013; Hawlitschka et al., 2013; Mehlan et al., 2016) as a possible therapeutic option in experimental PD-model. In hemi-PD rats established by unilateral injection of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (MFB; Ungerstedt, 1968; Ungerstedt and Arbuthnott, 1970; Meredith et al., 2008), intraperitoneally applied atropine antagonized profound PD-typical akinesia (Schallert et al., 1978) and in combination with L-DOPA suppressed pathological circling of hemi-PD rats (Schallert et al., 1979). In our previous studies (Wree et al., 2011) application of BoNT-A into the CPu ipsilateral to the dopaminergic depletion caused a long-term abolition of the pathological apomorphine-induced rotations. We hypothesized that due to the BoNT-A-application into the hypo-dopaminergic and hyper-cholinergic striatum of hemi-PD rats the cholinergic transmission is blocked thus leading to reduced pathological compensatory effects in this model.

In order to test our hypothesis that intrastriatal BoNT-A-application interferes with the 6-OHDA-induced hypercholinism (DeBoer et al., 1993) and/or the D_2-receptor upregulation (Creese et al., 1977) in the CPu, we injected in the present study the "effective" dose of BoNT-A (Wree et al., 2011) either into the CPu ipsilateral or contralateral to the dopaminergic deprivation. Here we show that at least some of the behavioral effects seen after ipsilateral BoNT-A-applications in hemi-PD rats should be reversed or even changed to the opposite by contralateral BoNT-A-injection in hemi-PD rats.

MATERIALS AND METHODS

Animals

Adult male Wistar rats, purchased at Charles River WIGA (Sulzfeld, Germany) and weighing 290–310 g at the time of first surgery were used. Rats were housed in standard cages at 22 ± 2°C under 12 h light/12 h dark cycle with free access of standard food and water. At the end of the experiments mean body weights were as follows: ipsilateral BoNT-A group 559.4 g ± 20.0; ipsilateral sham group 558.4 g ± 23.71; contralateral BoNT-A group 558.5 g ± 16.16; contralateral sham group 555.3 g ± 17.52.

Stereotactic Intervention of Animal Groups

All animals got a 6-OHDA-injection into the MFB of the right hemisphere (hemi-PD), and the successfully lesioned rats were divided into two groups: (1) 6-OHDA-lesioned animals receiving BoNT-A into the CPu of the right hemisphere (ipsilateral BoNT-A group); and (2) 6-OHDA-lesioned animals receiving BoNT-A into the CPu of the left hemisphere (contralateral BoNT-A group), each group added with respectively sham-injected animals. All groups were created by the outcome of the apomorphine-induced rotational behavior after 6-OHDA in that the means of the BoNT-A-injected and the vehicle-injected rats inside (1) and (2) did not differ significantly. All experiments were approved by the State Animal Research Committee of Mecklenburg-Western Pomerania (LALLF M-V/TSD/7221.3-1.1-003/13).

Surgery was conducted under aseptic conditions under ketamine (50 mg/kg body weight)/xylazine (4 mg/kg body weight) anesthesia using a stereotactic frame (David Kopf Instruments). For nearly complete lesion of the right side substantia nigra compact part 4 µl 6-OHDA solution (24 µg, Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1 M citrate buffer was injected over 4 min via a 26 gauge 5 µl Hamilton syringe into the MFB. The injection 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; **Figures 1A,B**). The success of the lesion was evaluated with apomorphine-induced rotations 1 month after surgery. All animals displayed more than four contralateral rotations/min, indicating a unilateral death of about 97% of the nigrostriatal DAergic neurons (Ungerstedt and Arbuthnott, 1970) and, therefore, were tested further. As reported previously

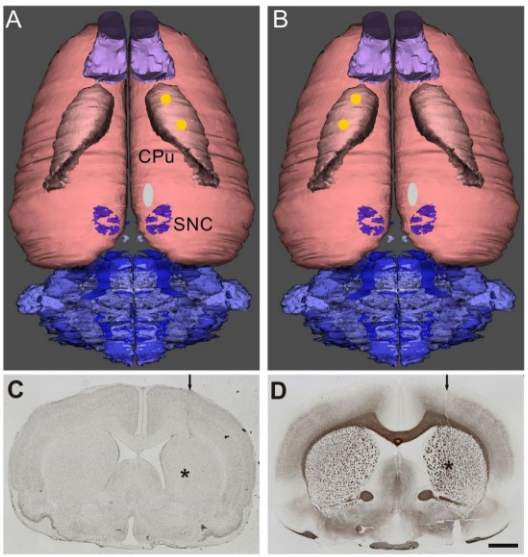

FIGURE 1 | Dorsal view of reconstructed rat brains. The location of the right side 6-hydroxydopamine (6-OHDA)-injection site in the medial forebrain bundle (MFB; white oval) anterior to the substantia nigra pars compacta (SNC) and the two injection sites of botulinum neurotoxin A (BoNT-A) or vehicle (yellow spots) in the caudate putamen (CPu) ipsilateral **(A)** and contralateral **(B)** to 6-OHDA are indicated. Adjacent 20 μm coronal sections of a rat brain stained for cell bodies **(C)** according to Merker or for myelin **(D)** according to Gallyas depict the needle tract (arrow) and the needle tip (asterisk) of a BoNT-A-injection ipsilateral to the 6-OHDA-application (9 months survival). Scale bar applies to **(C,D)**: 2 mm.

(Wree et al., 2011; Hawlitschka et al., 2013), 6 weeks after 6-OHDA-lesioning animals received injections of 2 × 1 μl BoNT-A solution (lot No. 13028A1A; List, Campbell, CA; purchased via Quadratech, Surrey, UK) containing a total of 1 ng BoNT-A dissolved in phosphate-buffered saline with 0.1% bovine serum albumin (PSA-BSA 0.1%) added into the right (ipsilateral) or left (contralateral) CPu. The coordinates with reference to bregma for the ipsilateral BoNT-A-application were: anterior = +1.3/−0.4 mm, lateral 2.6/3.6 mm to the right, and ventral −5.5 mm, respectively, those for the contralateral BoNT-A-application were: anterior = +1.3/−0.4 mm, lateral 2.6/3.6 mm to the left, and ventral −5.5 mm (**Figures 1A,B**). Coronal sections stained for cell bodies (Merker, 1983) and myelin (Gallyas, 1971, 1979) depict injection site at −0.4 mm, 3.6 mm and −5.5 mm (**Figures 1C,D**).

Behavioral Testing

Drug-Induced Rotation Tests (Apomorphine, Amphetamine)

Rotations were assessed using an automated, self-constructed rotometer system based on the design of Ungerstedt and Arbuthnott (1970) and defined as complete 360° turns and registered as net differences between the two directions per minute. Rotational behavior was induced by amphetamine and apomorphine in hemi-PD rats injected with BoNT-A. Tests were performed prior to (i.e., 4 weeks after 6-OHDA-lesion) and at five time points (2 weeks and 1, 3, 6 and 9 months) after intrastriatal application of BoNT-A (**Figure 2**). Animals were injected with d-amphetamine sulfate (2.5 mg/kg, s.c., Sigma Aldrich) and monitored for 60 min and in each case 3 days later with apomorphine (0.25 mg/kg, i.p.; Teclapharm, Germany), followed by registration of rotation for 40 min. In the hemi-PD rats, apomorphine-induced anti-clockwise rotations were expressed by positive values (**Figure 3**), whereas amphetamine-induced rotations in clockwise direction were expressed by negative values (**Figure 4**).

Spontaneous Motor Tests

Corridor task, stepping and open field (OF) tests were realized before 6-OHDA-lesion and 4 weeks thereafter, and 4 weeks,

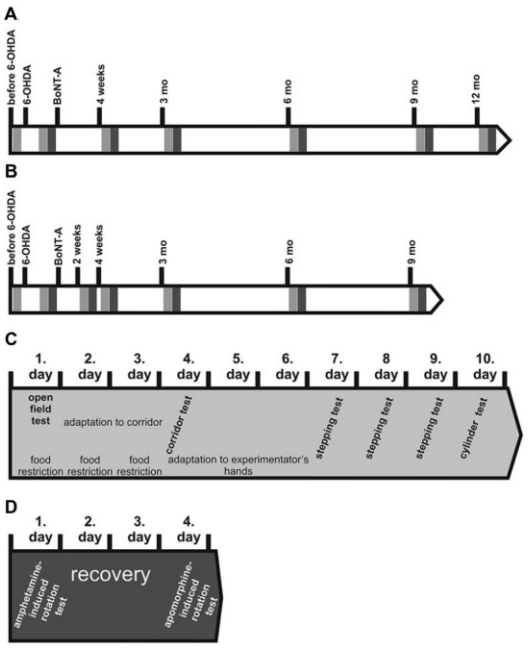

FIGURE 2 | Time points of lesions and behavioral tests in rats BoNT-A or sham-injected **(A)** ipsilateral or **(B)** contralateral to 6-OHDA. Light gray rectangles symbolize batteries of spontaneous behavior tests performed subsequently: open field (OF) test, corridor task, stepping and cylinder tests. Dark gray rectangles symbolize amphetamine-induced rotation test followed by an apomorphine-induced rotation test 3 days later. **(C)** The battery of spontaneous behavior tests were performed as follows: each behavior test series lasted 10 days. At the first day rats were tested for 10 min in the OF arena. Thereafter, rats were food restricted and adapted 2 days rats to the corridor task apparatus for 10 min each. One day later the final corridor test was carried out for 5 min. At the following 3 days rats were handled by the experimentator for 5 min each day. On the following 3 days rats underwent the stepping test twice a day. On the 10th day the forepaw usage was evaluated by the cylinder test. **(D)** Finally, drug-induced rotation tests were performed. First amphetamine rotation test was done for 60 min. Three days later rats were tested for apomorphine-induced rotations for 40 min.

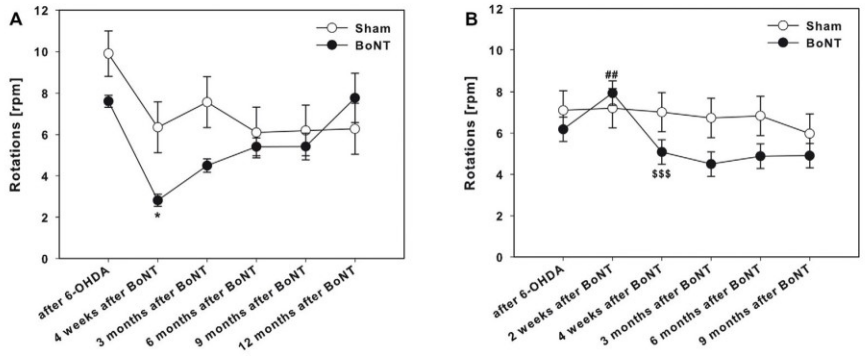

FIGURE 3 | Apomorphine-induced rotations in hemi-Parkinson's disease (PD) rats treated with intrastriatal BoNT-A- or vehicle-applied **(A)** ipsilaterally or **(B)** contralaterally. Ipsilateral BoNT-A-injections significantly decreased anti-clockwise rotations 4 weeks and 3 months compared to sham injection. Contralateral BoNT-A-injection caused a significant increase of the turning rate only 2 weeks after surgery. Asterisks indicate significant differences compared to the sham group ($^{*}P < 0.05$). Hashtags indicate significant differences compared to values before BoNT-A-injection ($^{\#\#}P < 0.01$). Paragraphs indicate significant differences compared to the previous value after BoNT-A-injection ($^{\S\S\S}P < 0.001$). Data are represented as mean ± SEM.

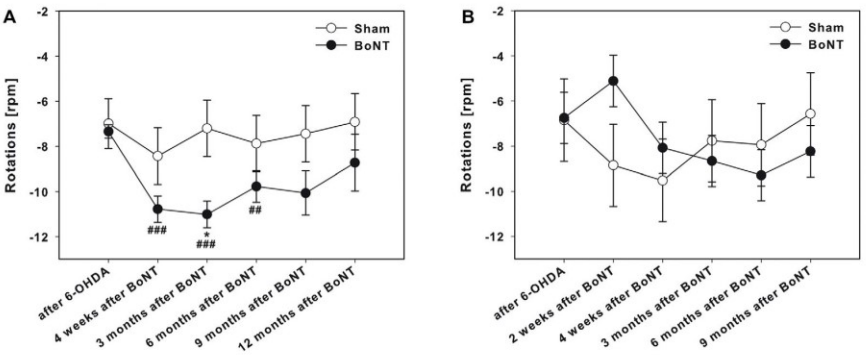

FIGURE 4 | Amphetamine-induced rotations in hemi-PD rats treated with intrastriatal BoNT-A-or vehicle-applied **(A)** ipsilaterally or **(B)** contralaterally. Ipsilateral BoNT-A-injection caused significantly increased rotations 4 weeks to 6 months after BoNT-A, while sham-injected rats did not change rotational behavior. Contralateral BoNT-A-injection in hemi-PD rats caused no significant change of rotational behavior compared to the sham group. Asterisks indicate significant differences compared to the sham group ($^{*}P < 0.05$). Hashtags indicate significant differences compared to values before BoNT-A-injection ($^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$). Data are represented as mean ± SEM.

3 and 6 months after ipsilateral intrastriatal injection of BoNT-A or vehicle, or 2 and 4 weeks, 3, 6 and 9 months after contralateral intrastriatal injection of BoNT-A or vehicle (**Figure 2**). Cylinder test was performed after 6-OHDA-lesion and 4 weeks, 3, 6, 9 and 12 months after ipsilateral intrastriatal injection of BoNT-A or vehicle, or before 6-OHDA-lesion and 4 weeks thereafter, and 2 and 4 weeks, 3, 6 and 9 months after contralateral intrastriatal injection of BoNT-A or vehicle (**Figure 2**).

Stepping Test

Forelimb akinesia was assessed using a modified version of a stepping test (Olsson et al., 1995) primary described as bracing test by Schallert et al. (1979) and has been established as a sensitive measure of bradykinesia in unilateral 6-OHDA-lesioned rats (Schallert et al., 1992; Lindner et al., 1997). We evaluated the adjusting steps. Rats were handled by the experimenter during 3 days to become familiar with the test procedure. Thereafter, tests were performed twice per day on three consecutive days. Briefly, the rat was held by the investigator with one hand softly blocking both its hind limbs and the not monitored forelimb, the unrestrained forepaw touching the table. In doing so the rat was moved slowly sideways across the table (0.9 m in 5 s) and the number of adjusting steps of the respective unrestrained left or right forepaw was counted while moving in the forehand and backhand directions. Finally, the means of forehand and backhand steps of left and right paws were calculated.

Cylinder Test

Forelimb preference was evaluated with the cylinder test in both groups. The use of the left and right forepaws during vertical exploration in a glass cylinder with a diameter of 20 cm was documented and analyzed with a video camera system (Sony) according to Schallert and Tillerson (2000) and Kirik et al. (2001). Thirty consecutive forepaw contacts with the glass cylinder were evaluated per animal by counting the initial contacts of the right or left paw and calculating the ratio of left and right forepaw use. To prevent subjective bias, contacts made by each forepaw with the cylinder wall were scored from videotapes by an observer blinded to the animals' identities.

Open Field Test

Spontaneous horizontal locomotor activity and anxiety were estimated via the OF test (Hall and Ballachey, 1932; Hall, 1934). Rats were placed in a square OF arena of 50 × 50 cm, which was positioned inside an isolation box (TSE-Systems, Bad Homburg, Germany). Rats were adapted for 1 h before the test at dark-phase in the examination room. Illumination of the OF test was provided by a white photo bulb at 450 lx and animals were monitored online by a video camera placed inside the isolation box and tracked using the VideoMot2 Software (TSE Systems). The OF was divided into 16 quadratic subfields in 12 peripheral and four central area by a grid in the tracking software. This paradigm mimicked the natural conflict in rats between the tendency to explore a novel environment and the tendency to avoid a brightly lit open arena (DeFries et al., 1966; Eikelis and Van Den Buuse, 2000). The rats were tested once in the arena for 10 min. Environmental odors were removed by cleaning the OF after each session to avoid influences of the behavior by odor trials. The total running distance of the animals, time spend in the center and in the edges of OF arena (Andringa et al., 2000), also the ration of center distance to total distance (Denenberg, 1969) were evaluated.

Corridor Task

Lateralized sensorimotor integration and neglect for the side contralateral to the 6-OHDA-lesion were examined using the adjacent version of corridor task according to Grealish et al. (2010). Before testing rats went onto a food restriction diet for 3 days and maintained at 90% of free-feeding bodyweight during habituation and testing (Schackel et al., 2013). Animals were adapted into the apparatus, a long, narrow self-constructed alleyway (240 cm long × 7 cm wide × 23 cm deep) for 10 min each on two consecutive days with some scattered sugar pellets (Ain-76A Rodent Tablet 20 mg TestDiet, Richmond, IN, USA) along the floor of the corridor and started from different ends of the corridor each day. On the test day, rats were first positioned individually in an identical, but empty corridor for 5 min for adaptation and then placed to the end of the testing corridor in which bowls (2 cm in diameter, distance between the bowls 15 cm) containing 5 pellets placed on the left and right sides. Rats were free to retrieve pellets from either side of their body for 5 min (Fitzsimmons et al., 2006). The number of ipsilateral (right side) and contralateral (left side) retrievals was counted and the data were expressed as the percentage of left or right side retrievals on the total number of retrievals. A "retrieval" involved a nose poke into a bowl, whether or not any pellets were taken from it, defining the side according to the rat's body axis (Dowd et al., 2005; Döbrössy and Dunnett, 2007; Grealish et al., 2010).

Statistics

Data of behavioral tests was subjected to two-way ANOVA with repeated measurements. A one-way repeated measures ANOVA is used for comparison of different time points in separate treatment groups which is essentially the same design as a paired *t*-test. The Holm-Sidak approach was used for adjustment for multiple testing for *post hoc* comparisons. A critical value for significance of $P \leq 0.05$ was used throughout the study. In case of non-normally distributed data, data were subjected to Kruskal-Wallis one- or two-way ANOVA on ranks. Dunn's test was used for *post hoc* comparisons after ANOVA on ranks to adjust for multiple testing. All statistical tests were done using SigmaPlot 11 Software (Supplementary Table S1).

RESULTS

Apomorphine-Induced Rotations

The right side hemi-PD rats showed apomorphine-induced anti-clockwise rotations of about 6–10 rotations per minute (**Figures 3A,B**).

Ipsilateral BoNT-A-Injection

Ipsilateral BoNT-A-injection caused a significant decrease in rotational behavior 4 weeks and 3 months after BoNT-A, thereafter rotations of BoNT-A-injected rats equaled those after sham-injection (**Figure 3A**).

Contralateral BoNT-A-Injection

The injection of 1 ng BoNT-A into the left striatum of right sided 6-OHDA-lesioned rats caused a significant increase of the turning rate 2 weeks after surgery. Remarkably, already 2 weeks later, i.e., 1 month after BoNT-A-administration, the turning rate decreased to a level, which was not significantly different from the turning rate prior to BoNT-A treatment (**Figure 3B**).

Amphetamine-Induced Rotations

The hemi-PD rats showed amphetamine-induced clockwise rotations of about seven turns per minute (**Figures 4A,B**).

Ipsilateral BoNT-A-Injection

Ipsilateral intrastriatal BoNT-A-injection caused a significantly increase of the turning rate 4 weeks to 6 months after BoNT-A (**Figure 4A**). Sham-injected rats did not change rotational behavior in hemi-PD rats (**Figure 4A**).

Contralateral BoNT-A-Injection

Contralateral intrastriatal BoNT-A-injection in hemi-PD rats caused a tentative decrease of rotational behavior only at 2 weeks

after BoNT-A (**Figure 4B**). At all other post-BoNT-A-injection time-points BoNT-A- and sham-injected rats did not differ in rotational behavior in hemi-PD rats (**Figure 4B**).

Stepping Test

Adjusting steps were measured on the unlesioned and lesioned sides for each group in the forward and backward directions. Before 6-OHDA-lesion no difference in the number of adjusting steps for the left and right forepaws in forehand and backhand directions for both experimental groups were observed (**Figures 5A–H**). Rats of both groups with their left and right forepaws made about 9–12 steps both in forward and backward directions. After 6-OHDA-lesion only the performance of the left forepaw (contralateral to 6-OHDA) was impaired in both the forehand (**Figures 5A,B**) and backhand (**Figures 5C,D**) directions.

Ipsilateral BoNT-A-Injection

Neither ipsilateral BoNT-A nor sham injections changed the impairments of hemi-PD rats. At the same time, side stepping movements of the right forepaw in 6-OHDA-lesioned rats in both the forehand and backhand directions were generally neither affected by ipsilateral BoNT-A nor sham injection up to 6 months (**Figures 5E,G**), with exception of single time points.

Contralateral BoNT-A-Injection

As compared to sham-injected rats, the BoNT-A-injected animals significantly improved left paw forehand steps from 2 weeks to 3 months after BoNT-A (**Figure 5B**), and backhand steps from 2 weeks until 9 months after BoNT-A (**Figure 5D**). In addition, we found significantly more adjusting steps of the right forepaw from 2 weeks up to 9 months after BoNT-A-application as compared to sham-injected rats, as well in forehand (**Figure 5F**) as in backhand (**Figure 5H**) directions. Thus, contralateral BoNT-A-injection positively influences forelimb usage in hemi-PD rats. Moreover, there was an age-related decrease in right paw stepping (**Figures 5F,H**).

Cylinder Test

Right side hemi-PD rats exhibited a significantly reduced use of the left forelimb, resulting in an about 50% decrease of the left/right ratio of forelimb usage (**Figures 6A,B**).

Ipsilateral BoNT-A-Injection

Intrastriatal ipsilateral treatment of hemi-PD rats with 1 ng of BoNT-A did not show any significant improvement of the left forelimb usage as assessed by the cylinder test. Also sham-treated rats showed no significant effect in forelimb usage of hemi-PD rats (**Figure 6A**).

Contralateral Injection

Interestingly, intrastriatal injection of BoNT-A contralateral to the lesioned side led 2 weeks after BoNT-A-application to a significant readjustment of the left and right forepaw usage. In the course of the following months this effect decreased (**Figure 6B**).

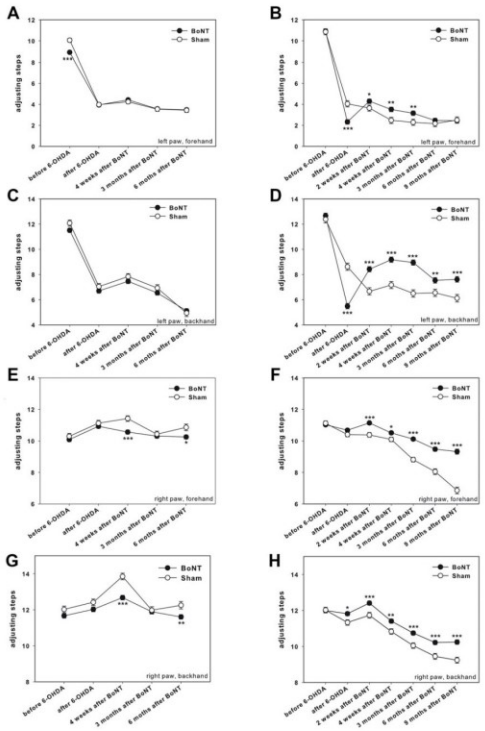

FIGURE 5 | Stepping test in right side hemi-PD rats treated with intrastriatal BoNT-A- or vehicle-applied **(A,C,E,G)** ipsilaterally or **(B,D,F,H)** contralaterally. In unlesioned rats 9–12 adjusting steps for the left **(A–D)** and right **(E–H)** forelimbs in forehand and backhand directions were seen. In hemi-PD rats the use of the left forepaw was impaired in both the forehand **(A,B)** and backhand **(C,D)** directions. Neither ipsilateral BoNT-A nor sham injection changed the impairments of left paw steps in hemi-PD rats **(A,C)**. The stepping of the right forelimb in hemi-PD rats in both the forehand **(E)** and backhand **(G)** directions were generally not affected by ipsilateral BoNT-A or sham injection. Contralateral BoNT-A-injected hemi-PD rats significantly improved left paw forehand steps from 2 weeks to 3 months after BoNT-A **(B)**, and backhand steps from 2 weeks until 9 months after BoNT-A **(D)**. Right forepaw adjusting steps significantly increased from 2 weeks up to 9 months after BoNT-A-application as well in forehand **(F)** as in backhand **(H)** directions compared to sham-injected rats. Asterisks indicate significant differences compared to the sham group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Data are represented as mean ± SEM.

Open Field

Hemi-PD rats of both BoNT-A- and sham-injected groups showed decreased locomotor activity compared to prelesion results (**Figure 7B**).

Ipsilateral BoNT-A-Injection

The total running distance (**Figure 7A**) and also the ratio of center distance to total distance (**Figure 7C**) were not significantly different between the BoNT-A and the sham group over time. Moreover, ipsilateral BoNT-A-injected rats and sham group spent the same time in the edges of the OF apparatus (data not shown).

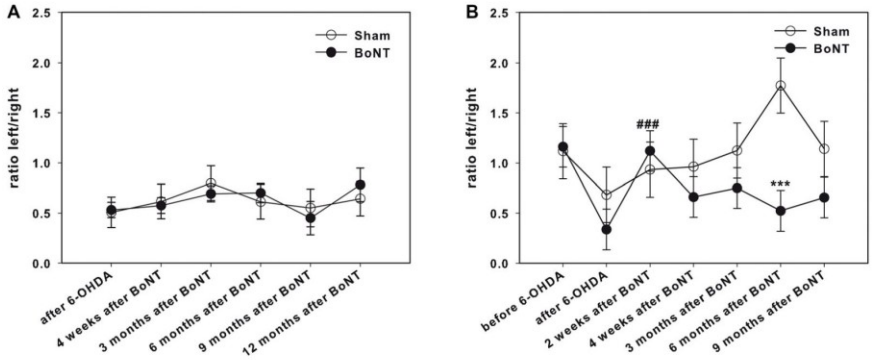

FIGURE 6 | Cylinder test in right side hemi-PD rats treated with intrastriatal BoNT-A- or vehicle-applied **(A)** ipsilaterally or **(B)** contralaterally. Hemi-PD rats exhibited a significantly reduced use of the left forelimb resulting in left/right ratio of about 0.5 **(A,B)**. Neither ipsilateral BoNT-A nor sham injection showed any significant improvement. Contralateral intrastriatal injection of BoNT-A only led at 2 weeks after BoNT-A to a significant readjustment of the right and left forepaw usage. Hashtags indicate significant differences compared to values before BoNT-A-injection ($^{###}P < 0.001$). Asterisks indicate significant differences compared to the sham group ($^{***}P < 0.001$). Data are represented as mean ± SEM.

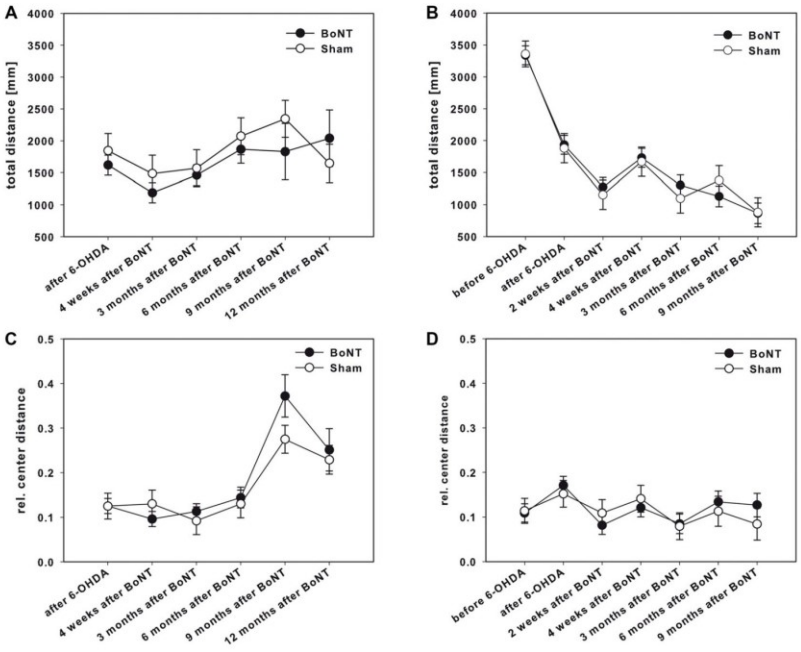

FIGURE 7 | OF test in right side hemi-PD rats treated with intrastriatal BoNT-A- or vehicle-applied **(A,C)** ipsilaterally or **(B,D)** contralaterally. Rats of both BoNT-A- and sham-injected groups showed decreased locomotor activity compared to prelesion results **(B)**. Neither ipsilateral BoNT-A nor sham injection showed any significant changes in total running distance **(A)** or the ratio of center distance to total distance **(C)**. The same is true for both contralaterally injected groups **(B,D)**. Data are represented as mean ± SEM.

Contralateral BoNT-A-Injection

The total running distance was similar in both experimental groups after contralateral application of BoNT-A or sham treatment up to 9 months (**Figure 7B**). Also, the relative center distance (**Figure 7D**) of the BoNT-A-injected animals did not differ significantly from the sham group over time as did the times spent in the edges of the OF (data not shown).

Corridor Task

Two weeks before lesion surgery, rats were tested in the adjacent version of the corridor task. This preoperative screening showed that all animals equally retrieved pellets from either left or right sides (**Figures 8A,B**). The right side 6-OHDA-lesion caused a significant neglect of the left corridor side; only about 5% of left retrievals were measured (**Figures 8A,B**).

Ipsilateral BoNT-A-Injection

Ipsilateral intrastriatal BoNT-A as well as sham injections in hemi-PD rats did not improve contralateral sensorimotor integration up to 6 months significantly (**Figure 8A**).

Contralateral BoNT-A-Injection

Left side sham-injected rats did not improve in this task over time. In contrast, contralateral intrastriatal BoNT-A-injection significantly reversed this bias 2 weeks to 9 months after treatment. Significant contralateral retrievals of about 40% were seen. However, 6 and 9 months after BoNT-A values declined again and equaled those of sham-injected rats (**Figure 8B**).

DISCUSSION

In our previous publications (Wree et al., 2011; Holzmann et al., 2012; Antipova et al., 2013; Hawlitschka et al., 2013; Mehlan et al., 2016), we described the effect of intrastriatal injection of BoNT-A in a rat model of hemi-PD. We used the 6-OHDA to induce hemi-PD, a widely utilized and highly reproducible toxin-based animal model of PD in rats (Ungerstedt et al., 1974; Schwarting and Huston, 1996; Deumens et al., 2002; Blandini and Armentero, 2012). Unilateral 6-OHDA-injection of the right MFB that conveys the efferent fibers from nigral cell bodies to the striatum, causes massive degeneration of the nigrostriatal pathway, highest level of nigral cell loss and striatal DA depletion (over 90%; Dauer and Przedborski, 2003; Blandini and Armentero, 2012). The resulting motor deficits at the side of the body contralateral to the lesion (Deumens et al., 2002) can be evaluated by spontaneous and drug-induced behavioral phenotypes. As described originally by Ungerstedt and Arbuthnott (1970), a 6-OHDA-lesion of the right SNC causes anti-clockwise, i.e., contraversive apomorphine-induced rotations (Ungerstedt et al., 1969). The most important finding in our prior studies with intrastriatally applied BoNT-A was the complete abolition of apomorphine-induced rotations up to 6 months, when the rats received 1 ng of BoNT-A into the right striatum 6 weeks after right side 6-OHDA-lesion (Wree et al., 2011; Antipova et al., 2013).

In continuation of previous studies and to enhance our knowledge of the BoNT-A-injection model, we here examined the effects of BoNT-A-injection into the striatum as well ipsilateral as contralateral to the hemisphere that received 6-OHDA before. For that reason we used a broad spectrum of different behavior tests, including spontaneous test, i.e., corridor task, stepping, cylinder and OF test and drug-induced motor tests, i.e., apomorphine- and amphetamine-induced rotations, to characterize and compare the effects of ipsilateral and contralateral intrastriatal BoNT-A-injections on the motor deficits, lateralized neglect, bradykinesia, emotionality and anxiety in hemi-PD rats.

We found a differentiated behavioral profile after ipsilateral or contralateral BoNT-A-application in hemi-PD rats. The effects of ipsilaterally injected BoNT-A were in line with those published previously (Wree et al., 2011; Holzmann et al., 2012; Antipova et al., 2013; Hawlitschka et al., 2013; Mehlan et al., 2016). Interestingly, the effects of contralaterally injected BoNT-A were either changed to the opposite compared to ipsilateral BoNT-A, or gave results not known yet. The disruption of presynaptic ACh as well as DA release by BoNT-A is transient and the synaptic signal transmission was shown by forming of new intact SNARE complexes within several months (Sweeney et al., 1989; Shapovalova, 1995; Washbourne et al., 1998; Weinstock et al., 2002). In the following paragraphs, the results seen in the various behavioral parameters are discussed with respect to assumed or hypothetical changes of transmitter and receptor concentrations in the CPu as a key structure of the basal ganglia loops induced by dopaminergic deprivation and BoNT-A-application.

Basal Ganglia Circuitry in Hemi-PD Rats

Dopaminergic deprivation of the striatum is followed by reactive adaptations of transmitter receptors, mainly by an upregulation of D2 receptors in the range of about 20%–30% (Reader and Dewar, 1999; Choi et al., 2012; Sun et al., 2013; Konieczny et al., 2017). The majority of inhibitory D2 receptors are located on cholinergic interneurons and medium spiny neurons (MSN) projecting to the external pallidum, i.e., being part of the indirect basal ganglia loop (Hurley and Jenner, 2006; Perreault et al., 2011; Bordia et al., 2016; Mamaligas et al., 2016; Rico et al., 2017). As in hemi-PD the tonically active cholinergic interneurons become hyperactive due to loss of D2-mediated DA inhibition, they strongly activate MSN by ACh. Thus, complementary to the loss of inhibitory D2-mediated DA inhibition the MSN increase their firing intensity also by cholinergic hyperstimulation. The increased GABAergic MSN projection reaches the external globus pallidus (EGP). As the EGP is thus strongly inhibited, its GABAergic projection to the internal globus pallidus (IGP = entopeduncular nucleus) and mainly to the subthalamic nucleus (STh) is strongly reduced compared to normal. As a result, the spontaneously active STh is less inhibited and thus more intensely firing, further stimulated by normal cortical glutamatergic afferents. As a consequence, the STh using glutamate as transmitter can stimulate the IGP more intensely. The massively stimulated IGP projects using GABA as transmitter to the ventrolateral thalamic nucleus (VL) that reacts with an inhibition of its neurons. Finally, the inhibited VL neurons send less activating glutamatergic stimuli to the premotor cortex that in turn reacts with a reduced initiation of movements of the contralateral body via its crossed motor efferents (Obeso et al., 2008a,b, 2014). These changes in basal ganglia circuitry seem to underlie the movement initiation deficits, the akinesia and

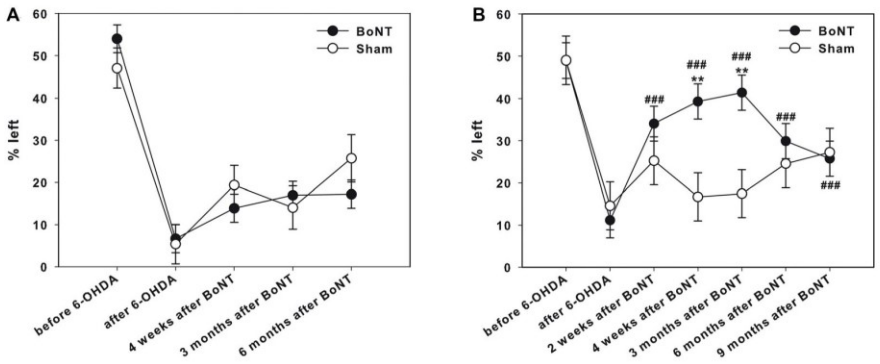

FIGURE 8 | Corridor task in hemi-PD rats treated with intrastriatal BoNT-A- or vehicle-applied **(A)** ipsilaterally or **(B)** contralaterally. Preoperative screening showed that all animals equally retrieved pellets from either left or right sides **(A,B)**. Right side hemi-PD rats significantly neglected of the left corridor side. Ipsilateral BoNT-A as well as sham injections did not improve contralateral sensorimotor integration in hemi-PD rats. Contralateral BoNT-A-injection reversed this bias in hemi-PD rats 2 weeks to 9 months. Significant contralateral retrievals of about 40% were seen. Left side sham-injected rats did not improve in this task over time. Asterisks indicate significant differences compared to the sham group ($^{**}P < 0.01$). Hashtags indicate significant differences compared to values before BoNT-A-injection ($^{###}P < 0.001$). Data are represented as mean ± SEM.

the reduced spontaneous use of the contralateral forelimb and changed walking pattern in hemi-PD rats (Blandini et al., 2000; DeLong and Wichmann, 2007; Braak and Del Tredici, 2008; Quiroga-Varela et al., 2013; Tremblay et al., 2015).

Drug-Induced Behavior

Apomorphine-Induced Rotations

Unilateral dopaminergic depletion led to apomorphine-induced contralateral rotations, ipsilateral intrastriatal BoNT-A reduced rotational behavior for at least 6 months, and contralateral intrastriatal BoNT-A increased rotational behavior only shortly at 2 weeks after BoNT-A in hemi-PD rats.

In hemi-PD rats apomorphine-induced rotations are due to binding of apomorphine to the upregulated D2 receptors in the dopaminergically deprived CPu neurons (Konieczny et al., 2017). Inhibitory D2 receptors are mostly located on cholinergic interneurons and MSN projecting to the EGP, i.e., being part of the indirect basal ganglia loop (Pisani et al., 2007; Tozzi et al., 2011; Lim et al., 2014; Bordia et al., 2016; Mamaligas et al., 2016). In hemi-PD rats apomorphine deactivates the hyperactive cholinergic interneurons via D2 receptors and also inhibits the D2-bearing MSN, forming the central parts of the indirect basal ganglia loop and projecting with reduced intensity to the EGP. As the EGP is disinhibited, its GABAergic projection to the STh is increased compared to normal and thus the more inhibited STh can stimulate the IGP less intensively. The less active IGP projects less intensively to the VL, and consequently, the disinhibited VL sends much more activating stimuli to the premotor cortex, which in turn reacts with an increased initiation of movements and muscle innervation of the contralateral body side. Thus, apomorphine induces contralateral rotations in hemi-PD rats.

Ipsilateral intrastriatal application of 1 ng BoNT-A in hemi-PD rats completely abolished apomorphine-induced contralateral rotation behavior for at least 6 months. BoNT-A is thought to block the ACh release of cholinergic interneurons, which are hyperactive in PD (Day et al., 2006; Obeso et al., 2008a,b). Seemingly, ipsilateral BoNT-A normalized ACh concentration in the injected CPu for a limited time, i.e., up to 6 months until BoNT-A is metabolized. Moreover, jet unpublished own results of BoNT-A-induced receptor concentration measurements speak in favor of a BoNT-A-induced reduction of D2 receptors in the respective striatum. Following the indirect basal ganglia loop the reduced activity of the D2 receptor bearing MSN result in a downregulation, i.e., normalization of the VL activity and the abolition of apomorphine-induced contralateral rotation behavior. These effects seemed to exist for at least 3 months, thereafter BoNT should be metabolized.

Contralateral intrastriatal application of BoNT-A shortly increased apomorphine-induced contralateral rotation behavior only at 2 weeks. It can be hypothesized that BoNT-A shortly reduced the D2 receptors in the contralateral striatum thus increasing the difference in the D2 receptor concentrations between both hemispheres and by this leads to an increase of the number of rotations.

Amphetamine-Induced Rotations

Unilateral dopaminergic depletion led to amphetamine-induced ipsilateral rotations, ipsilateral intrastriatal BoNT-A increased rotational behavior for at least 6 months, and contralateral intrastriatal BoNT-A tentatively decreased rotational behavior shortly at 2 weeks after BoNT-A in hemi-PD rats.

Amphetamine as a DA releaser causes strong DA delivery in the CPu contralateral to the 6-OHDA-lesion and a low one in the ipsilateral striatum because of the loss of DA afferents. Consequently, the strong DA imbalance activates the contralateral inhibitory dopaminergic system, influencing the

respective MSN via ACh, and by this causes ipsilateral rotations (about 7 per min). Moreover, the massive innervation of the D1 receptor bearing MSN in the CPu contralateral to the DA depletion also results in a stimulation of that hemisphere resulting in clockwise rotations of right side hemi-PD rats.

Ipsilateral intrastriatal application of 1 ng BoNT-A in hemi-PD rats increased amphetamine-induced ipsilateral rotation behavior for at least 6 months. Apparently, BoNT-A blocked the ACh release of cholinergic interneurons and reduced D2 receptor concentration in the respective striatum. Moreover, intrastriatal application of BoNT-A could have altered the concentration of other transmitters and their receptors, too (Bigalke et al., 1985; Ashton and Dolly, 1988; Poulain et al., 1990). As it is described that amphetamine and its derivates are able to bind directly to α-2 adrenergic receptors (Ritz and Kuhar, 1989) and activate serotonin receptors (Nichols et al., 1994; Schmidt et al., 1994), these receptors could also play a role in the BoNT-A-induced changes in amphetamine-induced rotation behavior.

Amphetamine-induced ipsilateral rotations were tentatively and shortly decreased at 2 weeks after contralateral intrastriatal BoNT-A-application. Possibly, BoNT-A temporally reduced DA release in the injected contralateral striatum thus decreasing the difference in the DA concentrations between both hemispheres and by this leading to a decrease of the number of rotations.

Spontaneous Motor Tests

Stepping Test

Unilateral right side dopaminergic depletion profoundly impaired stepping performance of the left paw (contralateral to 6-OHDA-lesion) in both forehand and backhand directions. Ipsilateral intrastriatal BoNT-A did not change the impairments seen in hemi-PD rats, while contralateral intrastriatal BoNT-A improved left paw forehand and backhand steps and positively influenced right forelimb usage.

In hemi-PD rats our results confirmed those of many others (Schallert et al., 1992; Mukhida et al., 2001; Kelsey et al., 2004; Tseng et al., 2005; Manfredsson et al., 2007; Pioli et al., 2008; Fang et al., 2010; Seeger-Armbruster and von Ameln-Mayerhofer, 2013; Shin et al., 2014; Tronci et al., 2015) showing the severe impairment of the contralateral paw in combination with unaltered stepping performance of the ipsilateral paw (Winkler et al., 1996; Kelsey et al., 2004; Pinna et al., 2010). The motor initiation deficits in the forelimbs, analogous to limb akinesia and gait problems in PD patients, were produced by DA depletion (Sabol et al., 1985; Fairley and Marshall, 1986; Salamone et al., 1993). As dopaminergic deprivation of the striatum is followed by increased GABAergic MSN projection to the EGP, resulting in a disinhibition of the spontaneously active STh and, as a result a more actively firing IGP, which inhibits VL. Finally, the inhibited VL neurons do not sufficiently activate the premotor cortex that in turn reduces initiation of movements of the contralateral body via its crossed motor efferents (Obeso et al., 2008a,b, 2014; Wree and Schmitt, 2015).

Ipsilateral intrastriatal application of 1 ng BoNT-A in hemi-PD rats did not change the impairments seen in hemi-PD rats. Obviously, the BoNT-A-induced changes in the DA-deprived CPu concerning extracellular ACh content, and DA and other transmitter receptors are not sufficient to improve spontaneous motor tasks. Importantly, several reports emphasized that it is the striatal DA depletion of over 80% that is responsible for decreased movement initiation (Sabol et al., 1985; Fairley and Marshall, 1986; Salamone et al., 1993; Chang et al., 1999; Seeger-Armbruster and von Ameln-Mayerhofer, 2013; Sun et al., 2013). As in our hemi-PD rats the DA depletion was nearly complete (Barnéoud et al., 2000; Kelsey et al., 2004), the ipsilateral intrastriatal BoNT-A-application is without effect on stepping tasks.

Contralateral intrastriatal application of 1 ng BoNT-A in hemi-PD rats improved the impairments of the left paw seen in hemi-PD rats for 3 (forehand steps) or 9 (backhand steps) months. This phenomenon is not fully understood. D1 and D2 receptor binding contralateral to unilateral 6-OHDA-lesion is reported unchanged to normal rats (Lawler et al., 1995; Pelled et al., 2002), whereas D2 receptor concentration is generally increased ipsilaterally (Araki et al., 1998; Reader and Dewar, 1999; Choi et al., 2012; Sun et al., 2013; Konieczny et al., 2017). This is corroborated by Capper-Loup et al. (2013) stating that contralateral to the 6-OHDA-lesion striatal D1R mRNA and D2R mRNA resemble those of naive rats, whereas ipsilaterally D1R mRNA is reduced and D2R mRNA is increased (Capper-Loup et al., 2013). With respect to DA concentration in the contralateral hemisphere contradicting measures were reported. Fox et al. (2016) found striatal DA release from the contralateral unlesioned substantia nigra equivalent in lesioned and control rats, whereas Zetterström et al. (1986) dialyzed more DA contralaterally to 6-OHDA than in unleased controls (Zetterström et al., 1986; Fox et al., 2016). Several groups hypothesized that if it is likely that the unilateral lesion of the nigrostriatal pathways affects both sides of the brain (Lawler et al., 1995; Fox et al., 2016), then it is not yet clear how the unlesioned side is influenced. It is discussed that Interhemispheric projections have functional significance (Fox et al., 2016; Schmitt et al., 2016). Lawler et al. (1995) combined a 6-OHDA with a corpus callosum transection in order to minimize interhemispheric projections on possible changes in the D1 and D2 receptors, DA and its metabolites in the non-lesioned striatum (Lawler et al., 1995). Bilateral cortico-striatal projections were traced in rats (Hassler et al., 1982; Berendse et al., 1992), however with ipsilateral predominance: both cortices project to both striata (Hassler et al., 1982; Berendse et al., 1992; Lieu and Subramanian, 2012; Schmitt et al., 2016), i.e., intact cortex to the lesioned striatum and cortex of the lesioned hemisphere to the intact striatum (Lieu and Subramanian, 2012) arguing that interhemispheric connections can influence behavioral responses to nigrostriatal lesioning. Unfortunately, there is no comprehensive summary of changes in neuroanatomical and electrophysiological properties in the so-called healthy hemisphere induced by unilateral lesion of the nigrostriatal dopaminergic system and its correlation to behavior.

Contralateral intrastriatal application of 1 ng BoNT-A in hemi-PD rats improved the use of the right paw in hemi-PD rats

for up to 9 months both in the number of forehand and backhand steps. In this case, BoNT-A was injected into a seemingly normal transmitter and receptor environment. BoNT-A is thought to block the ACh release of cholinergic interneurons and DA release of dopaminergic afferents (Bigalke et al., 1985; Ashton and Dolly, 1988), perhaps also reduces the D2 receptor concentration. As a result, the MSN decreased the GABAergic innervation of the EGP, resulting in an increased inhibition of the spontaneously active STh and followed by a less actively firing IGP, which inhibits VL to a lesser degree than normally. Finally, the disinhibited VL neurons considerably activated the premotor cortex that in turn increased the initiation of movements of the contralateral body, i.e., the forelimb ipsilateral to the DA deprivation, via its crossed motor efferents. In the end, the paw of the DA deprived body side is hyperactive as compared to the sham-injected hemi-PD rats. As already supposed by Olsson et al. (1995) we observed an age dependent decline of right paw forehand and backhand steps, both in sham- and BoNT-A-injected rats up to 9 months, suggesting some degree of habituation to the test.

Cylinder Test

Unilateral right side hemi-PD rats exhibited a significantly reduced use of the left forelimb. Intrastriatal ipsilateral BoNT-A or sham treatment did not show any significant improvement of the left forelimb usage. Interestingly, contralateral intrastriatal injection of BoNT-A led to a short term (at test point 2 weeks after BoNT-A) significant readjustment of forepaw usage.

In the hemi-PD rats our results are in line with others (Schallert and Tillerson, 2000; Kirik et al., 2001; Deumens et al., 2002; Cohen et al., 2003; Vercammen et al., 2006; Rauch et al., 2010; de Araújo et al., 2013), the impaired paw being relatively used by about 40% to 50% compared to the unimpaired. As for the stepping impairment the motor initiation deficit for voluntary movements in contralateral forelimb use is discussed as a result of DA depletion (Whishaw et al., 1986; Lundblad et al., 2002; Shi et al., 2004; Schallert and Woodlee, 2005; Wheeler et al., 2014; Sampaio et al., 2017). As dopaminergic deprivation of the striatum causes increased GABAergic MSN projection to the EGP, and via disinhibition of the spontaneously active STh a more actively firing IGP, the inhibited VL neurons do not sufficiently activate the premotor cortex. Consequently, there is a reduces initiation of movements of the contralateral body via its crossed motor efferents (Obeso et al., 2008a,b, 2014; Wree and Schmitt, 2015).

Ipsilateral intrastriatal application of BoNT-A or sham injection in hemi-PD rats did not change the impairments. Our explanation resembles that of the stepping test. For the execution of the voluntary forelimb movements a normal DA content of the CPu is essential (Salamone et al., 1993; Shi et al., 2004; Manfredsson et al., 2007; Woodlee et al., 2008; Plowman et al., 2011; Fleming et al., 2013; Mabandla et al., 2015). BoNT-A did not improve left forelimb use as it could increase DA concentration.

Contralateral intrastriatal application of 1 ng BoNT-A in hemi-PD rats shortly improved the use of the left paw in hemi-PD rats. Seemingly, BoNT-A for a limited time interacts with ACh release from cholinergic interneurons and DA release from dopaminergic afferents (Bigalke et al., 1985; Ashton and Dolly, 1988).

Open Field Test

Spontaneous horizontal locomotor activity and anxiety as measured by the OF test showed decreased locomotor activity in hemi-PD rats compared to prelesion results. Ipsilateral intrastriatal BoNT-A and sham injections neither changed total running distance nor the ratio of center distance to total distance significantly over time. Also contralateral intrastriatal BoNT-A or sham injection had a significant effect on locomotor activity of hemi-PD rats.

Unilateral 6-OHDA-injection decreased the overall spontaneous locomotor behavior as measured by total walking distance as already shown by others (Kirik et al., 2001; Tamás et al., 2005; Brown et al., 2011; Abedi et al., 2013; Capper-Loup et al., 2013; da Rocha et al., 2013; Sun et al., 2013; Machado-Filho et al., 2014; Chao et al., 2015; Kumari et al., 2015; Ximenes et al., 2015; Das et al., 2016; Sgroi et al., 2016). Reduced locomotion is tentatively interpreted as a result of depletion of striatal DA (Fearnley and Lees, 1991; Schwarting and Huston, 1996; Alam and Schmidt, 2002; Ferro et al., 2005; Carvalho et al., 2013), although there is no clear explanation why a unilateral DA depletion has such a massive effect (about 50% reduction) on total walking distance as a measure of whole body bradykinesia of the hemi-PD rats. Additionally, we could not observe that the impairments of hemi-PD rats were age and habituation dependent. It has been previously reported that locomotor activity declined in aging hemi-PD rats (Schulz et al., 2002, 2004; Jezek et al., 2003) or due to habituation (Gentsch et al., 1982; Bureš et al., 1983) in OF.

Corridor Task

Naive rats equally retrieved pellets from either left or right sides. The right side 6-OHDA-lesion caused a massive neglect of the left corridor side. Ipsilateral intrastriatal BoNT-A did not improve contralateral sensorimotor integration up to 6 months significantly. In contrast, contralateral intrastriatal BoNT-A-injection significantly reversed this bias, and significant contralateral retrievals of about 40% were seen.

Anatomical, biochemical and behavioral evidence indicate that striatum is heterogeneous with respect to function (Divac et al., 1967; Dunnett and Iversen, 1980; Pisa, 1988) and the intact nigrostriatal innervation is crucial for successful integration of sensory and motor function resulting in a coordinated goal-directed behavior (Turner, 1973; Dunnett and Iversen, 1982; Carli et al., 1985, 1989; Fairley and Marshall, 1986). Thus, the corridor task is a sensitive behavioral test for unilateral 6-OHDA-lesion (Boix et al., 2015) measuring the lateralized sensorimotor proprioception and neglect.

Unilateral striatal DA depletion results in a polymodal "neglect" characterized by a failure to orient to contralateral stimuli (Marshall et al., 1971; Ljungberg and Ungerstedt, 1976; Fairley and Marshall, 1986; Brown et al., 2011) and hemi-PD rats failed to orient to tactile, visual or olfactory stimuli presented on the contralateral side of the body (Dowd et al., 2005).

Respective abnormalities have already been reported in PD patients (Berardelli et al., 2001). Brown and Robbins suggested that the "striatal neglect" was not due to a failure to localize the stimuli in contralateral space but, rather, resulted from a deficit in directing responses in contralateral space (Brown and Robbins, 1989). Accordingly, Carli et al. (1985) stated that the neglect is not primarily sensory in nature but is evident only when a contralateral response initiation, in our experiments the retrieval of pellets, is required.

In our study naive animals made equivalent numbers (about 50%) of retrievals from both the left or right corridor sides as shown previously by others (Dowd et al., 2005; Fitzsimmons et al., 2006; Döbrössy and Dunnett, 2007; Kerkerian-Le Goff et al., 2009; Jouve et al., 2010). Hemi-PD rats largely neglect sugar pellets encountered on their contralateral side, demonstrating a pronounced ipsilateral retrieval bias (Torres et al., 2008; Cordeiro et al., 2010; Kaindlstorfer et al., 2012; Naughton et al., 2016). As discussed with stepping and cylinder tests, hemi-PD rats showed a severe impairment of motor initiation of the contralateral body including forelimbs and especially in head orientation. Although the hemi-PD rat seemingly detected the contralateral stimulus (sugar pellets in bowl), it could not react due to the impaired initiation, and, consequently, food pellets were retrieved by the unaltered paw of the opposite side. The motor initiation deficits in the forelimbs were discussed as caused by DA depletion (Sabol et al., 1985; Fairley and Marshall, 1986; Salamone et al., 1993; Dowd et al., 2005; Fitzsimmons et al., 2006) with the known consequences in basal ganglia circuitry.

Intrastriatal ipsilateral BoNT-A-injection did not alter the striatal neglect in hemi-PD rats. This is not surprising, as the DA deficit underlying the neglect was not changed by BoNT-application.

As a key finding in the present study, we obtained a significantly positive effect of intrastriatal contralateral BoNT-A-injection on the contralateral neglect in hemi-PD rats. As discussed with the BoNT-induced changes in the stepping test, we have no comprehensive interpretation of the BoNT-induced behavioral benefit. Taken together, as the molecular mechanism underlying the jet unclear lesion-induced compensatory mechanisms in the hemisphere contralateral to the 6-OHDA-lesion is not further substantiated, we cannot explain the beneficial effect in the contralateral intrastriatal BoNT-application on spontaneous motor task of the hemi-PD rats.

CONCLUSION

As hypothesized, some of the behavioral effects seen after ipsilateral BoNT-A-application in hemi-PD rats were reversed or even changed to the opposite by contralateral BoNT-A-injection in hemi-PD rats.

In hemi-PD rats, intrastriatal ipsilateral BoNT-A-injection massively influenced the outcome of drug-induced tests. We found a significant decrease in apomorphine-induced rotations and an increase in amphetamine-induced rotations. Moreover, ipsilateral intrastriatal application of BoNT-A showed no effect on the forelimb usage and akinesia, lateralized sensorimotor integration and spontaneous locomotor activity.

However, contralateral intrastriatal BoNT-A-injection preferentially affected spontaneous motor behavior reducing striatal neglect, and also maintained a significant reduction of the akinesia in stepping test. Furthermore, contralateral intrastriatal application of BoNT-A exhibited a short influence on the apomorphine-induced rotations and led to a transient re-adjustment of right and left forepaw usage in cylinder test. Amphetamine-induced rotations and locomotor activity in OF test stayed unaltered after contralateral BoNT-A-injection.

These temporally restricted effects of BoNT-A-application suggest that intrastriatally applied BoNT-A acted not only as an inhibitor of ACh release but also had impact on transmitter receptors, especially D2 receptor expression and thereby on the basal ganglia circuitry. Evaluation of receptor concentrations of all important striatal transmitters are subject of ongoing studies of our group.

AUTHOR CONTRIBUTIONS

CH, OS and AW designed the study. VAA, AW and AH performed experiments. CH did the statistics and the figures. VAA, CH, AW, OS and AH wrote the manuscript.

FUNDING

Rostock University Medical Center internal funding FORUN 889005 + 889014.

ACKNOWLEDGEMENTS

The technical assistance of Susann Lehmann and Frauke Winzer is gratefully acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnbeh.2017.00119/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Brain Research 1676 (2017) 46–56

Contents lists available at ScienceDirect

Brain Research

journal homepage: www.elsevier.com/locate/bres

Research report

Intrastriatally injected botulinum neurotoxin-A differently effects cholinergic and dopaminergic fibers in C57BL/6 mice

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ARTICLE INFO

Article history:
Received 12 April 2017
Received in revised form 10 August 2017
Accepted 12 September 2017
Available online 15 September 2017

Keywords:
Botulinum neurotoxin-A
Striatum
Basal ganglia
Mouse brain
Unbiased stereology
Interneuron

ABSTRACT

Unilateral intrastriatal BoNT-A injection abolished apomorphine-induced rotational behavior in a rat model of hemiparkinsonism (hemi-PD) up to 6 months. It was hypothesized that the beneficial effect of botulinum neurotoxin-A (BoNT-A) grounded on the reduction of the Parkinson's diseases (PD) associated striatal hypercholinism. Intrastriatal injection of BoNT-A was not cytotoxic in rat brain, but neuronal fiber swellings in the BoNT-A infiltrated striata appeared and named BoNT-A-induced varicosities (BiVs). In the rat BiVs were immunoreactive (ir) either for choline acetyltransferase (ChAT) or tyrosine hydroxylase (TH). In the present study the structural effect of unilateral intrastriatal BoNT-A injection in the naïve mouse brain was analyzed to extend possible therapeutic BoNT-A applications to genetical Parkinsonian strains.

We investigated the effect of a single dose of 25 pg BoNT-A injected into the right caudate-putamen (CPu) for up to 9 months, and of increasing doses up to 200 pg on striatal volume, number of ChAT-ir interneurons, and numeric density and volume of the ChAT-ir BiVs in comparison to the uninjected hemisphere.

Intrastriatal BoNT-A injection did not alter the number of ChAT-ir interneurons irrespective of survival time and dosage tested. However, the numeric density of the ChAT-ir BiVs at a dose of 25 pg increased from 1 to 3 months after BoNT-A, followed by a time dependent decrease. In parallel, with increasing BoNT-A survival time, the mean BiV volume increased as the number of small BiVs decreased. Interestingly, in contrast to rats we did not find TH-ir BiVs in BoNT-A injected mouse striatum.

1. Introduction

Parkinson's disease (PD) is an age-related, chronic and slowly progressive neurodegenerative movement disorder (Dauer and Przedborski, 2003; Fahn and Sulzer, 2004). The neuropathological hallmarks of PD are progressive loss of dopaminergic neurons in substantia nigra pars compacta (SNpc) (Agid and Blin, 1987; Hornykiewicz, 1998), deficit of dopamine (DA) (Drui et al., 2014; Yuan et al., 2010) in the striatum and an increased release of acetylcholine (ACh) by tonically active intrastriatal interneurons (Ding et al., 2006; Obeso et al., 2008; Pisani et al., 2007). The resulting changes in basal ganglia circuitry (Benazzouz et al., 2014; Coffield and Yan, 2009) leads to motor deficits including tremor at rest, muscle rigidity, bradykinesia and postural instability (Obeso et al., 2010; Shin et al., 2014). Pharmacotherapy of PD is mainly based on l-3,4-Dihydroxyphenylalanine (l-DOPA) (Marsden and Parkes, 1977; Sander et al., 2012), possible alternatives are dopamine agonists (Devos et al., 2013; Schapira, 2002). Anticholinergic drugs, used to treat PD beyond l-DOPA, have a good antiparkinsonian effect, nevertheless, they after long term intake exhibit a lot of central and peripheral adverse effects (Clarke, 2002; Connolly and Lang, 2014).

Abbreviations: ACh, acetylcholine; BiVs, Botulinum neurotoxin-A-induced varicosities; BoNT-A, botulinum neurotoxin-A; BoNT-B, botulinum neurotoxin-B; BoNT-C, botulinum neurotoxin-C; ChAT, choline acetyltransferase; CPu, caudate-putamen; DA, dopamine; hemi-PD, hemisparkinsonism; ir, immunoreactive; PD, Parkinson's disease; SNAP-25, synaptosomal-associated protein of 25-kDa; SV2, synaptic vesicle glycoprotein 2; TH, tyrosine hydroxylase; SN, substantia nigra; SNpc, substantia nigra pars compacta; L-DOPA, L-3,4-Dihydroxyphenylalanine; 6-OHDA, 6-hydroxydopamine.

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http://dx.doi.org/10.1016/j.brainres.2017.09.016
0006-8993/

In order to reduce undesirably effects we used in our study a local injection of botulinum neurotoxin-A (BoNT-A) into caudate-putamen (CPu) as an anticholinergic therapy of PD (Antipova et al., 2013; Hawlitschka et al., 2013; Holzmann et al., 2012; Mehlan et al., 2016; Wree et al., 2011). BoNT-A produced by anaerobic bacteria Clostridium botulinum (Matak and Lacković, 2014; Montecucco and Molgó, 2005) is a metalloprotease that enters peripheral motor nerve terminals and blocks the release of ACh via the specific cleavage of the synaptosomal-associated protein of 25-kDa (SNAP-25) (Caleo and Schiavo, 2009; Rossetto et al., 2014). Therefore, BoNT-A is used therapeutically in the treatment of human syndromes associated with cholinergic hyperfunction or movement dysfunctions (Montecucco and Molgó, 2005; Ney and Joseph, 2007).

The direct injection of BoNT-A into CPu was introduced as a local anticholinergic therapy in the hemisparkinsonian (hemi-PD) rat model (Antipova et al., 2013; Hawlitschka et al., 2013; Holzmann et al., 2012; Mehlan et al., 2016; Wree et al., 2011). Ipsilateral intrastriatal injection of 1 ng of BoNT-A in the rat 6-hydroxydopamine (6-OHDA) model abolished apomorphine-induced rotations up to 6 months (Antipova et al., 2013; Wree et al., 2011). Morphologically, intrastriatal BoNT-A led to axonal swellings named botulinum neurtoxin-A-induced varicosities (BiVs). BiVs were immunoreactive (ir) either for ChAT or for TH (Mehlan et al., 2016; Wree et al., 2011). In addition we showed that in the rats intrastriatal BoNT neither significantly changed the volume of the CPu and the total number of striatal neurons nor the number of ChAT-ir interneurons (Antipova et al., 2013; Mehlan et al., 2016; Wree et al., 2011).

As it is known that mice are very sensitive to botulinum toxins and, additionally, differ in many parameters from rats (Corchs et al., 2015; Ellenbroek and Youn, 2016; Jaramillo and Zador, 2014; Klein et al., 2012; Lazarov and Hollands, 2016; Lindström and Korkeala, 2006; Overgaard et al., 2013; Sesardic and Das, 2008; Wheeler et al., 2009) we here studied the effect of intrastriatal BoNT-A application on striatal morphology in näive mice. We evaluated striatal volumes, the number of ChAT-ir neurons as well as the numeric density and the size of the BiVs dependent on post BoNT injection survival and dosage.

In a first experiment, 25 pg BoNT-A was unilaterally injected into the CPu and the long-term effect from 1 month until 9 months after BoNT on various parameters examined. Secondly, the dose dependent efficacy of increasing dosage up to 200 ng BoNT-A at a constant post injection survival of 6 months was studied.

2. Results

2.1. Body and brain weight

Body weight was not significantly influenced by intrastriatal application of 25 pg BoNT-A (Fig. 1A). From 1 to 9 months after BoNT-A body weight slightly but insignificantly increased from 26.70 ± 0.33 g (mean ± SEM) at 1 month, to 29.25 ± 0.67 g at 3 months, to 32.46 ± 0.54 g at 6 months and to 31.62 ± 0.80 g at 9 months. The values 9 months after BoNT-A were not different from those 9 months (34.27 ± 0.63 g) after vehicle application (Fig. 1A). However, the body weight of the mice 6 months after

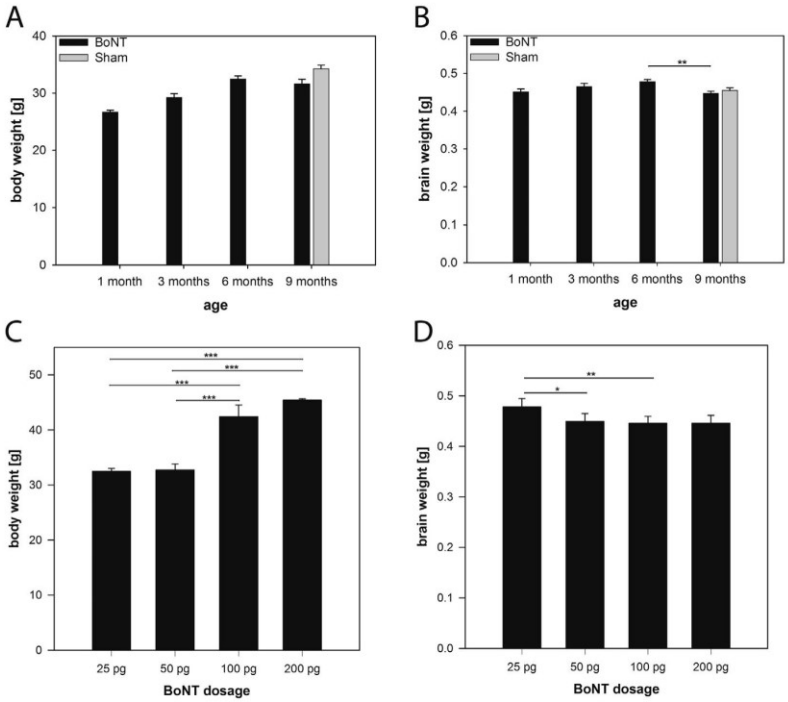

Fig. 1. (A) Body weight and (B) brain weight of mice injected with 25 pg BoNT-A into the right striatum evaluated 1 month, 3, 6 and 9 months after BoNT-A or vehicle (sham) application. (B) Brain weighs of mice 1 to 9 months after 25 pg BoNT-A application were nearly constant. (C) Body weights and (D) brain weights of the mice 6 months after different BoNT-A dosage. (C) Mice injected with 100 pg and 200 pg BoNT-A had higher body weights than mice receiving 25 pg or 50 pg. (D) The brain weights of mice 6 months after dosage of 50 pg and 100 pg BoNT-A showed significantly lower weights compared with those receiving 25 pg BoNT-A. All data are presented as mean values ± SEM. Asterisks indicate significant difference ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

higher BoNT-A dosages differed. 25 pg BoNT-A mice weighted 32.46 ± 0.54 g (mean ± SEM), 50 pg BoNT-A mice 32.73 ± 1.10 g, 100 pg BoNT-A mice 42.41 ± 2.11 g, and 200 pg BoNT-A mice 45.44 ± 0.22 g at 6 months. Mice with dosages of 100 pg and 200 pg BoNT-A had significantly higher body weight than those receiving 25 pg or 50 pg (Fig. 1C).

The brain weight of mice 1–9 months after 25 pg BoNT-A injection were nearly constant: 0.451 ± 0.008 g (mean ± SEM) at 1 month, 0.465 ± 0.008 g at 3 months, to 0.478 ± 0.005 g at 6 months and to 0.439 ± 0.009 g at 9 months. Only the brain weight of the mice 9 months after 25 pg BoNT-A was significantly lower than that after 6 months (Fig. 1B). Brain weight 9 months after BoNT-A was similar to that after the respective vehicle application (0.455 ± 0.007) (Fig. 1B).

The brain weight of mice 6 months after dosages of 50 and 100 pg BoNT-A showed significantly lower measures as compared with those receiving 25 pg BoNT-A. (Fig. 1D). Compared to 25 pg BoNT-A mice having brain weight of 0.478 ± 0.005 g (mean ± SEM), 50 pg BoNT-A mice had 0.450 ± 0.006 g, 100 pg BoNT-A mice had 0.446 ± 0.006 g, and 200 pg BoNT-A mice had 0.446 ± 0.011 g (Fig. 1D).

2.2. CPu volume

The volume of the right and left CPu of mice receiving 25 pg BoNT-A into the right striatum measured at 4 time points (1 month, 3, 6 and 9 months) after BoNT-A or vehicle injection (9 months survival) did not differ significantly between the hemispheres (Fig. 2A). In details, one month after intrastriatal BoNT-A injection (right hemisphere, 25 pg), the volumes of the left CPu were 9.959 ± 0.200 mm^3 (mean ± SEM) and of the BoNT-A injected right CPu were 9.401 ± 0.200 mm^3. Three months after BoNT-A injection, the volumes of the left CPu were 8.826 ± 0.224 mm^3 and of the right CPu 8.429 ± 0.224 mm^3, after 6 months measures for the left CPu were 8.934 ± 0.224 mm^3 and for the right CPu 8.499 ± 0.224 mm^3. Nine months after BONT-A injection, the volumes were for the left CPu 8.422 ± 0.224 mm^3 and for the right CPu 8.095 ± 0.224 mm^3 (Fig. 2A).

However, the CPu volume of mice 6 months after the higher BoNT-A dosage differed: the CPu of mice injected with 50 pg and 200 pg BoNT-A showed significantly reduced volumes compared with the respective contralateral hemisphere (Fig. 2B): 6 month after intrastriatal injection of 25 pg BoNT-A the volumes of the left CPu were 8.934 ± 0.224 mm^3 (mean ± SEM) and for the right CPu 8.499 ± 0.224 mm^3. Mice injected with 50 pg BoNT-A had volumes of the left CPu of 10.751 ± 0.583 mm^3 and of the right CPu of 8.039 ± 0.583 mm^3, those injected with 100 pg BoNT-A had volumes of the left CPu of 8.662 ± 0.583 mm^3 and of the right CPu of 8.023 ± 0.583 mm^3. In the 2 mice composing the 200 pg BoNT-A group CPu volumes were in the left hemisphere 10.314 ± 0.923 mm^3 and in the right 6.918 ± 0.923 mm^3, the latter being significantly smaller than the 25 pg BoNT-A injected striatum.

2.3. Number of ChAT-ir neurons

Inspection of ChAT reacted sections revealed no obvious changes of cholinergic perikarya in the BoNT-A injected CPu (Figs. 3 and 4A–D, F–H) compared with the contralateral hemisphere (Figs. 3 and 4E).

The number of striatal ChAT-ir neurons of mice injected with 25 pg BoNT-A into the right CPu estimated after a survival of 1 month, 3, 6 and 9 months after BoNT-A or vehicle did not significantly differ between the right and left CPu over the whole time span of the experiment (Fig. 5A). One month after 25 pg BoNT-A into the right CPu, the number of ChAT-ir neurons was

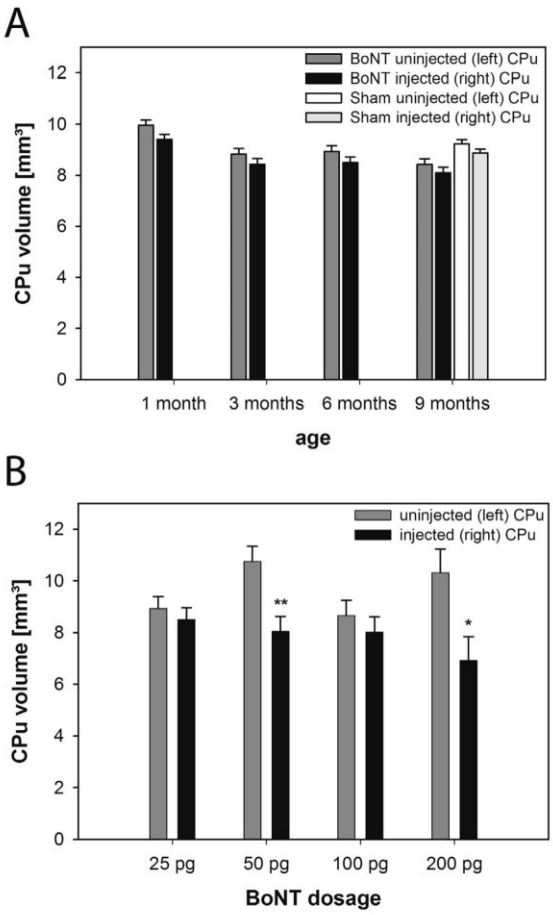

Fig. 2. (A) Volumes of the left and right CPu of the mice injected with 25 pg BoNT-A into the right striatum measured 1 month, 3, 6 and 9 months after BoNT-A or vehicle (sham) application. Volumes were not significantly different between the both hemispheres irrespective of survival time. (B) CPu volumes of mice that received 25 pg, 50 pg, 100 pg and 200 pg BoNT-A into the right striatum measured 6 months later. CPu of mice injected with 50 and 200 pg BoNT-A showed significantly reduced CPu volumes compared with the contralateral hemisphere. All data are presented as mean values ± SEM. Asterisk indicate significant difference ($^{*}P < 0.05$, $^{**}P < 0.01$).

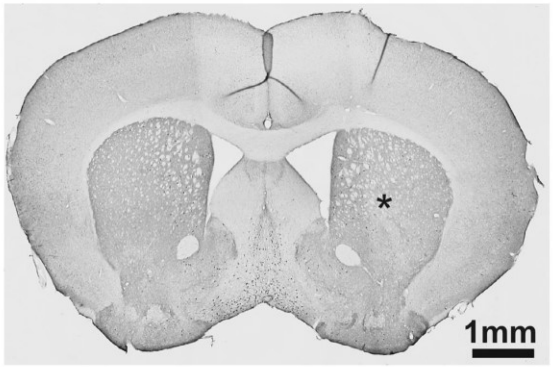

Fig. 3. Frontal section immunohistochemically reacted for ChAT. The asterisk indicates the position of the tip of the BoNT- injection cannula.

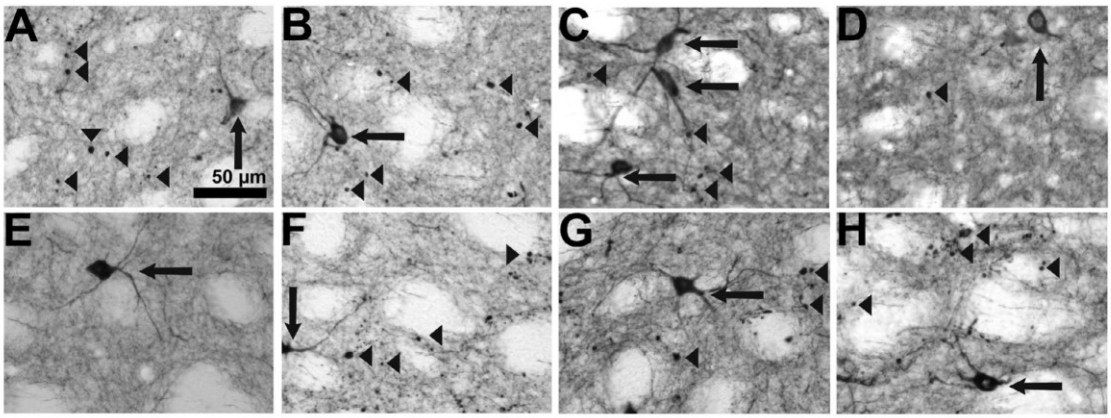

Fig. 4. Photomicrographs of immunohistochemical stainings for ChAT of mice striata: cholinergic interneurons are marked by black arrows and ChAT-ir BiVs are indicated exemplarily by black arrow heads. (A-D) Mice received 25 pg BoNT-A per striatum. Animals survived 1 month (A), 3 (B), 6 (C) and 9 months (D). (E) CPu was injected with the vehicle substance. (F-H) Mice survived intrastriatal injection of 50 pg (F), 100 pg (G) and 200 pg (H) BoNT-A for 6 months.

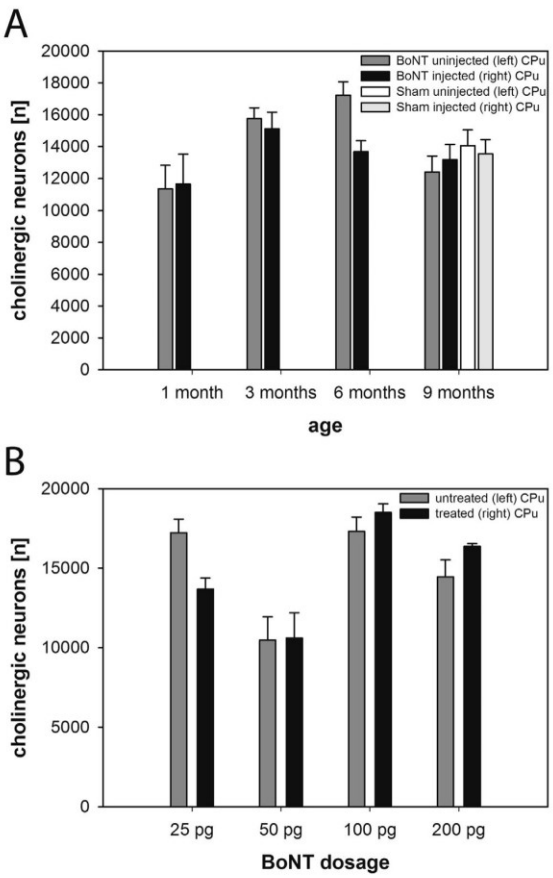

Fig. 5. (A) Number of striatal ChAT-ir neurons of mice injected with 25 pg BoNT-A into the right CPu estimated 1 month, 3, 6 and 9 months after BoNT-A or vehicle (sham), and (B) number of ChAT-ir neurons from mice receiving 25–200 pg BoNT-A into the right striatum and sacrifice 6 months later. Irrespective of dosage and survival times the quantity of ChAT-ir neurons in the CPu was unaltered. All data are presented as mean values ± SEM.

11338 ± 1137 (mean ± SEM) in the intact left CPu and 11645 ± 1137 in the BoNT-A injected hemisphere. Three months after injection of BoNT-A, the number of ChAT-ir neurons were 15749 ± 1271 in the left CPu and 15111 ± 1271 in the right CPu. Six months after BoNT-A injection we counted 17223 ± 1271 ChAT-ir neurons in the left hemisphere and 13676 ± 1271 neurons in the right CPu. Nine months after BoNT-A, the CPu of the left hemisphere contained 12399 ± 1271 and that of the right hemisphere 13174 ± 1271 ChAT-ir neurons (Fig. 5A). Right side vehicle-injected animals exhibited in the left CPu 14066 ± 932 and in the right hemisphere 13540 ± 932 ChAT-ir neurons (Fig. 5A).

The number of ChAT-ir neurons in mice receiving increasing BoNT-A up to 200 pg into the right CPu and sacrifice 6 months later were predominantly constant. The right CPu of the 50 pg animal group contained 10617 ± 3513 and the left CPu 10474 ± 3290 neurons. The 100 pg group exhibited in the right CPu 18498. ± 1216 ChAT-ir neurons and 17309 ± 2015 in the contralateral CPu. Animals, injected with 200 pg BoNT-A had 16368 ± 235 on the right side and 14455 ± 1511 ChAT-ir neurons on the left side (Fig. 5B). Moreover, it can be seen that the number of ChAT-ir neurons in the striatum generally showed a considerable interindividual variability (Fig. 5A and B).

2.4. Botulinum neurotoxin-A-induced varicosities (BiVs)

2.4.1. Numeric density of ChAT-ir BiVs

In the ChAT reacted sections BiVs were seen in all BoNT injected striata, however, varying in number and size (Fig. 4A–D, F–H). BiVs were never seen in the contralateral and in vehicle injected striata (Fig. 4E). After intrastriatal injection of 25 pg BoNT-A into the right CPu we counted the number of BiVs per mm^3 (Fig. 4A–D, F–H) and found 2094 ± 367 BiVs/mm^3 (mean ± SE) after 1 month, 3230 ± 307 BiVs/mm^3 after 3 months, 1908 ± 165 BiVs/mm^3 after 6 months, and 749 ± 74 BiVs/mm^3 after 9 months (Fig. 6A). Thus, the numeric density of BiVs was maximal at 3 months after BoNT and thereafter significantly decreased (Fig. 6A).

The numeric density of ChAT-ir BiVs from mice receiving increasing BoNT-A dosage from 25 to 200 pg into the right CPu and sacrificed 6 months later were as follows: 25 pg BoNT-A induced 1908 ± 165 BiVs/mm^3, 50 pg induced 5066 ± 464 BiVs/mm^3, 100 pg induced 4056 ± 732 BiVs/mm^3, and 200 pg induced 3148 ± 767 BiVs/mm^3, respectively (Fig. 7A). Thus, the numeric density of BiVs was maximal 6 months after BoNT at a dose of

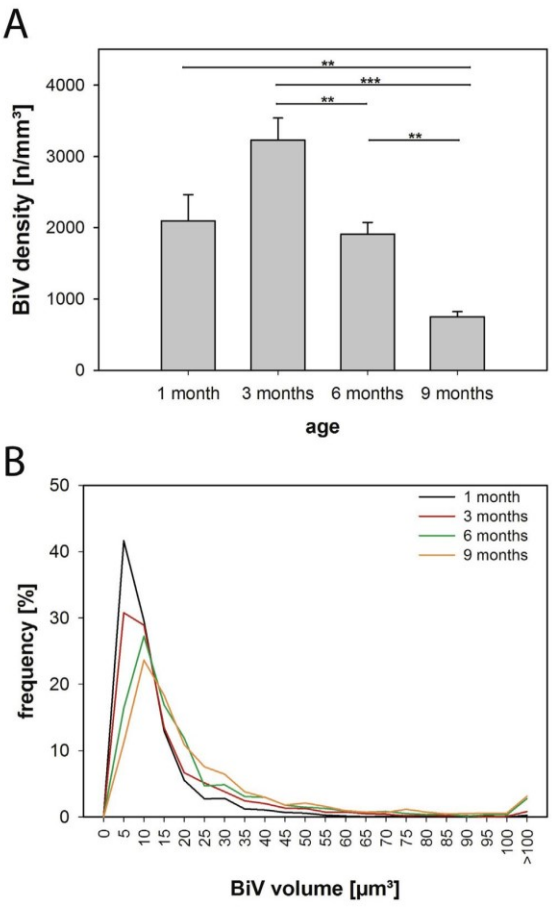

Fig. 6. Numeric density, volumes of single ChAT-ir BiVs and their relative volume distribution from mice receiving 25 pg BoNT-A into right CPu measured 1 month, 3, 6 and 9 months after BoNT-A injection. (A) The maximal number of ChAT-ir BiVs was found 3 months post injection and then decreased over time. Data are presented as mean values ± SEM. Asterisks indicate significant difference ($^{**}P < 0.01$, $^{***}P < 0.001$). (B) Histogram of ChAT-ir BiV volume distribution. Percentage of distinct single BiV volumes of the all measured BiVs per time group. The percentage of volume categories in 5 μm^3 steps was created. The rate of single BiVs with small volumes up to 15 μm^3 is highest in striata after 1 month survival and declined with longer post injection times.

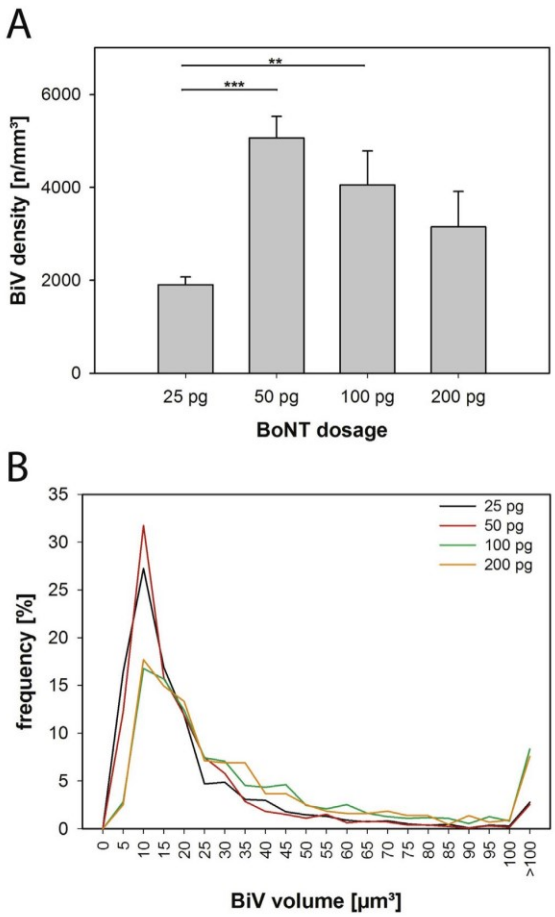

Fig. 7. Numeric density, volumes of single ChAT-ir BiVs and their relative volume distribution from mice that received 25 pg, 50 pg, 100 pg and 200 pg BoNT-A into the right striatum measured 6 months later. (A) The maximal number of ChAT-ir BiVs was found in mice injected with 50 pg BoNT-A. Data are presented as mean values ± SEM. Asterisks indicate significant difference ($^{**}P < 0.01$, $^{***}P < 0.001$). (B) Histogram of ChAT-ir BiV volume distribution. The percentage of volume categories in 5 μm^3 steps is shown. The rate of single BiVs with small volumes up to 15 μm^3 is highest in striata injected with the lowest dose of 25 pg BoNT-A, and decreased with increasing dosage.

50 pg BoNT and then declined with higher BoNT-A dosage of 100 pg and 200 pg (Fig. 7A).

2.4.2. Volume of ChAT-ir BiVs

Injection of 25 pg BoNT-A resulted in a median BiV volume of 5.87 μm^3 after 1 month, 7.82 μm^3 after 3 months, 11.42 μm^3 after 6 months, and 13.70 μm^3 after 9 months (data not shown). The median volume of single ChAT-ir BiVs increased significantly with increasing survival time up to 9 months (data not shown).

The rate of single BiVs with small volumes up to 15 μm^3 was highest in striata 1 month and lowest in those 9 months after BoNT-A (Fig. 6B). The ratio completely inverted for larger BiVs. For BiV volumes from 30 to 40 μm^3, the ratio decreased with increasing survival times (Fig. 6B).

In the mice 6 months after having received different amounts of BoNT-A into the right CPu the median volumes of single ChAT-ir BiVs increased dose-dependently from the 25 pg to 200 pg. The median volumes were 11.42 μm^3 following 25 pg BoNT-A, 11.62 μm^3 after 50 pg, 21.66 μm^3 after 100 pg, and 20.82 μm^3 after 200 pg (data not shown).

The respective histogram of perceptual distribution of BiV volumes revealed (Fig. 7B) that with higher dosage the relative quantity of small BiVs up to 15 μm^3 was reduced, while the larger ones with more than 25 μm^3 increased.

2.4.3. TH-ir BiVS

Serial sections from all mouse brains were also immunoreacted with TH-antisera, but contrary to ChAT staining (Fig. 4A–D, F–H) we never found TH-ir BiVs after intrastriatal BoNT-A application, although a dense TH-ir fiber network was clearly present in the CPu of mice. Therefore, in order do directly compare the occurrence of ChAT- and TH-ir BiVs in rats and mice, we injected exactly the same amount of BoNT-A (100 pg) unilaterally into the CPu of both species and reacted brain slices 6 month later. In the rat brain

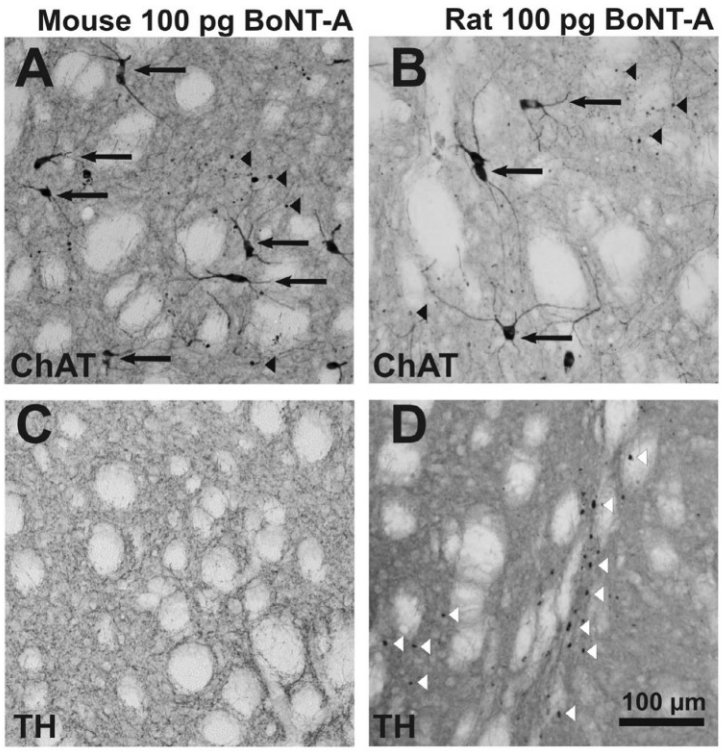

Fig. 8. Photomicrograph of immunohistochemical stainings of striata treated with 100 pg BoNT-A. Survival time was 6 months. (A) and (C) are from a mouse striatum, (B) and (D) are from a rat. (A) and (B) were immunoreacted for ChAT, (C) and (D) for TH. Black arrows indicate cholinergic interneurons. Black arrow heads mark exemplarily cholinergic BiVs, white arrow heads TH-ir BiVs.

100 pg BoNT-A induced ChAT-ir BiVs (Fig. 8B) as well as TH-ir BiVs (Fig. 8D). In the BoNT-A injected mouse striata comparable ChAT-ir BiVs were seen (Fig. 8A), but no TH-ir BiVs were demonstrable in a dense TH-ir fiber network (Fig. 8C).

3. Discussion

Our group previously studied the effect of intrastriatal injection of BoNT-A against the background of BoNT-A as a therapeutic option for a local anticholinergic therapy in the hemi-PD rat model (Antipova et al., 2013; Hawlitschka et al., 2013; Holzmann et al., 2012; Mehlan et al., 2016; Wree et al., 2011). In the present study we wanted to extend our knowledge of intrastriatal BoNT-A injections in a first step to the naive mouse. Mice seemed important since in experimental Parkinson research especially mouse lineages with mutations or knockouts of relevant PD-associated genes are under consideration (Bezard et al., 2013; Cremer et al., 2015a,b; Deng and Yuan, 2014; Pickrell et al., 2013). Moreover, as rat and mouse brains differ in several major aspects (Bartelle et al., 2016; Klein et al., 2012; Overgaard et al., 2013), i.e. rats are not simply "big mice", possible difference in the reaction to BoNT-A application seemed interesting (Ellenbroek and Youn, 2016; Francis et al., 2014).

As a first step, the effective and well tolerable BoNT-A dose for intrastriatal injection in mice was detected. In rat brain Antonucci et al. injected the superior colliculus with 225 pg BoNT-A (Antonucci et al., 2008), our group applied 1 ng into the rat CPu (Wree et al., 2011). In mice, the median lethal dose for different intraperitoneally injected BoNT serotypes was in the range 0.1–1.0 ng toxin/kg body weight (Gill, 1982). Subsequently, for a mouse weighing 25 g we calculated a dosage of 25 pg for intrastriatal injection. Others used different dosage: in adult C575L/6N mice Antonucci et al. (Antonucci et al., 2008) applied 0,2 µl of a 10 nM BoNT-A solution (=300 pg) into the hippocampus. Luvisetto et al. injected BoNT-A and BoNT-B into the lateral cerebral ventricle of CD1 mice (Luvisetto et al., 2003) and extrapolated the LD50 for BoNT-A and BoNT-B to 0.5–1.0 ng/kg body weight. Doupling of the dosage led to death of the mice.

3.1. Body and brain weight

Following 25 pg BoNT-A injection body weight was not significantly influenced up to 9 months survival. However, mice injected with 100 pg and 200 pg BoNT-A showed increased weight compared to mice receiving lower dosage of 25 or 50 pg BoNT-A after a survival of 6 months. Possibly, in the high dose mice, the injected BoNT-A had diffused into the region of the hypothalamus. There it could have cleaved SNAP-25 that is known to be essential for exocytosis of leptin (Mora and Pessin, 2002; Stuber and Wise, 2016). Subsequently, due to lower leptin levels, mice developed obesity. In the rat an effect of 1 ng of intrastriatal BoNT-A on the body weights was not measured in our experiments (data not shown) perhaps because the rat brain is fifth fold larger than the mouse brain, and by this BoNT-A diffusion did not reach the hypothalamus. Alternatively, mice were generally more sensitive to BoNT-A than rats (Lindström and Korkeala, 2006; Sesardic and Das, 2008; Wheeler et al., 2009).

The brain weight of mice 1–9 months after 25 ng BoNT-A injection persisted nearly constant resembling sham injected mice. The small but significant 6% reduction of brain weight in mice with

dosage from 50 to 200 pg BoNT as compared to the 25 pg BoNT-A mice speak in favor of a mild degeneration process. Tentatively, that could also be seen in Fig. 3 as the lateral ventricle on the injected hemisphere seems slightly widened.

3.2. CPu volume

The volumes of the right CPu and left CPu of mice receiving 25 pg BoNT-A into the right striatum measured during 9 months after BoNT-A or vehicle did not differ significantly between the hemispheres. Corresponding to the respective brain weight, the CPu volume in mice with dosage of 50 and 200 pg BoNT was slightly reduced as compared to the 25 pg BoNT-A mice. Thus, it can be speculated that the reduction in CPu volume in part caused the reduction in brain weight. As yet brain weight of mice after intracerebral injection of various BoNTs was not determined (Antonucci et al., 2008, 2009; Luvisetto et al., 2003), measures could only be discussed with respect to quantitative data obtained by our group in rats (Antipova et al., 2013). In rats 1 ng BoNT-A injected intrastriatally did not change striatal volume significantly (Antipova et al., 2013) again speaking in favor of marked susceptibility of mice to intracerebral BoNT application.

3.3. Number of ChAT-ir neurons

The number of striatal ChAT-ir neurons showed a considerable interindividual variability ranging between 11000 and 15000. As to our knowledge no data jet exist on the total number of ChAT-ir neurons in mouse CPu, we for comparison took data from rat brain. Assuming that as in the rat about 1–2% of all striatal neuron were ChAT-ir (Kemp and Powell, 1971; Phelps et al., 1985), our data well correspond to Rosen and Williams (Rosen and Williams, 2001) who published the total number of all neurons in the various mouse strains in the CPu ranging from 1.4 to 2.5 million and specifically in C57BL/6J mice being 1.720 ± 0.015 million per hemisphere.

Intrastriatal BoNT-A injection in mice did not alter the number of ChAT-ir interneurons irrespective of survival time and dosage tested. These data obtained in mice reinforced the results found after 1 ng BoNT-A injection into the rat CPu where the total number of striatal neurons as well as the ChAT-ir neurons therein were unaffected (Antipova et al., 2013; Mehlan et al., 2016). We can conclude that intrastriatal BoNT-A did not cause death of ChAT-ir neurons in mouse and rat striata.

3.4. Numeric density and volume of ChAT-ir BiVs

The ChAT-ir BiVs previously described in BoNT-A injected striata of rats (Mehlan et al., 2016; Wree et al., 2011) were also found in mice. The appearance of BiVs was discussed as a neuropathologic phenomenon (Mehlan et al., 2016; Wree et al., 2011). Due to the BoNT induced cleaving of the SNARE protein SNAP-25 we hypothesized that the exocytosis of neurotransmitter-containing synaptic vesicles is blocked (Costantin et al., 2005; Schiavo et al., 2000), and thus unreleased vesicles accumulated together with other material in neuronal processes (Wree et al., 2011). However, the exact mechanisms by which the BiVs were generated by BoNT-A are still obscure. It has still to be clarified whether there is a relationship of BiVs with the known anterograde, retrograde and transsynaptic transport of BoNT-A or it's cleavage products (Antonucci et al., 2008; Hong et al., 2017; Restani et al., 2011). Interestingly, in BoNT-C treated short term cultured cerebellar granule cells Berliocchi et al. described enlarged varicosities and blebs in the form of pearl like structures appearing along neurites as signs of neurite degeneration with cytoskeletal and mitochondrial disarray (Berliocchi et al., 2005).

We found that 1 month after 25 pg BoNT-A injection a medium number of small BiVs the majority of BiVs ranging in size from 5 to 15 μm^3. Three months after BoNT-A the numeric density of BiVs increased, the median volume increased and the relative percentage of small BiVs decreased. Combining the 3 parameters we assume BoNT-A induced time dependent accumulation of vesicles and possibly degeneration products in the neurites. After even longer survival times the combination of a decrease in number of BiVs, an increase in median volume and a decrease in relative percentage of small BiVs speaks for a teardown of small BiVs and a further accumulation of larger ones that survive longer. Further assuming that the effect of BoNT-A as a functional blocker of synaptic transmission is correlated to the number of BiVs our data nicely fit with our results in behavioral changes of BoNT-A treated hemi-PD rats. Maximal therapeutic effect was seen 1 and 3 months after BoNT-A, 6 months following BoNT-A rotation behavior was less influenced, and 9 months after BoNT-A the therapeutic effect was reversed (Antipova et al., 2013; Wree et al., 2011). Taking also into account the duration of the BoNT effect application in humans up to about 4 months (Chen, 2012; Orsini et al., 2015; Rossetto et al., 2001) evidence at least in the peripheral nervous system exist that the degenerated and subsequently removed BiVs were replaced by intermittent axonal sprouting and reformation of functional presynaptic structures (Benecke and Dressler, 2007; de Paiva et al., 1999).

Investigating the dose-depending efficacy of BoNT-A on the numeric density and volume of BiVs 6 months after BoNT-A revealed that the numeric density following dosage of 50–200 pg exceeded that after 25 pg and that the median volume increased with increasing dosage. It could be speculated that the toxic effect of BoNT-A increased with higher doses leading to more and larger BiVs. The results obtained in mice are in line with measures from rat studies (Wree et al., 2011): In the rat the numeric density of BiVs drastically increased with increasing BoNT-A dose. Furthermore, respective functional data showed that increasing BoNT-A dosage increased the therapeutic effect on rotation behavior (Wree et al., 2011) and forelimb usage (Antipova et al., 2013).

3.5. Absence of TH-ir BiVs in C57BL/6 mice

In mice TH-ir BiVs were never found following intrastriatal BoNT-A application. This was in contrast to respective BoNT-A applications in rat CPu where always plenty of TH-ir BiVs were seen. In order to look for further possible differences in the reaction of the dopaminergic system of mice and rats we evaluated the substantia nigra (SN) TH-ir neurons in both hemispheres of mice and rats 9 months after having received well tolerated BoNT-A dosage into the right CPu (Fig. 9A–C). In mice (n = 7) injected with 25 pg BoNT-A the number of TH-ir neurons per section in the SN did not differ between the hemispheres (left: 159.3 ± 13.9, right: 171.4 ± 9.8). In rats (n = 7) injected with 1 ng BoNT-A the number of TH-ir neurons per section in the SN also did not differ between the hemispheres (left: 218.2 ± 16.4, right: 220.0 ± 21.2) (Fig. 9D). Thus, neither in mice nor rats striatal BoNT-A affected the number of TH-ir neurons in the SN. The absence of TH-ir BiVs in the mouse brain seemed astonishing as BoNT-A was shown to block synaptic transmission equally in mouse and rat not only of ACh (Bigalke et al., 1985; Gasior et al., 2013; Weisemann et al., 2016), but also of other neurotransmitters including GABA, DA, serotonin, glutamate, glycine and norepinephrine (Ashton and Dolly, 1988; Bozzi et al., 2006; Mahrhold et al., 2006). The obvious difference of the BoNT-A effect between mouse and rat might relay on the SV2C receptor being mainly responsible for the internalization of BoNT-A (Dong et al., 2006; Kroken et al., 2016). Although much is known about the SV2C receptor, unfortunately no data exist concerning differences in affinity or susceptibility in rat and mouse

striatum (Dardou et al., 2013, 2011; Dong et al., 2006; Kroken et al., 2016). The relationship of BoNT-A and BiVs should be clarified in long time cell culture experiments in which cholinergic and dopaminergic neurons of mice and rats are exposed to different concentrations of BoNT-A. Theoretically, neuron- or transmitter-specific differences would be not surprising, as rats and mice considerably differ in many aspects of the nervous system (Klein et al., 2012; Lazarov and Hollands, 2016; Overgaard et al., 2013). Further experiments will have to examine whether the structural alterations induced by BoNT-A in the dopaminergic terminals in mice and rats are reflected by behavioral differences. Furtermore, the present experiments show that extending the results up to now after intrastriatal BoNT-A obtained in experimental hemiparkinsonian rats (Wedekind et al., 2017; Wree et al., 2011) probably cannot simply transferred to genetical Parkinsonian strains.

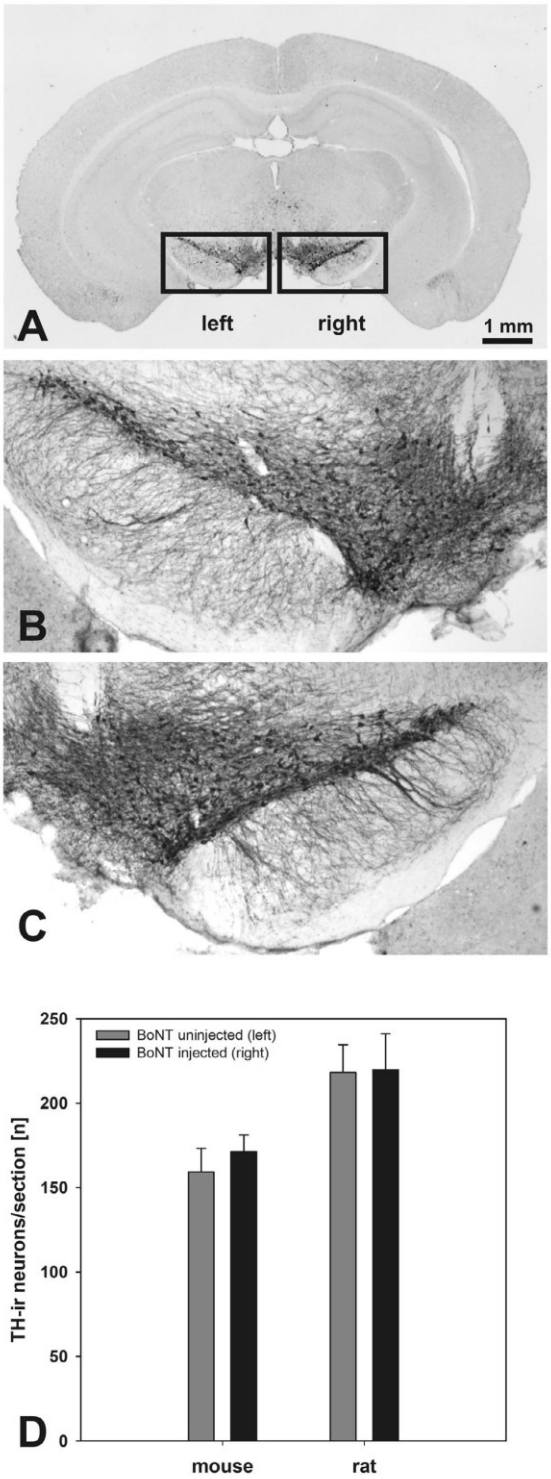

Fig. 9. (A-C) Immunohistochemical staining for TH of mouse SN 9 months after right side intrastriatal BoNT-A injection. (A) Overview of a frontal section. (B) and (C) Higher magnifications of the left and right SNs. (D) Semiquantitative estimation of the number of TH-ir neurons in selected 30 μm thick sections of the SN in both hemispheres of mice and rats 9 months after right side intrastriatal BoNT-A injection. Mice were injected with 25 pg, rats with 1 ng BoNT-A. Data are presented as mean values ± SEM.

4. Conclusion

25 pg BoNT-A injected into the striatum of mice had no significant effect on the volume of mouse striatum and the number of ChAT-ir interneurons therein. Dosage of 50–200 pg BoNT-A mildly reduced CPu volume but did not influence the number of ChAT-ir neurons. Intrastriatal BoNT-A induced ChAT-ir BiVs exhibited the highest numeric density 3 months after BoNT-A. With longer post injection survival time the number of BiVs decreased, whereas following increasing BoNT-A dosage the median size of the solitaire ChAT-ir BiVs increased. TH-ir BIVs were never detected in BoNT-A injected striata.

The present results extended the knowledge about the effect of intrastriatal BoNT-A injection. Also in naïve mice intrastriatal BoNT-A injection had long-lasting morphological effects on cholinergic structures, however, dopaminergic terminals were spared. Further experiments will have to evaluate structural and behavioral consequences of therapeutic BoNT-A applications in mice of lineages with mutations or knockouts of relevant PD-associated genes.

5. Experimental procedures

5.1. Animals

Jung adult male C57BL/6-mice (Charles River Wiga, Sulzfeld, Germany) weighing 18–24 g at the time point of the surgery were used. All mice (n = 53) were housed in standard cages in a temperature-controlled room (22 °C ± 2 °C) and under 12 h light/12 h dark conditions with free access of food and water. All procedures were approved by the State Animal Research Committee of Mecklenburg-Western Pomerania (LALLf M-V/TSD/7221.33-1.1-053/08, LALLf M-V/TSD/7221.3-1.1-003/13).

5.2. BoNT-A application

All surgery was done under deep anesthesia with ketamine (75 mg/kg, bela-pharm Vechta, Germany)/xylazine (5.8 mg/kg, Rompun®, Bayer, Germany) using a stereotactic frame (Kopf®, Tujunga, CA, USA) equipped with a mouse adaptor from Stoelting (Wood Dale, USA). Mice received an injection of 1 μl BoNT-A solution (Lot No. 13028A1A; List, Campbell, USA, purchased via Quadratech Diagnostics, Surrey, United Kingdom) containing a

total of 25 pg, 50 pg, 100 pg or 200 pg BoNT-A dissolved in PBS + 0.1% BSA into the right CPu delivered over 4 min (Fig. 3). The control group received 1 μl BoNT-A vehicle solution. The injection coordinates with reference to bregma were: ap +0.65 mm, lateral −1.6 mm and vertical −3.0 mm from dura, respectively (Paxinos and Franklin, 2001). In young adult male rats, a total dose of 1 ng BoNT-A was injected at the following coordinates according to bregma: anterior-posterior =+1.3 mm/−0.4 mm, lateral = −2.6 mm/−3.6 mm and ventral = −5.5 mm/−5.5 mm (right CPu) (Paxinos and Watson, 2005; Wree et al., 2011).

5.3. Histochemistry

For histological examination, animals were killed with overdose of ketamine/xylazine and transcardially perfused with 0.9% sodium chloride, followed by 3.7% paraformaldehyde (PFA) solved in phosphate-buffered saline (pH 7.4). Brains were immediately removed from the skull, postfixed in 3.7% PFA overnight (4 °C), cryprotected in 20% sucrose in PBS for 1 day (4° C), and frozen at −50 °C. Frontal 30 μm thick brain slices were cut serially with cryostat (Leica, Germany).

Different series were stained with cresyl violett (Nissl stain) or using diaminobenzidin (DAB)-based immunohistochemistry for ChAT or TH. Briefly, cholinergic structures were immunostained using a polyclonal goat anti-ChAT affinity purified antibody (Millipore, Schalbach, Germany 1:200), followed by incubation of rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA, 1:250). Staining of catecholaminergic structures occurred by incubation of a monoclonal sheep anti-TH antibody (Sigma-Aldrich, St. Louis, MO, USA 1:1000) followed by incubation of biotinylated anti-sheep IgG (Vector Laboratories, Burlingame, CA, 1:200). The methods of staining have previously been described (Wree et al., 2011).

5.4. Unbiased stereological analysis

The immunohistochemically stained brain slices were examined stereologically using the program Stereo Investigator 8.0. Striatal volumes, number of cholinergic neurons and the numeric density of BiVs where calculated by unbiased counting by means of the optical fractionator principle.

5.5. Number of ChAT-ir neurons, numeric density and volume of ChAT-ir BiVs

In mice (n = 43) that received 25 pg of BoNT-A (n = 34) or vehicle (n = 9) unilaterally into the right CPu we measured the number of ChAT-ir neurons at 4 time points (1 month (n = 10), 3 (n = 8), 6 (n = 8) and 9 months (n = 8)) after BoNT-A in the striata of both hemispheres. Furthermore, we analyzed the influence of different unilateral intrastriatally applied BoNT-A dosages (50 pg (n = 5), 100 pg (n = 5), and 200 pg (n = 2)) on the number of ChAT-ir neurons in both CPu in mice 6 months post BoNT injection. The numeric density and volume of ChAT-ir BiVs was measured in the right CPu as they were not present contralaterally.

5.6. Volumetric analysis

In all mice the volumes of the left and right CPu of each animal were assessed in Stereo Investigator 8.0 using the tracing data from each CPu and the measured section thickness. Region volumes of all sections of a subject were added to obtain the total volume of the CPu.

5.7. Semiquantitative estimation of TH-ir neurons

In mice and rats nine months after right side intrastriatal injection of 25 pg or 1 ng BoNT-A the number of TH-ir neurons in the SN were counted in left and right brain stem, evaluating two to three representative sections per animal.

5.8. Statistical analysis

For stereology, a one-way ANOVA was used to analyze the morphometric quantities with the post hoc Bonferroni and Student-Newman-Keuls tests. $P < 0.05$ was considered statistically significant. The mean coefficient of error of optical fractionator estimates was calculated according to the method of Gundersen and Jensen (Gundersen and Jensen, 1987) and was <0.05 in all analyses.

Prior to usage of parametric statistical tests data were checked for equal variance using the Levene Median Test and subjected to the Kolmogorov-Smirnov test (with Lilliefors' correction) to test data for normality. In case of normal distribution data were then subjected to one-way or two-way ANOVA. The Holm-Sidak multiple comparison approach was used to determine which groups were different. A critical value for significance of $P \leq 0.05$ was used throughout the study. In case of non-normally distributed data, data were subjected to Kruskal-Wallis one- or two-way ANOVA on ranks. Dunn's test was used for post hoc comparisons after ANOVA on ranks to adjust for multiple testing. All statistical tests were done using SigmaPlot 11 Software.

Acknowledgement

We gratefully acknowledge Ms. Susan Lehman and Ms. Frauke Winzer for perfect technical support.

Conflict of Interest

The authors declare no conflict of interest.

Individual contribution of authors

AH, OS, VA and AW designed the study, AH, VA and AW performed the experiments, SW, JS, AMN and OS made the stereological evaluations, CH did the statistics and the figures, AH, CH, OS, AW and VA wrote the manuscript.

Funding statement

Rostock University Medical Center internal funding FORUN 889005 and 889014.

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Article

Repeated Intrastriatal Botulinum Neurotoxin-A Injection in Hemiparkinsonian Rats Increased the Beneficial Effect on Rotational Behavior

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Received: 22 August 2018; Accepted: 8 September 2018; Published: 11 September 2018

Abstract: Injection of botulinum neurotoxin-A (BoNT-A) into the striatum of hemiparkinsonian (hemi-PD) rats reduced apomorphine-induced rotation behavior significantly, for at least 3 months. Thereafter, rotation behavior increased again. We injected hemi-PD rats with 1 ng BoNT-A twice, the second injection following 6 months after the first one and tested the rats for apomorphine-induced rotations and spontaneous motor behaviors, i.e., corridor task and stepping test. To test the hypothesis that BoNT-A reduced striatal hypercholinism in hemi-PD rats, the acetylcholinesterase inhibitor donepezil was injected prior to separate apomorphine-induced rotation tests. In hemi-PD rats, the first BoNT-A injection led to a clear reduction of the apomorphine-induced rotations, and the second BoNT-A injection to a more massive and prolonged reaction. In hemi-PD rats whose apomorphine-induced rotation behavior was strongly reduced by an intrastriatal BoNT-A, subsequent donepezil injections led to significant increases of the rotation rate. Concerning corridor task and stepping test, neither first nor second BoNT-A injections changed hemi-PD rats' behavior significantly. The data give evidence for the possibility of repeated intrastriatal administrations of BoNT-A, for treatment of motor symptoms in experimental hemi-PD over a longer time.

Keywords: botulinum neurotoxin-A; striatum; basal ganglia; donepezil; acetylcholine; 6-OHDA

Key Contribution: In hemiparkinsonian rats the temporal positive effect of repeated intrastriatal BoNT-A application on apomorphine-induced rotations and spontaneous motor behaviors was studied for the first time.

1. Introduction

Parkinson's disease (PD) is the most frequent neurodegenerative movement disorder, which mainly affects movement ability. PD is associated with a loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), leading to a lack of dopaminergic inhibition of tonically active cholinergic interneurons in the striatum (CPu) (Figure 1A,B). This causes a hypercholinism in the CPu, which is thought to contribute to the majority of motor symptoms in PD [1–5] (Figure 1B). Classic anticholinergic treatments ameliorate motor symptoms of PD, but acting systemically they also entail numerous unwanted side effects [6–9].

Botulinum neurotoxin-A (BoNT-A) cleaves synaptosomal-associated protein-25 (SNAP-25), a component of the vesicle fusion apparatus of the cholinergic presynaptic membrane. Therefore,

it inhibits the release of acetylcholine (ACh) in the peripheral nervous system [10,11], and as we hypothesized in the central nervous system as well [12] (Figure 1C). Intrastriatal injection of 1 ng BoNT-A in hemiparkinsonian (hemi-PD) rats reverses apomorphine-induced rotations for at least 3 months [12–17]. During a time frame of 12 months after BoNT-A treatment, hemi-PD rats showed a gradual recurrence of the apomorphine-induced rotation rate [12–14].

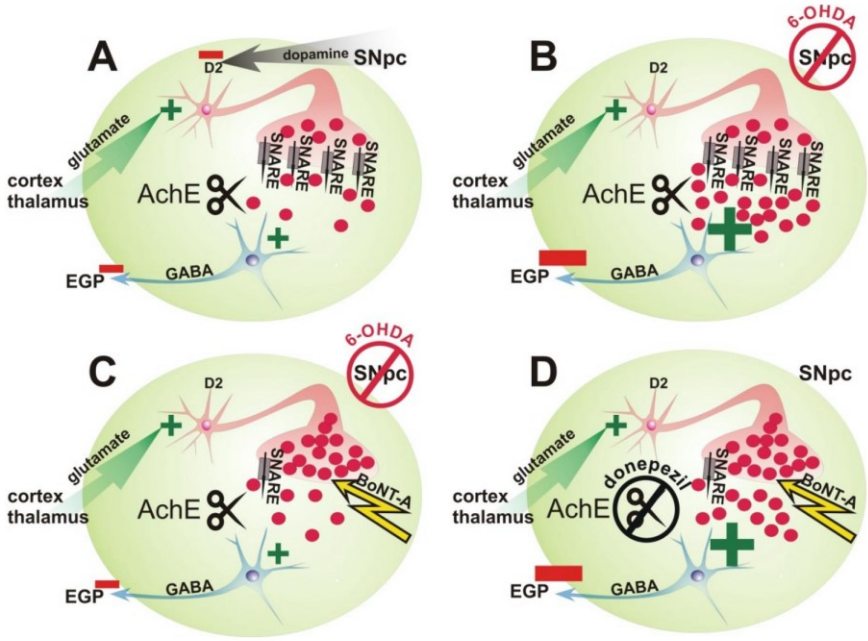

Figure 1. Theoretical concept of the ACh-dependency of the reduction of the apomorphine-induced rotation rate after unilateral BoNT-A treatment of hemiparkinsonian (hemi-PD) rats. (**A**–**D**) Scheme of the indirect pathway of the basal ganglia circuitry. Neurons, efferences and afferences of the striatum (green oval area) relevant for our experiments are shown. A large cholinergic interneuron is depicted with a stylized presynapse (red), the red circles representing ACh. The rectangles with an arrow (SNARE) symbolize the entirety of the *N*-ethylmaleimide-sensitive-factor attachment receptor complex, which conveys the fusion of transmitter vesicles with the presynaptic membrane. The scissors named with "AChE" represent acetylcholinesterase which cleaves ACh, and the blue neuron a medium sized spiny projection neuron (MSN), which inhibits the globus pallidus externus (EGP) neurons by GABA. (**A**) Under normal conditions tonically active cholinergic interneurons excite MSNs by release of ACh. These cholinergic interneurons are excited by glutamatergic input of motor, sensory, and prefrontal cortices and thalamus. Owing to their D_2 receptors, cholinergic interneurons are inhibited by dopaminergic input from the substantia nigra pars compacta (SNpc). (**B**) After lesion of the SNpc with 6-OHDA the dopaminergic input to the striatum, and consequently, the inhibition of cholinergic interneurons is reduced, leading to a striatal extracellular hypercholinism, symbolized by a higher number of red circles. Therefore, GABA-ergic projection neurons to the EGP become overactive. (**C**) The injection of BoNT-A directly into the striatum cleaves the SNAP-25, an essential component of the SNARE complex in cholinergic presynaptic boutons. In consequence, the extracellular amount of ACh and the inhibition of the EGP should normalize. (**D**) After administration of the blood-brain barrier passing acethylcholinesterase inhibitor donepezil, the BoNT-A-induced reduction of extracellular ACh in hemi-PD rat striatum should be at least partially reversed, and the extracellular ACh concentration in the striatum increased again.

In this study, two aspects of hemi-PD and BoNT treatment were evaluated. First, we tested whether repeated intrastriatal BoNT-A injections were able to improve motor behavior in hemi-PD rats for a longer time period, resembling used clinical practice for BoNT-A treatments [18,19]. Thus, hemi-PD rats were intrastriatally injected with 1 ng BoNT-A 1 month and 7 months following 6-hydroxydopamine (6-OHDA) lesion (Figure 2), and underwent the apomorphine-induced rotation test, as well as the stepping test and corridor task for spontaneous motor behaviors.

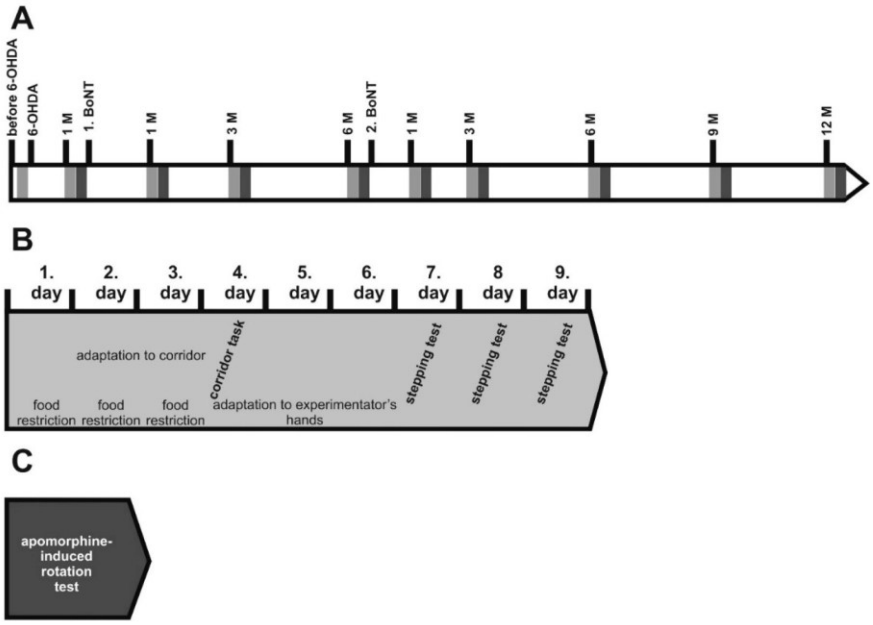

Figure 2. (**A**) Time points of 6-OHDA lesion and behavioral tests in rats after first (1. BoNT) and second (2. BoNT) BoNT-A or sham injection. Light grey rectangles symbolize batteries of corridor task and stepping test, dark grey rectangles apomorphine-induced rotation test. (**B**) The spontaneous behavior tests were performed as follows: Each series lasted 9 days. During the first 3 days, rats were food restricted and then 2 days adapted to the corridor task apparatus for 10 min each. Next day, the final corridor task was performed for 5 min. At the following 3 days, rats were handled by the experimenter for 5 min each day, and on the following 3 days, rats underwent the stepping test twice a day. (**C**) Finally, apomorphine-induced rotation test was performed for 40 min.

In the second set of experiments, the effect of donepezil on apomorphine-induced rotation behavior in hemi-PD rats was investigated. Doing so, we wanted to test whether the decrease of the apomorphine-induced rotation rate after intrastriatal BoNT-A injection in hemi-PD rats was due to a reduction of extracellular ACh content in the CPu. We supposed that the observed decrease in the apomorphine-induced turning rate in BoNT-A-injected hemi-PD rats, was attributed to a reduction of ACh content in the treated CPu. However, direct evidence for a decrease of the extracellular ACh concentration in the CPu in hemi-PD rats after BoNT-A application, and a correlation between the hypothesized ACh decrease and the reduction of the apomorphine-induced turning rate, was not provided yet.

Assuming the observed beneficial effect of BoNT-A was a result of a reduction of striatal extracellular ACh content, an increase of the extracellular ACh should abrogate this effect. Experimentally, such an increase of ACh in the synaptic clefts of the CPu was provoked by application of the blood-brain barrier passing acetylcholinesterase (AChE) inhibitor donepezil (Figure 1D) [20]. Hemi-PD BoNT-A-treated rats underwent the apomorphine-induced rotation test up to 9 months after

BoNT-A, and each again 72 h later following injection of donepezil 1 or 24 h ahead. Rotations of both tests were compared to separate the donepezil effect.

There is consistent information about the pharmacological half-life of donepezil in man, but not in rats. In the product information of donepezil-based medications (Aricept®, Full Prescribing Information; 2016 http://www.aricept.com/prescribing-and-patient-info), as well as in several publications [20–25], the half-life ($t_{1/2}$) is denoted at about 70 h in man. However, in rats, divergent half-lifes were reported either in the range of 2 up to 3.5 h [26–28] or about 24 h [29]. To circumvent the divergent information about the $t_{1/2}$ of donepezil, we studied experimental groups which were administered donepezil or sham donepezil 24 h or 1 h prior to the second rotation test at each monitoring point (Figures 7 and 9).

2. Results

2.1. Repetitive Intrastriatal BoNT-A Injection in Hemi-PD Rats

2.1.1. Body Weight

Both intrastriatal applications of BoNT-A were tolerated well by the animals and did not result in health problems. Thus, the development of body weight in hemi-PD rats was neither significantly altered by the first nor second BoNT-A injections, compared with sham-injected controls ($F_{1, 20} = 1.091$, $p = 0.31$). (Figure 3).

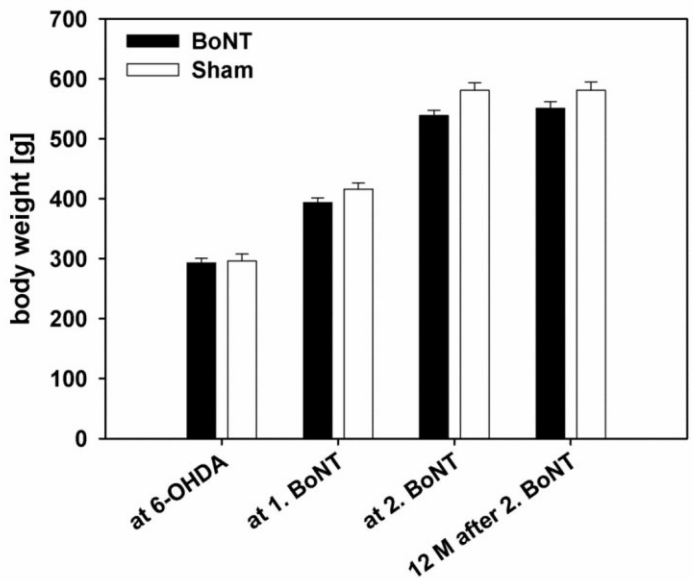

Figure 3. Body weights of both groups evaluated at the following time points: day of 6-OHDA injection, day of first (1. BoNT) and second (2. BoNT) BoNT-A or vehicle injections, as well as 12 months after the second BoNT-A or vehicle applications. Repetitive BoNT-A injection did not alter body weights, compared with vehicle injection in hemi-PD rats. All data are represented as mean ± SEM.

2.1.2. Apomorphine-Induced Rotation Test

Hemi-PD rats intrastriatally treated with BoNT-A had less apomorphine-induced rotation, compared with sham-injected animals ($F_{1, 20} = 4.385$, $p = 0.048$). Hemi-PD rats 4 weeks after 6-OHDA, showed about 6 apomorphine-induced turns per minute. In the present study, hemi-PD rats 1 month after BoNT-A showed about 2 apomorphine-induced rotations per minute, and thereby significant

lower values compared with sham-injected hemi-PD rats (p = 0.001). Thereafter, a progressive resurgence of the BoNT-A-induced reduction of the apomorphine-induced rotation rate occurred, reaching values identical to sham-injected rats at 6 months (Figure 4). However, 6 months after the second BoNT-A injection using the same coordinates as before, an even more pronounced and significant decline of the turning rate to about 2 was found, resembling the 1-month value of the first BoNT-A injection (Figure 4). To fit the rotation data by a regression line, the parameter rpm/day was calculated for every post BoNT-A test point after the first and second application, and both slopes (m) of the regressions were calculated. The time dependent slope of the apomorphine-induced rotation rates after the first BoNT-A injection was m = 0.0296 rpm d^{-1}, which after the second BoNT-A injection was significantly flatter (m = 0.0136 rpm d^{-1}) (t_{20} = 2.531, p = 0.02) (dashed lines in Figure 4).

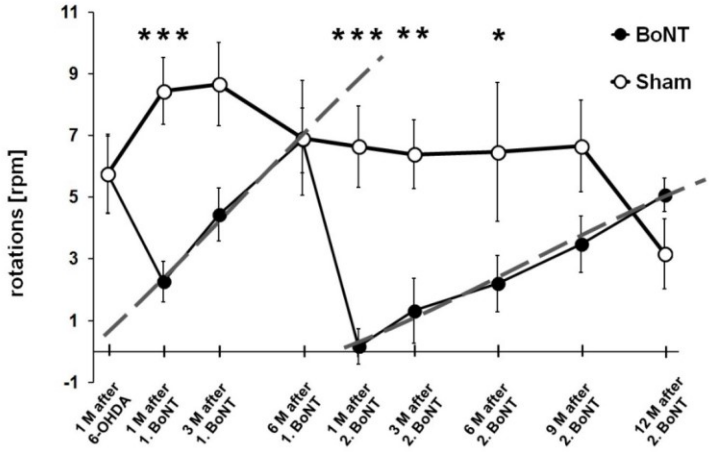

Figure 4. Apomorphine-induced rotation rates of hemi-PD rats repetitively treated with intrastriatal BoNT-A (n = 11, black dots) or vehicle (n = 6, light dots) 1 month and 7 months after 6-OHDA lesion. Vehicle solution has no significant effect on apomorphine-induced rotation behavior in hemi-PD rats. First BoNT-A reduces the turning rate significantly and temporally, the effect diminishing after 6 months. The effect of the 2. BoNT-A injection is more distinct and lasts longer than the 1. BoNT. Dashed grey lines mark linear regressions of the respective time dependencies of the apomorphine-induced rotation rates after 1. and 2. BoNT-A. Asterisks indicate significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data are represented as mean ± SEM.

2.1.3. Spontaneous Motor Tests

Adjusting Steps

The stepping test was used to evaluate forelimb akinesia, which is most likely due to bradykinesia of the affected limb [30–33]. Adjusting steps were measured on the non-lesioned and lesioned sides, for each animal group (BoNT-A- and sham-treated hemi-PD rats), in the forehand and backhand directions.

Before 6-OHDA lesion, no difference in the number of adjusting steps for the left and right forepaws, in forward and backward directions, was observed in either group (Figure 5A–D). All rats made about 9–12 steps in forward and in backward directions, with their left and right forepaws.

Right side 6-OHDA lesion induced impairment in the performance of the left forelimb (contralateral to 6-OHDA, Figure 5A,C) during the adjusting step when the rats were moved forward and backward by the experimenter, the right forelimb (ipsilateral to 6-OHDA lesion) being unaffected (Figure 5B,D). No significant improvement or worsening of the left forepaw movements was monitored after the first, as well as after the second intrastriatal BoNT-A or sham injection (Figure 5A,C). At the same time, in 6-OHDA-lesioned rats, side stepping movements of the right forepaw in both forehand

and backhand directions, were generally neither affected by ipsilateral BoNT-A nor sham injection up to 12 months (Figure 5B,D), with exception of single time points.

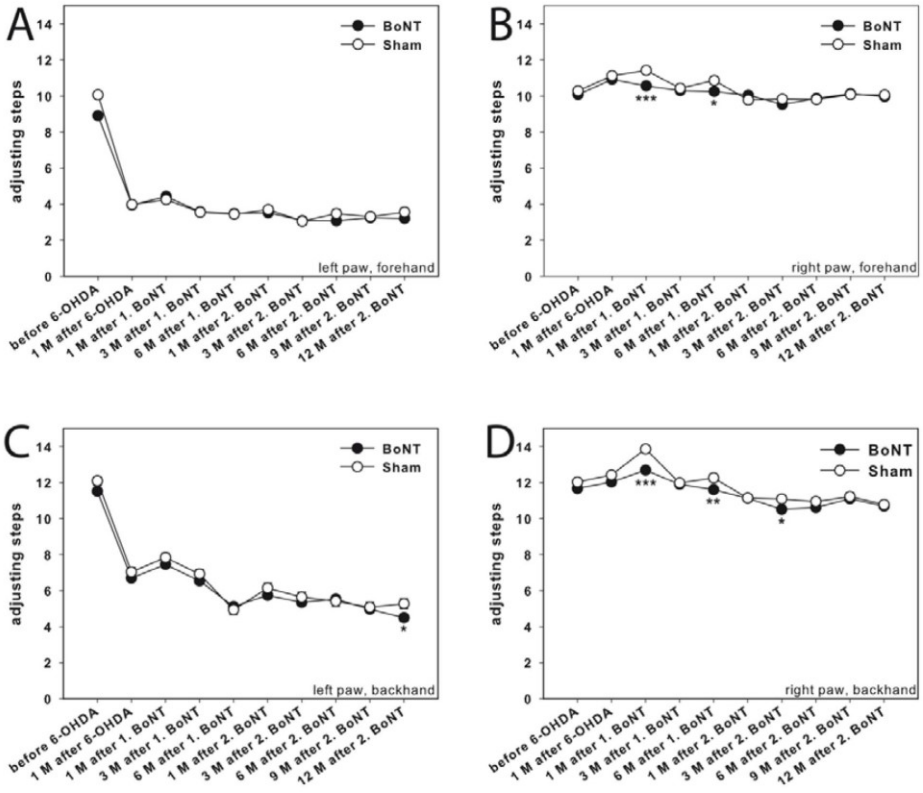

Figure 5. Stepping test in hemi-PD rats treated with repetitive intrastriatal BoNT-A or vehicle. In unlesioned rats, about 10–12 adjusting steps for the left (**A**,**C**) and right (**B**,**D**) forelimbs in forehand and backhand directions were counted. In hemi-PD rats, the use of the left forepaw was impaired in both the forehand (**A**) and backhand (**C**) directions. Neither 1. nor 2. BoNT-A nor sham injection changed the impairment of left and right forelimb steps in both the forehand (**A**,**C**) and backhand (**B**,**D**) directions in hemi-PD rats. Asterisks indicate significant differences compared to the sham group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data are represented as mean ± SEM.

Corridor Task

Two weeks before the 6-OHDA injection, rats were tested in the adjacent version of the corridor task. Preoperative screening showed that all animals performed an equivalent number of retrievals from each side of the corridor (about 50% of the total retrievals from either side) (Figure 6). Animals with right side hemi-PD exhibited a strong neglect of the left corridor side (contralateral to lesion): Only about 5% of retrievals were measured on the left side (Figure 6).

Neither the first ipsilateral intrastriatal BoNT-A nor sham injections in hemi-PD rats, improved a contralateral sensorimotor integration up to 6 months significantly (Figure 6). Moreover, the second intrastriatal injection of BoNT-A or vehicle demonstrated non-significant effects on sensorimotor (spatial) neglect for the side contralateral to the lesion during the next 12 months post-injection (Figure 6).

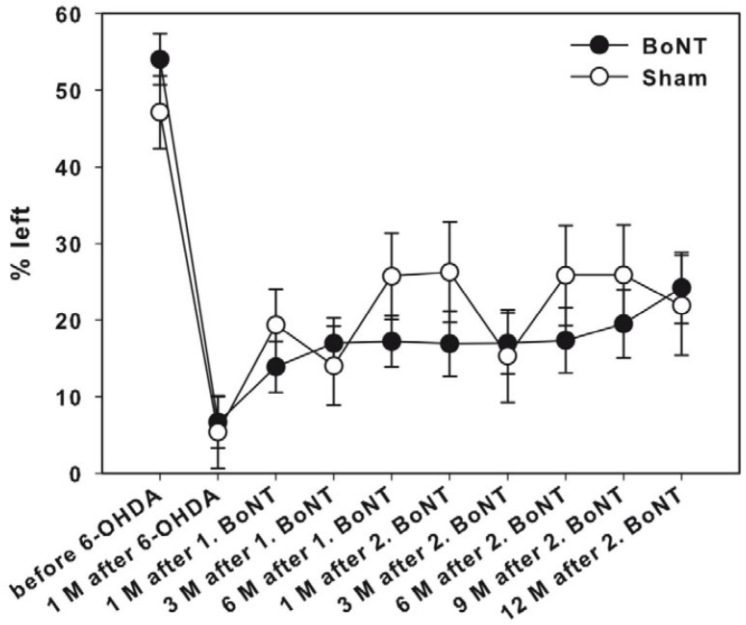

Figure 6. Corridor task in hemi-PD rats repetitively treated with intrastriatal BoNT-A or vehicle. Before 6-OHDA lesion, all animals equally retrieved pellets from either the left or right sides of the corridor apparatus. Right side hemi-PD rats significantly neglected the left corridor side. Neither 1. nor 2. BoNT-A nor sham injection improved contralateral sensorimotor integration in hemi-PD rats. Data are represented as mean ± SEM.

2.2. Influence of Donepezil on Apomorphine-Induced Rotation Behavior

In the second experiment, we investigated whether it could be the reduced extracellular ACh concentration that caused the reduction of the BoNT-A-related apomorphine-induced rotation rate in hemi-PD rats. We compared the results of two apomorphine-induced rotation tests performed with an interval of 72 h, whereby the first was performed without additional donepezil, and the second following injection of donepezil (2 mg kg^{-1} body weight (BW)), either 24 h or 1 h before apomorphine.

2.2.1. Reaction of Rats to Donepezil

Animals' reaction to 2 mg kg^{-1} BW donepezil was qualitatively evaluated. Starting about 8–10 min after i.p. injection, rats showed donepezil-related gnawing. This donepezil-induced gnawing behavior lasted approximately for about 2.5 h. We also measured a decline of the body temperature of approximately 1 °C, 1 h after donepezil administration. Tremor, salivation, lacrimation, and increased defecation, indicative for threefold higher donepezil dosages used by others were not seen [23,27,34–36]. General motor activity seemed uninfluenced by donepezil. Moreover, no fasciculations mentioned after daily oral application of 2.5 mg kg^{-1} [37] were seen after a single dose applied i.p., as used in our study.

2.2.2. Donepezil Injection 24 h Prior to the Second Rotation Test

Two groups of hemi-PD rats were used. One group was intrastriatally injected with 1 ng BoNT-A (group 6-OHDA + BoNT-A), and the other with the solvent of BoNT-A (group 6-OHDA + sham BoNT-A). At 1, 2, 3, and 4 months after BoNT-A or vehicle application, the apomorphine-induced rotation rate was measured, and at each time point 48 h later, 2 mg kg^{-1} BW donepezil was injected i.p., and a further 24 h later, the second apomorphine-induced rotation test was performed (Figure 7).

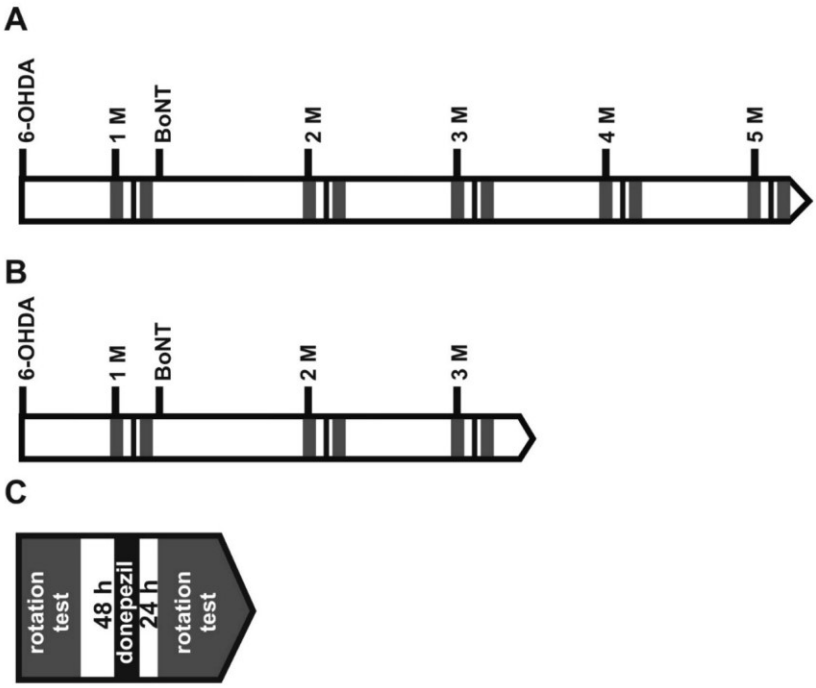

Figure 7. Time points of repetitive apomorphine-induced rotations (dark grey rectangles) in hemi-PD rats, before and after BoNT-A or sham injection, under the influence of donepezil (**A**) or sham donepezil (**B**). (**C**) The two apomorphine-induced rotations were performed as follows: Apomorphine rotations were tested 72 h apart. Furthermore, 24 h before the second rotation test, donepezil or vehicle was injected.

As expected, intrastriatal BoNT-A reduced apomorphine-induced rotations in hemi-PD rats (Figure 8A). Seventy-two h later the same rats having received donepezil 24 h before, showed significantly increased rotation rates (Figure 8A).

However, a comparable donepezil effect in apomorphine-induced rotations was also found in sham BoNT-A-treated hemi-PD rats (Figure 8A). Significant differences between the two consecutive rotation tests—first without and second with donepezil—were found for the monitoring points at 1 and 2 months after the BoNT-A or sham BoNT-A administration (Figure 8A).

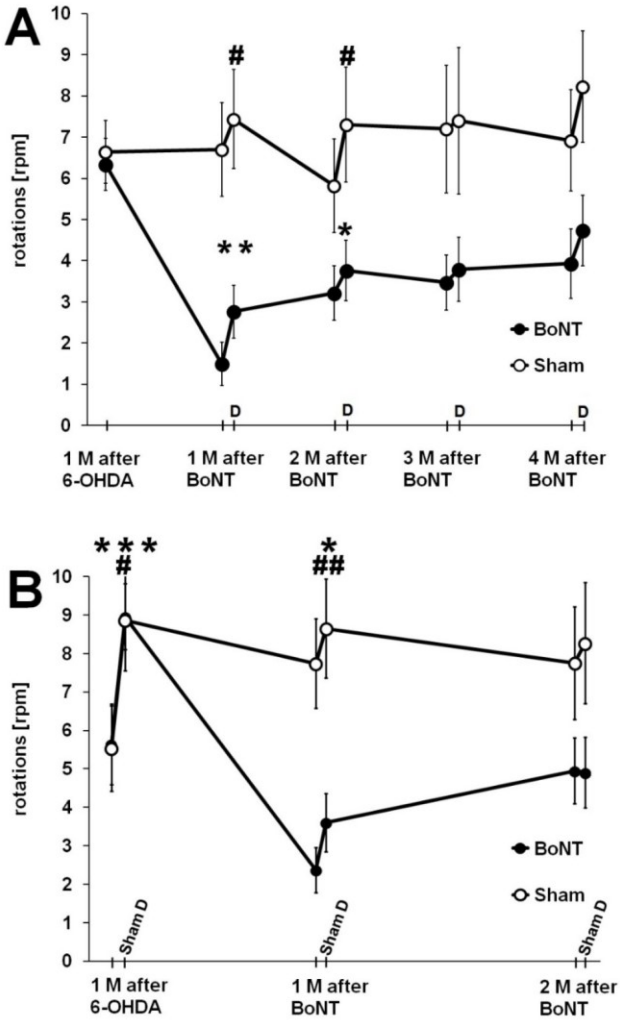

Figure 8. (**A**) Hemi-PD rats treated with intrastriatal BoNT-A (black dots) or vehicle (light dots) underwent apomorphine-induced rotation tests, before and 1 to 4 months after BoNT-A or sham BoNT-A. In the second apomorphine-induced rotation test under the influence of donepezil (2 mg kg^{-1} BW) injected 24 h ahead, the rotation rate significantly increased at time points 1 and 2 months after BoNT-A, as well as after sham BoNT-A. Asterisks mark significant donepezil-induced changes of the turning rate between the two associated rotations in the group which was treated with BoNT-A, hashes mark those in the group treated with sham BoNT-A. (**B**) Hemi-PD rats treated with intrastriatal BoNT-A (black dots) or vehicle (light dots) underwent apomorphine-induced rotation test, before and 1 to 2 months after BoNT-A or sham BoNT-A. In the second apomorphine-induced rotation test under the influence of sham donepezil injected 24 h ahead, rotation rates significantly increased at time points 1 month after 6-OHDA and 1 month after BoNT-A. Asterisks mark significant sham donepezil-induced changes of the turning rate between the two rotation tests of the BoNT-A-treated group, hashes mark significant changes in the group treated with sham BoNT-A. Asterisks and hashes indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; # $p < 0.05$, ## $p < 0.01$). Data are represented as mean ± SEM.

2.2.3. Sham Donepezil Injection 24 h Prior to the Second Rotation Test

Using a comparable experimental design, except for the donepezil injection prior to the second rotation, rats were injected with 0.9% NaCl solution 24 h prior to the second rotation test at the monitoring points 1 month after the 6-OHDA, and 1 and 3 months after BoNT-A injection in hemi-PD rats (Figure 8B). Remarkably, in these experiments we measured significant increases in the apomorphine-induced rotations 24 h after sham donepezil injection, 1 month after 6-OHDA lesion, as well as 1 month after BoNT-A injection or sham BoNT-A injection (Figure 8B).

2.2.4. Donepezil Injection 1 h Prior to the Second Rotation Test

Three experimental groups of hemi-PD rats were studied (Figure 9): The first group (n = 6) was BoNT-A-treated 1 month after the 6-OHDA and received donepezil 1 h prior to the second rotation tests at every monitoring point (Figure 10A). The second group (n = 6) was sham BoNT-A-treated 1 month after 6-OHDA and received donepezil 1 h prior to the second rotation tests (Figure 10B). The third group (n = 6) was BoNT-A-treated 1 month after induction of hemi-PD and received sham donepezil (0.9% NaCl) 1 h prior to the second rotation tests at every monitoring point (Figure 10C). One h after injection of 2 mg kg^{-1} BW donepezil, sham BoNT-A-treated hemi-PD rats showed a significant and considerable decrease of the apomorphine-induced rotation rate of about 3 to 4 rpm (rotations in anti-clockwise direction), compared to values measured 72 h before (Figure 10B). One h after donepezil injection BoNT-A-treated hemi-PD rats only tentatively showed a decline of the turning rate, these changes being mostly insignificant (Figure 10A). Only 9 months after BoNT-A treatment, additional donepezil affected rotation behavior significantly, when the BoNT-A effect had nearly vanished (Figure 10A). Regarding BoNT-A-treated hemi-PD rats, which were injected with sham donepezil 1 h prior, a second rotation test at neither monitoring point showed significant changes in the turning rates (Figure 10C).

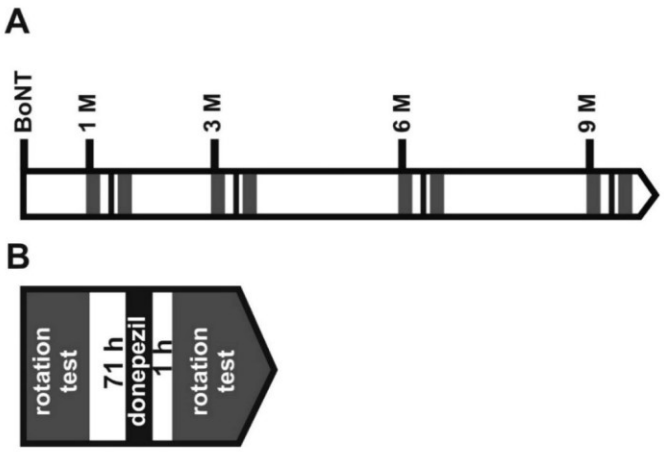

Figure 9. (**A**) Time points of repetitive apomorphine-induced rotations (dark grey rectangles) in hemi-PD rats, before and after BoNT-A or sham injection, under the influence of donepezil or sham donepezil. (**B**) The two apomorphine-induced rotations were performed as follows: Apomorphine rotations were tested 72 h apart. One h before the second rotation test, donepezil or vehicle was injected.

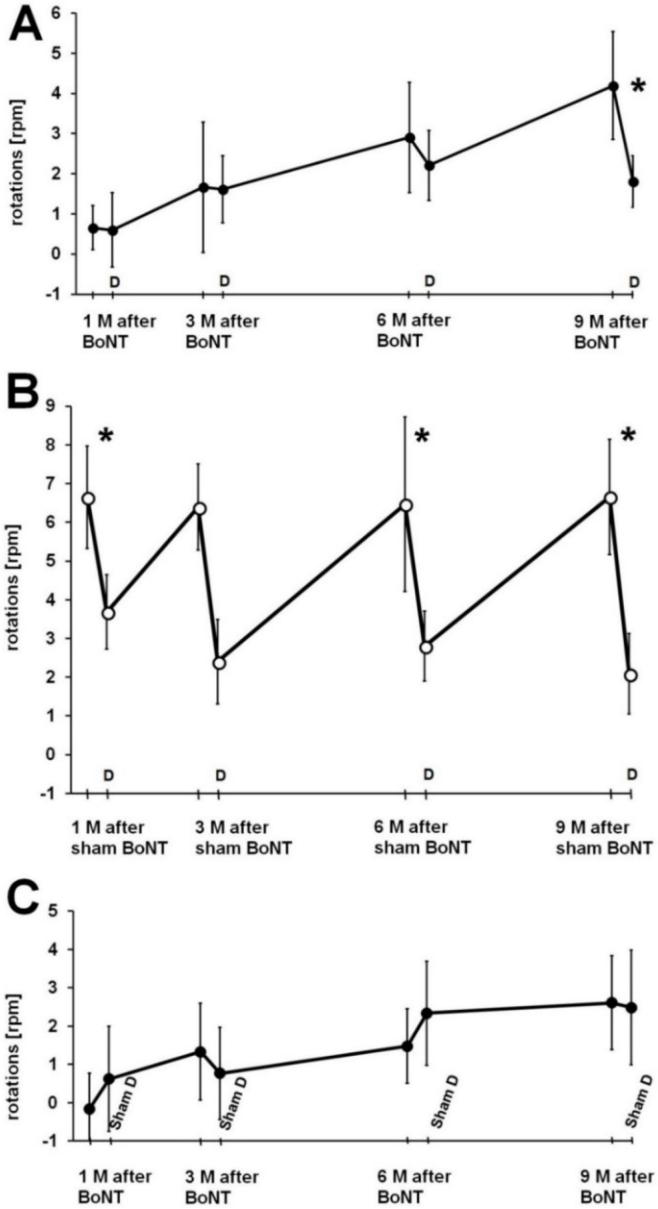

Figure 10. (**A**,**B**) Hemi-PD rats treated with intrastriatal BoNT-A (black dots) or vehicle (light dots) underwent repetitively apomorphine-induced rotation tests, before and 1 to 9 months after BoNT-A (**A**) or sham BoNT-A (**B**). In the second apomorphine-induced rotation test, under the influence of donepezil injected 1 h ahead, rotation rates were unchanged in the BoNT-A group (**A**) but were significantly increased in the sham BoNT-A group up to 9 months (**B**). Sham donepezil injections 1 h prior to the second apomorphine-induced rotation tests in BoNT-A-treated hemi-PD rats did not influence their rotational behavior (**C**). Asterisks indicate significant differences (* $p < 0.05$). Data are represented as mean ± SEM.

3. Discussion

Injection of 1 ng BoNT-A into the striatum of hemi-PD rats abolishes apomorphine-induced rotations for at least 3 months [12–17]. Thereafter, the apomorphine-induced rotation rate reverses [12–14,38]. Analogous to clinical practice in the use of BoNT-A, we evaluated the effect of repetitive BoNT-A injections (1 and 7 months after 6-OHDA) in the hemi-PD rat model using a battery of tests, including apomorphine-induced rotations and spontaneous behavior tests.

In the striatum, BoNT-A is thought to act in two main directions: ACh release of the tonically active cholinergic interneurons is blocked [1,4,5]; and the concentrations of the dopamine D_2 receptor is reduced as shown in hemi-PD rats with D_2 receptor upregulation, as in BoNT-A-injected normal rats [38,39].

3.1. Repetitive Intrastriatal BoNT-A Injection in Hemi-PD Rats

3.1.1. Body Weight

Neither first nor second intrastriatal BoNT-A injections significantly influenced the body weight of the rats. Even 12 months after the second BoNT-A application, body weights of BoNT-A- (551 ± 11 g; mean ± SEM) or sham-injected rats (581 ± 14 g) did not differ significantly. Thus, repetitive intrastriatal BoNT-A had no negative effect on the health of the rats, being in line with unaltered measures after the single BoNT-A application [12,39].

3.1.2. Apomorphine-Induced Rotation Test

It was shown that intrastriatal injection of 1 ng BoNT-A in hemi-PD rats ameliorated apomorphine-induced rotations for at least 3 months, and during 12 months after BoNT-A treatment, hemi-PD rats showed a gradual recurrence of the apomorphine-induced rotation rate [12–17]. We demonstrated that repetitive intrastriatal injections of BoNT-A in hemi-PD rats were well tolerated, and the second injection had an effect significantly exceeding the behavioral outcome of the first injection. The second BoNT-A injection led to a more distinct reduction of the apomorphine-induced rotation rate after 1 month: Rotation rate 1 month after first BoNT-A was 2.27 ± 0.86 rpm, and 0.17 ± 0.58 rpm (mean ± SEM) after second BoNT-A. The time dependent slope of apomorphine-induced rotations was flatter after the second BoNT-A injection: Rotation rate 3 months after first BoNT-A was 4.44 ± 0.86 rpm, and 1.32 ± 1.06 rpm after second BoNT-A. Rotation rate 6 months after first BoNT-A was 6.85 ± 1.05 rpm, and 2.20 ± 0.91 rpm after second BoNT-A. The 3 months effect of the first BoNT-A injection equaled that of the 12 months effect of the second BoNT-A application (5.08 ± 0.54 rpm). These results suggested that although 6 months after the first BoNT-A injection, the rotation rate retoured to the pre-BoNT-A value (6 months after first BoNT-A = 6.85 ± 1.05 rpm, pre-BoNT-A = 5.75 ± 1.25 rpm), a residual impact of the first BoNT-A application seemingly persisted.

Our results concerning the more profound effect of the second intrastriatal BoNT-A injection, were in line with the prolongation of the therapeutic intervals observed following intramuscular BoNT-A injections. Rogozhin et al. [40] demonstrated in the mouse epitrochleoanconeus muscle that after repeated BoNT-A administrations, the functional recovery of neuromuscular transmission occurred slower than after a single BoNT-A injection. After a single BoNT-A injection, quantal ACh release from motor nerve terminals reached 50% of control about 6 weeks after injection; however, after repetitive BoNT-A this level was reached about 11 to 15 weeks after the last injection. Accordingly, striking structural abnormalities of neuromuscular junctions and intramuscular nerves were more pronounced after repetitive injections. Moreover, in human therapeutic BoNT-A application, more distinct and longer lasting effects, resulting in an increase in the mean duration of efficacy with the number of injections are well known [41–45].

We suppose that 6 months after the first intrastriatal BoNT-A injection, morphological and/or physiological parameters had not fully recovered, so that the second BoNT-A application caused

a more distinct and cumulative effect, seen in the longer and more pronounced reduction of the apomorphine-induced turning rate. This phenomenon is seemingly a specific BoNT-A effect, as the apomorphine-induced rotation rate was not dependent on the time after 6-OHDA lesion, and even not influenced by sham injection experiments [38].

3.1.3. Spontaneous Motor Tests

Unilateral right side 6-OHDA lesion induced marked and long-lasting stepping deficits with the left forepaw (contralateral to lesion), in both forehand and backhand directions. Neither the first nor the second ipsilateral intrastriatal BoNT-A injection changed the impairments seen in hemi-PD rats. The same was true for sensorimotor integration behavior. In the corridor task, right side hemi-PD resulted in a massive neglect of the left corridor side. The first, as well as the second ipsilateral intrastriatal BoNT-A injection, did not improve contralateral sensorimotor integration significantly.

The outcomes of both behavioral tests reflected the motor initiation deficits of the forelimb, depending on contralateral striatal DA depletion [46–51]. DA deprivation of the striatum leads to increased GABAergic MSN projection to the EGP, which results in a disinhibition of the spontaneously active STh. As a result, a more intensely firing IGP inhibits VL. Finally, the inhibited VL neurons do not adequately activate the premotor cortex, which in turn reduces initiation of movements of the contralateral body via its crossed motor efferents [52–55]. Intrastriatal ipsilateral BoNT-A injection of 1 ng BoNT-A did not change the impairments seen in hemi-PD rats. Probably, the BoNT-A-induced changes in the DA deprived CPu concerning extracellular ACh content [56–58], and receptors of DA and others transmitters [59–61] were not adequate to improve spontaneous motor behavior. The degree of bradykinesia is associated with the degree of depletion of DA neurons [62,63]. Only animals with more than 80% striatal DA depletion exhibited similar stepping deficits [62,64–66], whilst animals with less depletion did not [33,67]. As in hemi-PD rats, the DA deprivation was almost complete [68–71], and consequently, not influenced by BoNT-A applications. It is not surprising that stepping behavior and sensorimotor integration were changed, neither by the first nor the second ipsilateral intrastriatal BoNT-A application.

3.2. Influence of Application of 2 mg kg^{-1} BW Donepezil Prior to Apomorphine-Induced Rotation Test

Hemi-PD rats treated intrastriatally with BoNT-A and injected with 2 mg kg^{-1} BW donepezil 24 h prior an apomorphine-induced rotation test, showed a significant increase of their turning rate, compared to a rotation test 72 h before. This seemingly proved our working hypothesis that the BoNT-A-induced reduction of apomorphine-induced rotation rate of hemi-PD rats was due to a reduction of the striatal ACh content. However, in sham BoNT-A-treated hemi-PD rats, we found comparable results. Moreover, time matched sham donepezil-injected BoNT-A-treated hemi-PD rats showed a significantly increase in turning rate, respectively. Therefore, we concluded that the similar increases of the rotation rate were not due to specific donepezil-induced increase of extracellular ACh content in the striatum. According to the microdialysis, studying ACh in the cortex and hippocampus of freely-moving rats following i.p. injection of 4 μmol kg^{-1} donepezil, extracellular ACh concentrations steeply increased, and showed maximal values 1 to 2 h after injection and reached baseline levels again after about 4 to 5 h [72–74]. Thus, apomorphine injections at an interval of 72 h were seemingly unaffected by donepezil, but could tentatively have caused a behavioral sensitization, discussed in repetitive apomorphine applications [75,76].

Remarkably, an injection of 2 mg kg^{-1} BW donepezil 1 h prior to an apomorphine-induced rotation test, in contrast to a donepezil injection 24 h prior to a rotation test, leads to a significant reduction of the turning rate in sham BoNT-A-treated rats, and to a tendential reduction of the turning rate in BoNT-A-treated rats. Obviously, the effect of donepezil in rats is limited to some hours after its systemic application, because we registered strong effects on the apomorphine-induced rotation behavior 1 h after injection, but we saw no specific alterations 24 h after application. So our results remain in line with those reports postulating a $t_{1/2}$ of donepezil of few hours [26–29], and shown by

others [72–74,77]. Contradicting our working hypothesis, the application of donepezil did not lead to a jump of the turning rate in BoNT-A-treated hemi-PD rats. We suppose that this is due to the systemic action of donepezil. Babiloni et al. (2014) investigated the effect of donepezil on EEG markers and motor activity in mice during short post-administration periods up to 3 h. Donepezil normalized motor activity, compared to vehicle-induced increased motor activity [78]. Comparable results were seen in a traumatic brain injury model, where Shaw et al. (2013) subsequent to daily donepezil injection (19 days) did not find motor improvement, but stated significantly impaired beam-balance time and beam-walk time at the dosage used in our experiments [79].

Although donepezil was shown as beneficial with respect to cognition in mild to moderate dementia [80–83], and also moderate dementia with Parkinson's disease, donepezil therapy did not change motor PD symptoms [84,85]. Furthermore, donepezil treated dementia with Lewy body, without relevant worsening of extrapyramidal symptoms [86]. However, others stated that donepezil treatment could cause adverse motor effects in a subset of patients with parkinsonian dementia [87–90]; worsened activities of daily living mobility in patients with progressive supranuclear palsy [91]; and deteriorated motor behavior in patients with dystonic reactions [92].

It can be speculated that the reductions of apomorphine-induced rotations, following 1 h after donepezil, were not caused by an increase of extracellular ACh in the striatum, but by a general depression of the rats' motor activity. The donepezil-related reduction in motor activity was measured in the sham-treated hemi-PD rats, as a reduction in apomorphine-induced rotations (Figure 10B). Seemingly, no significant donepezil-induced (Figure 10A) or sham donepezil-induced (Figure 10C) reductions in motor activity were measured in the BoNT-A-treated hemi-PD rats, as those rats performed fewer rotations per se.

We conclude, that the systemic administration of donepezil, irrespective of the time prior to an apomorphine-induced rotation test, was not a qualified method to prove the ACh dependence of the effect of intrastriatal injection BoNT-A. Application of donepezil 24 h ahead of apomorphine had no specific effect, as the temporal increase of extracellular ACh in the brain was already abrogated. Application of donepezil 1 h before apomorphine likely did not have the hypothetically expected effect, i.e., reversing the BoNT-A-induced reduction of apomorphine-induced rotation behavior, caused by the increase of extracellular ACh, as the general motor-depressive effect of donepezil exceeded that of the increase of extracellular ACh in the striatum.

4. Conclusions

Firstly, data showed that repeated intrastriatal BoNT-A injections in the hemi-PD rat model were possible and well tolerated. In hemi-PD rats, the second intrastriatal BoNT-A injection had a more intense and longer lasting effect on the reduction of apomorphine-induced rotations, but like the first one, it did not affect forelimb akinesia and lateralized sensorimotor integration. Secondly, systemic donepezil injection prior to testing the apomorphine-induced rotation behavior is not qualified to prove the dependency of striatal extracellular ACh content for turning rate reductions after BoNT-A treatment.

These different effects of BoNT-A application suggest that intrastriatally applied BoNT-A acts both as an inhibitor of ACh release and influences transmitter receptors, especially D_2 receptor expression, and thereby, affects the basal ganglia circuitries. Therefore, the evaluation of receptor densities of all important striatal transmitters is the subject of ongoing studies of our group.

5. Materials and Methods

5.1. Animals

All experiments were started with 2.5 months old, male Wistar rats (strain Crl:WI BR, Charles River Wiga, Sulzfeld, Germany) weighing 295–305 g. Animals were housed in standard cages at 22 °C ± 2 °C under a 12 h light/dark cycle, with free access to water and food. All procedures used in

the present study complied with the guidelines on animal care. The experiments were approved by the local Animal Research Committee of the state of Mecklenburg-Western Pomerania (LALLF M-V 7221.3-1.1-003/13 from 26 April 2013.

5.2. Induction of Hemiparkinsonism

All surgeries were carried out under aseptic conditions and deep anesthesia (50 mg kg^{-1} BW) ketamine and 4 mg kg^{-1} BW xylazine). To induce an experimental hemi-PD syndrome, a unilateral injection of 24 µg 6-OHDA dissolved in 4 µL 0.1 M citrate buffer was performed into the right medial forebrain bundle (MFB), using a stereotactic frame (Stoelting, Wood Dale, IL, USA). The injection coordinates with reference to bregma were: AP = −2.3, L = 1.5, V = −9.0, as described in Reference [93]. The success of the lesion was verified by measurement of the apomorphine-induced rotation rate [94,95], 4 weeks after 6-OHDA injection. Rats (n = 45) that displayed an apomorphine-induced rotation rate of at least 4 rotations per minute to the left side, were successfully lesioned, indicating unilateral death of about 97% of the nigrostriatal dopaminergic neurons [95]. Only those rats were used for further experiments.

5.3. Body Weight

Body weights were measured at the following time points: 6-OHDA injection, first and second BoNT-A or vehicle injections, as well as at every monitoring point and 12 months after the second BoNT-A or vehicle applications.

5.4. Injection of BoNT-A into the Striatum

About 6 weeks after the 6-OHDA injection, the animals underwent a next stereotactic surgery (Figure 2). Either a solution of BoNT-A dissolved in phosphate-buffered saline supplemented with 0.1% bovine serum (prepared from BoNT-A powder, Lot No. 13028A1A; List, Campbell, CA, USA; purchased via Quadratech, Surrey, UK) or vehicle (sham BoNT-A) solution was injected into the right CPu at two sites [12,14,15]. Hemi-PD rats were treated with either 1 ng BoNT-A (n = 29) or vehicle (n = 16). The respective coordinates with reference to bregma were: AP = +1.3/−0.4 mm, L = 2.6/3.6 mm to the right, and V = −5.5 mm. Animals received a 2 × 1 µL BoNT-A solution containing a total of 1 ng BoNT-A or vehicle solution. Animals for the repetitive BoNT-A injection experiment underwent a second BoNT-A or a sham injection with vehicle solution, 6 months after the first one, respectively (Figure 2).

5.5. Apomorphine-Induced Rotation Test

The rate of apomorphine-induced rotations, served as a measure for the extent of the basal ganglia circuit disturbance by the unilateral lesion of the SNpc [94,95].

The apomorphine-induced turning rate was ascertained 4 weeks after the 6-OHDA lesion, and 1, 3, and 6 months after the first BoNT-A injection, as well as 1, 3, 6, 9, and 12 months after the second BoNT-A treatment. Two experimental groups were studied: hemi-PD rats treated with BoNT-A (n = 16), and hemi-PD rats injected with vehicle solution (n = 8) (Figure 2).

Apomorphine was injected s. c. (0.25 mg kg^{-1}) and the animals' turns registered on a self-constructed automated rotometer device over 40 min. In right-sided hemi-PD rats, the apomorphine-induced complete anti-clockwise 360° turns were expressed as positive values (Figures 4, 8 and 10).

5.6. Spontaneous Motor Tests

Stepping test and corridor task were performed before 6-OHDA lesion, 4 weeks thereafter, and 1, 3, and 6 months after the first ipsilateral intrastriatal injection of BoNT-A or sham BoNT-A; and 1, 3, 6, 9, and 12 months after the second BoNT-A or vehicle injection (Figure 2).

5.6.1. Adjusting Steps

Before evaluating adjusting steps, rats were handled by the experimenter during 3 days to adapt to the test procedure [33,96,97]. Tests were performed twice per day on 3 successive days. Briefly, the rat was held with one hand softly blocking both its hind limbs and the restrained forelimb, with the unrestrained forepaw touching the table. The rat was moved slowly sideways across the table (90 cm in 5 s) and the number of adjusting steps of the unrestrained left or right forelimb was counted, whilst moving in the forehand and backhand directions. Subsequently, the forehand and backhand steps of left and right paws were evaluated using the video recorded sessions, which allowed counting of the number of adjusting steps by an investigator blinded to the state of the rats.

5.6.2. Corridor Task

Lateralized response selection was examined using the version, according to Grealish et al. [98]. Prior to testing rats were food restricted for 3 days, and maintained at about 90% of free-feeding BW during habituation and testing, as described in Reference [99]. Rats were adapted to the self-constructed alleyway (240 cm long × 7 cm wide × 23 cm deep) for 10 min each on 2 successive days, with some scattered sugar pellets (Ain-76A Rodent Tablet 20 mg TestDiet, Richmond, IN, USA) along the floor of the corridor. Each day, the animals started from different ends of the corridor. For final testing, on day 1, rats were first placed in an identical but empty corridor for 5 min for adaptation, and then to the end of the testing corridor. In the testing corridor, bowls (2 cm in diameter, distance between the bowls 15 cm) were placed on the left and right sides, containing 5 pellets each. Rats were allowed for 5 min to retrieve pellets from either side of their body, as detailed in References [50,100]. The retrievals of the right side (ipsilateral) and left side (contralateral) were counted, and the data expressed as the percentage of left- or right-side retrievals on the total number of retrievals. The side is defined according to the rat's body axis. A "retrieval" involved a nose poke into a bowl, whether or not pellets were taken from it, as outlined in References [98,101,102].

5.7. Donepezil Modifying Apomorphine-Induced Rotations

In different experiments the possible effect of donepezil on apomorphine-induced rotation rates was evaluated. Hemi-PD rats intrastriatally injected with BoNT-A (1 ng) or sham BoNT-A were twice tested for apomorphine-induced rotations, at an interval of 72 h. At 24 h or 1 h prior to the second test for apomorphine-induced rotations, donepezil (2 mg kg^{-1} BW) or sham donepezil (0.9% NaCl) was injected i.p. (Figures 8 and 10).

Thus, a total of 7 groups were tested: (i) hemi-PD + BoNT-A + 24 h donepezil ($n = 15$); (ii) hemi-PD + sham BoNT-A + 24 h donepezil ($n = 9$); (iii) hemi-PD + BoNT-A + 24 h sham donepezil ($n = 14$); (iv) hemi-PD + sham BoNT-A + 24 h sham donepezil ($n = 7$); (v) hemi-PD + BoNT-A + 1 h donepezil ($n = 6$); (vi) hemi-PD + sham BoNT-A + 1 h donepezil ($n = 6$); and (vii) hemi-PD + BoNT-A + 1 h sham donepezil ($n = 6$)—(not done: hemi-PD + sham BoNT-A + 1 h sham donepezil). Groups i–iv were tested 1 to 4 months after BoNT-A or sham BoNT-A, and groups v–vii 1 to 12 months after BoNT-A or sham BoNT-A.

5.8. Data Analysis

The results were presented as means ± SEM. Computations and statistics of donepezil modifying apomorphine-induced rotations were performed with Excel®. For comparison of rotation rates and slopes of rotation rates between two animal groups at the same monitoring point, the unpaired Student's *t*-Test was performed. For investigation of rotation rates and slopes of the rotation rate of one animal group at different monitoring points, the paired Student's *t*-Test was performed. In all cases, p values ≤ 0.05 were considered significant and p values < 0.01 were considered highly significant.

Data of body weights and spontaneous motor tests, i.e., stepping test and corridor task, were subjected to two-way ANOVA using SigmaPlot 11 Software (Systat Software, Inc., San Jose,

CA 95110, USA). The Holm-Sidak approach was used for adjustment for multiple testing, for post hoc comparisons. A critical value for significance of $p \leq 0.05$ was used.

Author Contributions: Conceptualization, A.H., A.W., and V.A.; Formal analysis, C.H.; Funding acquisition, A.H. and A.W.; Investigation, A.H., A.W., and V.A.; Methodology, V.A.; Supervision, A.H. and A.W.; Visualization, A.H. and C.H.; Writing—original draft, A.H., C.H., A.W., and V.A.

Funding: Rostock University Medical Center internal funding FORUN 889005 and 889014.

Acknowledgments: The technical assistance of Susann Lehmann and Frauke Winzer is gratefully acknowledged.

Conflicts of Interest: The authors declare no conflict of interest regarding the material in the manuscript.

Abbreviations

6-OHDA	6-hydroxydopamine
ACh	acetylcholine
AChE	acetylcholinesterase
BoNT-A	botulinum neurotoxin-A
BW	body weight
CPu	caudate-putamen (striatum)
DA	dopamine
D_2	dopamine D_2 receptor
EGP (=LGP)	external (lateral) globus pallidus
GABA	gamma-aminobutyric acid
hemi-PD	hemiparkinsonian
IGP (=MGP)	internal (medial) globus pallidus
M	slope/drawdown
MFB	medial forebrain bundle
MSN	medium spiny neuron
PD	Parkinson's disease
PPN	pedunculopontine nucleus
RPM/d	(revolutions per minute)/day
SNAP-25	synaptosomal-associated protein-25
SNARE	*N*-ethylmaleimide-sensitive-factor attachment receptor
SNpc	substantia nigra pars compacta
STh	subthalamic nucleus
VL	ventrolateral thalamic nucleus

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Article

Unilateral Botulinum Neurotoxin-A Injection into the Striatum of C57BL/6 Mice Leads to a Different Motor Behavior Compared with Rats

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Received: 5 July 2018; Accepted: 15 July 2018; Published: 17 July 2018

Abstract: Different morphological changes in the caudate-putamen (CPu) of naïve rats and mice were observed after intrastriatal botulinum neurotoxin-A (BoNT-A) injection. For this purpose we here studied various motor behaviors in mice (n = 46) longitudinally up to 9 months after intrastriatal BoNT-A administration as previously reported for rats, and compared both outcomes. Apomorphine- and amphetamine-induced rotational behavior, spontaneous motor behavior, as well as lateralized neglect were studied in mice after the injection of single doses of BoNT-A into the right CPu, comparing them with sham-injected animals. Unilateral intrastriatal injection of BoNT-A in mice induced significantly increased contralateral apomorphine-induced rotations for 1 to 3 months, as well as significantly increased contralateral amphetamine-induced rotations 1 to 9 months after injection. In rats (n = 28), unilateral BoNT-A injection also induced significantly increased contralateral apomorphine-induced rotations 3 months after injection, but did not provoke amphetamine-induced rotations at all. Lateralized sensorimotor integration, forelimb preference, and forelimb stepping were significantly impaired on the left side. The differences in motor behaviors between rats and mice may be caused by different BoNT-A effects on cholinergic and catecholaminergic fibers in rat and mouse striata, interspecies differences in striatal receptor densities, and different connectomes of the basal ganglia.

Keywords: botulinum neurotoxin-A; basal ganglia; interspecies differences in motor behavior; mouse; rat; interneurons

Key Contribution: We investigated the effect of intrastriatal BoNT-A application on motor behavior in naïve mice for the first time. To interpret the differences of the BoNT-A effect in mice and rats, multi-receptor fingerprints of Wistar rats and C57Bl/6 mice as well as their basal ganglia connectomes were compared.

1. Introduction

Parkinson's disease (PD) is a common chronic progressive age-related neurodegenerative movement disorder characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta and, subsequently, of dopamine in the caudate-putamen (CPu) [1–3]. Dopamine deficit causes a profound impairment of neuronal circuits in the basal ganglia [4–7], and particularly an increased release of acetylcholine by tonically active striatal interneurons [8–12]. Anticholinergic drugs are used to antagonize striatal hypercholinism in PD, but this treatment often elicits adverse side effects [13–15]. In order to circumvent general anticholinergic drug effects, we tested a local intrastriatal injection of botulinum neurotoxin-A (BoNT-A) [16–20].

Indeed, in the experimental 6-OHDA-induced hemiparkinsonian (hemi-PD) rat model, intrastriatal application of BoNT-A abolished apomorphine-induced rotational behavior—most probably by blocking acetylcholine, the release of cholinergic terminals, and/or inducing changes in receptor densities [16–23]. Moreover, the unilateral intrastriatal BoNT-A injection induced rotational behavior in naïve rats without 6-OHDA-induced hemi-PD. Following the injection of 1 ng BoNT-A into the right CPu, rats showed 2–4 apomorphine-induced rotations per min in the direction of the injection site for two months, and rotated tentatively to the contralateral side thereafter [19].

To date, behavioral effects of intrastriatal BoNT-A injections were studied in rats, but not in mice. This would be interesting, as comparative morphological studies of the striata of BoNT-A-injected rats and mice striata showed obvious differences in the structural changes of choline acetyltransferase-immunoreactive (ChAT-ir) and tyrosine hydroxylase-immunoreactive (TH-ir) fibers [19,20,24], in addition to similarities concerning unchanged CPu volume and the number of cholinergic interneurons. Rats and mice differed in the induction of BoNT-A-induced varicosities: both kinds of these varicosities were found in rats [16,17,19–21], whereas only ChAT-ir BoNT-A-induced varicosities were observed in mice [24].

As naïve mice and rats reacted morphologically in a different manner following intrastriatal BoNT-A injection, we sought to determine whether these species differences would also hold true for motor behavior. Thus, mouse motor behavior was scored after unilateral BoNT-A injection into the right CPu using apomorphine- and amphetamine-induced rotations. Spontaneous behavior was tested using the cylinder and stepping tests, lateralized sensorimotor activity by the corridor task, and cerebellar ataxia by hindlimb clasping. To evaluate temporally restricted effects, mice were tested up to 9 months after BoNT-A injection. A potential dose-dependency was evaluated by using dosages of 25 and 50 pg BoNT-A.

It is known that mice are very sensitive to botulinum toxins and also differ in many respects from rats [25–33]. However, there are currently no studies evaluating the behavioral outcome of intrastriatally applied BoNT-A in naïve mice. Moreover, the effect of BoNT-A in naïve mice must be understood prior to evaluation of intrastriatal BoNT-A application in mice models of hemi-PD and genetic PD. The BoNT-A dosage used for intrastriatal injection for the treatment of experimental hemi-PD in rats was based on the LD50 and recent intracerebral BoNT-A injections [34–38]. In Wistar rats we found a well-tolerated and effective dosage at 1 ng BoNT-A per CPu, whereas 5 ng were mortal [19]. In the C57BL/6 mice, dosages of 25–50 pg BoNT-A per striatum were found to be appropriate [24].

Studying the behavioral effect of unilateral intrastriatal BoNT-A application in naïve mice also seemed to be important, since the effect of therapeutic BoNT-A applications is explored in increasingly studied genetic Parkinsonian mouse models [39–45].

2. Results

2.1. Body Weight

Changes in body weight were evaluated as an index of general adverse effects of BoNT-A application. The mice weighed 18–24 g at the time of BoNT-A or vehicle injection. Thereafter, body

weight increased in all experimental groups (Figure 1). Increase in body weight over time was significantly ($F_{2, 50}$ = 15.5999, $p < 0.001$) lower in mice injected with BoNT-A than in those receiving vehicle substance. However, 9 months after BoNT-A injection, the 25 pg BoNT-A mice weighed 31.85 ± 0.37 g (mean ± SEM), the 50 pg BoNT-A mice 33.72 ± 0.40 g, and thus did not differ from the sham group, which weighed 34.66 ± 0.35 g (Figure 1).

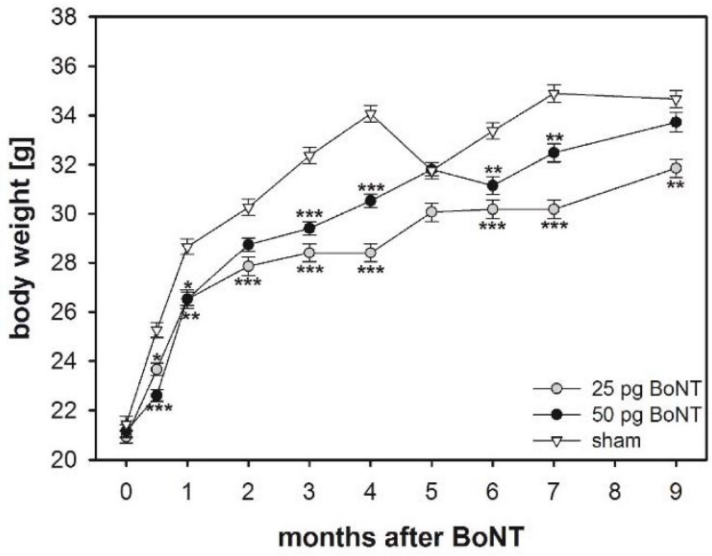

Figure 1. Body weight over time. Asterisks indicate significant differences (25 pg, n = 15; 50 pg, n = 20) compared to the sham group (n = 11) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data are means ± SEM.

2.2. *Apomorphine-Induced Rotations*

2.2.1. Mice

In order to test the effect of a unilateral intrastriatal BoNT-A injection on drug-induced motor behavior we used apomorphine- and amphetamine-induced rotation tests. Half a month after injection of 25 or 50 pg BoNT-A or vehicle into the right CPu mice showed no apomorphine-induced rotation behavior (Figure 2A). Vehicle application did not induce rotational behavior in the subsequent 9 months. However, both applications of 25 and 50 pg BoNT-A resulted in significant ($F_{2, 50}$ = 6.150, p = 0.004) anti-clockwise, i.e., contralateral, apomorphine-induced rotations with net rotations of about 2 per minute after a post-injection survival of 1 and 3 months (Figure 2A). Six and 9 months after BoNT-A, rotational behavior decreased to values similar to those observed in the vehicle-treated group.

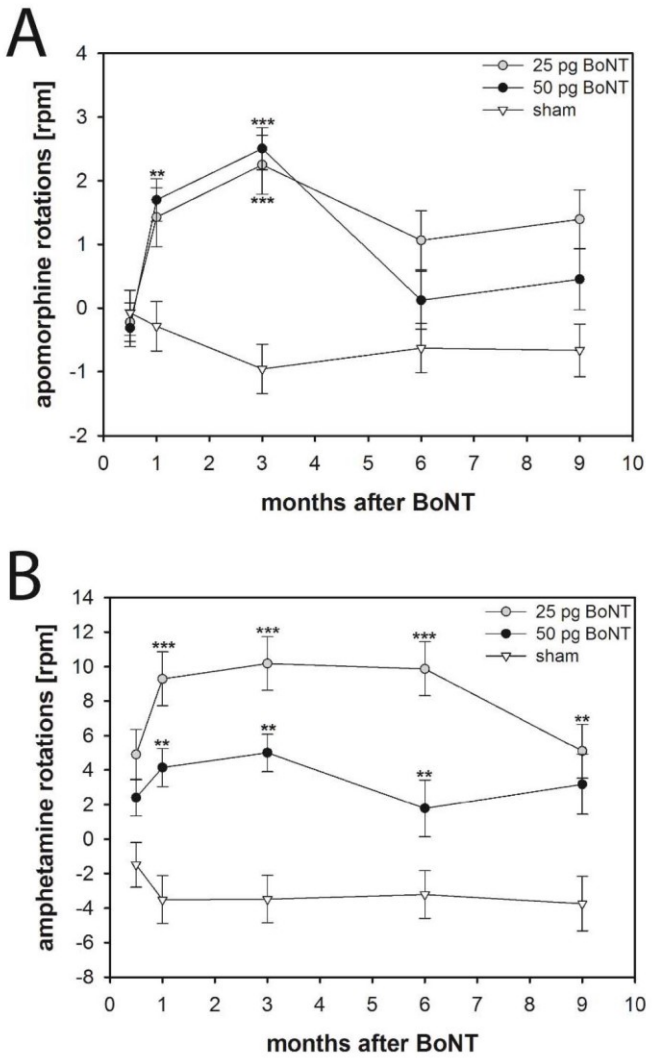

Figure 2. (**A**) Apomorphine- and (**B**) amphetamine-induced rotations in mice treated with intrastriatal BoNT-A (25 pg, $n = 15$; 50 pg, $n = 20$) or vehicle ($n = 11$). (**A**) BoNT-A in either dosage caused a significant increase of the apomorphine-induced turning rate 1 and 3 months after injection. (**B**) BoNT-A in both dosages resulted in significantly increased amphetamine-induced rotations 1–9 months after injection, while sham injections did not change rotational behavior. Asterisks indicate significant differences compared with the sham group (** $p < 0.01$, *** $p < 0.001$). Data are means ± SEM.

2.2.2. Rats

Following apomorphine injection rats significantly rotated contralaterally to the intrastriatal BoNT-A application with rotations of approximately 1.5 to 2 per minute 3 months after 1 ng BoNT-A (Figure 3A).

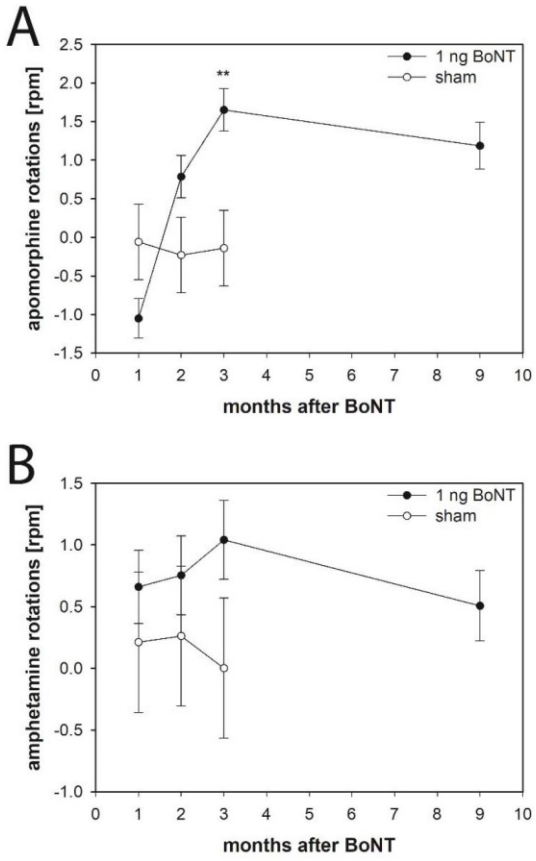

Figure 3. (**A**) Apomorphine- and (**B**) amphetamine-induced rotations in rats intrastriatally injected with BoNT-A (n = 22) or vehicle (n = 6). (**A**) 1 ng BoNT-A caused a significant anti-clockwise apomorphine-induced turning 3 months after injection, (**B**) but did not influence amphetamine-induced rotation behavior significantly 1–9 months after BoNT-A as compared to the sham group. Asterisks indicate significant differences compared with the sham group (** $p < 0.01$). Data are means ± SEM.

2.3. Amphetamine-Induced Rotations

2.3.1. Mice

After intrastriatal injection of 25 or 50 pg BoNT-A, the mice showed significantly increased ($F_{2, 48}$ = 15.360, $p < 0.001$) amphetamine-induced rotations compared to the vehicle group from 1 to 9 months post injection (Figure 2B). Whereas application of BoNT-A was associated with strong amphetamine-induced anti-clockwise net rotations of approximately 4–10 per min, vehicle injection resulted in clockwise rotations of approximately 3 per min (Figure 2B).

2.3.2. Rats

In rats, amphetamine application revealed no significant effect of unilateral intrastriatal BoNT-A as compared with vehicle injection (Figure 3B).

2.4. Cylinder Test

The cylinder test evaluates asymmetries in spontaneous forelimb use during exploratory activity in a novel environment. Sham injection did not induce any laterality of forepaw usage during the whole testing period up to 9 months; left and right forepaws were used equally often in the sham group (Figure 4A). In contrast, BoNT-A-injected mice, irrespective of the dosage and survival time, exhibited a significantly reduced ($F_{2, 52} = 31.971$, $p < 0.001$) use of the left forepaw of about 40% (Figure 4A).

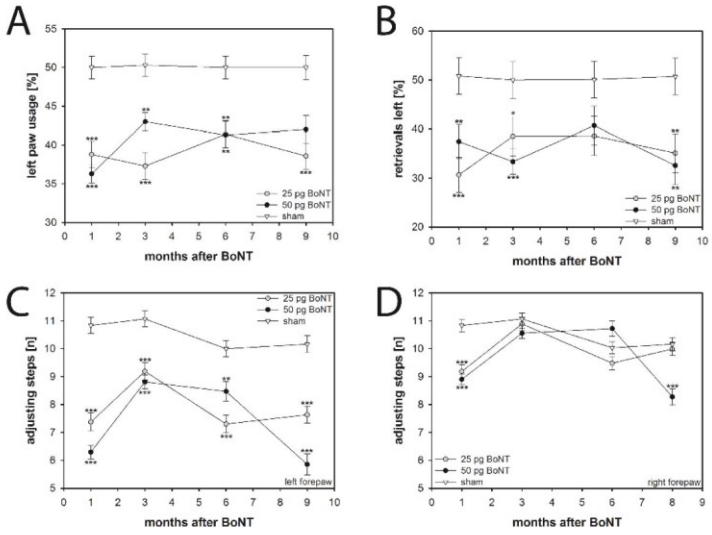

Figure 4. (**A**) The cylinder test of BoNT-A-injected mice (25 pg, $n = 15$; 50 pg, $n = 20$) of both dosages revealed a significantly lower use of the left forepaw at all time points compared with sham-injected mice ($n = 11$). (**B**) In the corridor task, BoNT-A-injected mice of both dosages (25 pg, $n = 11$; 50 pg, $n = 12$) retrieved pellets significantly less often from the left side compared to the sham group ($n = 10$). (**C**,**D**) The stepping test revealed constant adjusting steps of sham-treated mice ($n = 10$). (**C**) Right side BoNT-A-injected mice of both dosages (25 pg, $n = 11$; 50 pg, $n = 10$) displayed significantly decreased left forepaw adjusting steps 1–9 months after surgery, whereas (**D**) the number of adjusting steps of the right forepaw only differed significantly from the sham group one month after BoNT-A injection. However, the 50 pg BoNT-A group also showed significantly fewer right forepaw adjusting steps 9 months after injection. Asterisks indicate significant differences compared with the sham group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data are means ± SEM.

2.5. Corridor Task

Lateralized sensorimotor integration was assessed using the corridor test, which depends on the rodent's ability to retrieve food from either side of its body. Animals of the vehicle group made an equivalent number of retrievals (about 50% of total retrievals from both the left and right sides of the corridor) over the whole testing period (Figure 4B). However, mice of both BoNT-A groups showed a significant neglect ($F_{2, 40} = 11.506$, $p < 0.001$) of the corridor side contralateral to the BoNT-A injection side. These mice retrieved only about 35% of the sugar pellets from the left corridor side during the whole observation period (Figure 4B).

2.6. Stepping Test

The motor activity of the forelimbs was estimated using a mouse-friendly version of the stepping test. Adjusting forepaw steps were measured on the injected (right) and noninjected (left) sides. Mice of the sham group made approximately 10–11 steps with either forepaw during the whole testing time

(Figure 4C,D). Mice of both BoNT-A groups exhibited significantly fewer adjusting steps ($F_{2, 42} = 99.773$, $p < 0.001$) with their left forepaws up to 9 months after BoNT-A (Figure 4C). A reduction in adjusting steps with the right forepaw was seen in BoNT-A-injected mice of both dosages 1 month after BoNT-A (Figure 4D).

2.7. Hindlimb Clasping

We evaluated neurological abnormalities using the hindlimb clasping test. Mice of the two BoNT-A groups and the sham group did not show any pathological hindlimb clasping during the whole testing period from 1 to 9 months; the hindlimbs of all mice were consistently splayed outward and away from the abdomen resulting in an assigned score of 0.

3. Discussion

Previous studies of unilateral injections of BoNT-A into the CPu of male C57BL/6 mice showed that the number of ChAT-ir interneurons was unaltered in the CPu, and ChAT-ir BoNT-A-induced varicosities were visible. Both results are comparable with findings in the rat [16–20]. However, in contrast to rats, there were no TH-ir BoNT-A-induced varicosities in BoNT-A-injected mouse. As BoNT-A application seemed to have a different influence on cholinergic and dopaminergic axons in the mouse striatum [24], the CPu-associated motor behavior was studied. Moreover, a possible temporally occurring BoNT-A effect as seen in previous studies in rats [16–21] was evaluated by testing mice up to 9 months after BoNT-A injection. Dose dependency was studied by using dosages of 25 and 50 pg BoNT-A. As a significant dose dependency within the evaluated parameters was not obvious, the results of mice receiving dosages of 25 and 50 pg BoNT-A will be discussed together.

We found that unilateral intrastriatal BoNT-A injection in naïve mice led to significant contralateral amphetamine- and apomorphine-induced rotations as well as to significant impairments of the left (i.e., contralateral) side with respect to lateralized sensorimotor integration, forelimb usage, and forelimb stepping.

3.1. Basal Ganglia Circuitry after BoNT-A Injection

Locally injected BoNT-A is thought to act in two main ways in the striatum, where it blocks the acetylcholine release of the tonically active cholinergic interneurons [46–48], counteracts the D_2 receptor upregulation found in hemi-PD rats, and reduces D_2 receptor concentrations in naïve rats [22,23]. The assumed BoNT-A-induced reduction of acetylcholine concentration in the injected CPu reduces the firing activity of medium spiny neurons projecting to (i) the external globus pallidus, i.e., those which are part of the indirect basal ganglia loop, and (ii) to the internal globus pallidus, i.e., those which are part of the direct basal ganglia loop [49–53].

The effects of BoNT-A on the reduction of cholinergic transmission and of D_2 receptor concentrations seemingly underlie movement initiation deficits, akinesia and reduced spontaneous use of the contralateral forelimbs [54–57].

3.2. Body Weight

Body weight increased in all three experimental groups from two weeks up to 9 months after intrastriatal BoNT-A or vehicle injection. However, mice injected with either of the BoNT-A dosages weighed less compared to the sham group two weeks after injection onwards, although the differences diminished after 9 months. These differences might point at a mild temporal toxicity of BoNT-A after the stereotactic injection.

3.3. Spontaneous Motor Tests

3.3.1. Spontaneous Forelimb Use

The cylinder test evaluates locomotor asymmetry and forelimb use in rodent models of central nervous system disorders by assessing the innate drive to explore a novel environment by rearing and leaning their forepaw against the wall of the glass cylinder [58]. Unilaterally sham-injected mice used the left and right paws symmetrically, i.e., about 50% each. Right side intrastriatal application of BoNT-A significantly reduced the use of the left paw.

Seemingly, the impairment of motor initiation deficit for voluntary movements of the contralateral forelimb is a specific result of BoNT-A injection into the CPu since it decreases firing of GABAergic medium spiny neurons to the internal globus pallidus in the direct loop, and therefore inhibits the ventrolateral thalamic nucleus. On the other hand D_2 receptor-bearing medium spiny neurons increase inhibition of the external globus pallidus in the indirect loop, and resulted in a more actively firing internal globus pallidus via reduced inhibition of the spontaneously active subthalamic nucleus. Therefore, the inhibited neurons of the ventrolateral thalamic nucleus are not able to sufficiently activate the premotor cortex via both loops.

3.3.2. Sensorimotor Integration

The corridor task, originally designed to study unilateral sensorimotor integration impairments in rats [59,60], was adapted for experiments in mice [61]. Mice injected with 25 or 50 pg BoNT-A into the right striatum retrieved pellets significantly less often from the left side during the testing period up to 9 months after BoNT-A, whereas sham-injected mice behaved symmetrically. As is the case for the forepaw preference studied in the cylinder test, the motor initiation deficit for voluntary movements of the contralateral forelimb is probably a specific result of the BoNT-A injection into the CPu. Both the reduction of striatal cholinergic transmission and the reduction of D_2 receptor density causes changes in the basal ganglia circuitry resulting in a reduced initiation of movements of the contralateral body via crossed motor efferents [57,62,63].

3.3.3. Forelimb Adjusting Steps

In our study, we used the mouse-friendly version of the stepping test described by Blume et al. [64] and modified by Heuer et al. [65]. Right sided intrastriatal application of 25 and 50 pg BoNT-A clearly reduced the stepping frequency of the left forepaw up to 9 months after injection compared to sham group. A reduced initiation of movements of the paw contralateral to the BoNT-A application via crossed motor efferents is reasonable for the outcome of this behavioral test [66–68]. Interestingly, the intrastriatal BoNT-A injection into the right CPu also results in a short-term reduction of right paw adjusting steps. One month after vehicle injection mice made 10.8 ± 0.2 steps; at the same time the 25 pg BoNT-A group made 9.2 ± 0.2, and the 50 pg BoNT-A group 8.9 ± 0.2 steps. This phenomenon is not fully understood, as the right forepaw steps should be unaltered. The mouse-friendly design of the stepping test has also been applied in the mouse MTPT model [64] resulting in bilateral impairment, and also following different right side models of dopaminergic lesioning to induce unilateral parkinsonian-like symptoms [65,69,70]. Unilateral dopaminergic depletion, however, led to contradicting results in the same test. Heuer et al. [65] stated that the number of steps is reduced in both paws to about 80% compared to sham-injected mice irrespective of the experimental approach inducing unilateral or bilateral striatal dopamine reduction. In contrast, Glajch et al. [69] reported a ratio of contralateral-to-ipsilateral steps of about 0.2 after unilateral 6-OHDA lesion of the medial forebrain bundle. Boix et al. [70] reported a ratio of approximately 6 to 27%, depending on the 6-OHDA dosage used. Thus, both groups show a clear unilateral effect. However, neither Glajch et al. [69] nor Boix et al. [70] mention the absolute number of steps made by the ipsilateral paw, which would be important for comparison with our results.

3.3.4. Hindlimb Clasping

Hindlimb clasping has been shown to occur in various neurodegenerative mouse models [71,72] including PD models [73,74]. All our mice, irrespective of BoNT-A or vehicle injection, never showed pathological hindlimb clasping during the whole testing period from 1 to 9 months. We interpret the absence of this pathological behavior in our model as indirect evidence that the BoNT-A dosages used are not generally toxic in the striatum.

3.4. Drug-Induced Rotation Tests

3.4.1. Apomorphine-Induced Rotations

There is an apomorphine-induced rotational behavior of BoNT-A-treated mice and rats. Mice with 25 and 50 pg BoNT-A injected intrastriatally showed significant contralateral apomorphine-induced rotations after a survival of 1 and 3 months with rotations of about 2 per minute. Thereafter, rotational behavior decreased to values not significantly different from those of the vehicle group. These measurements corroborate data obtained in rats (Figure 3A), which also showed a significantly altered behavior with rotations of approximately 1.5 to 2 per minute 3 months after intrastriatal injection of 1 ng BoNT-A.

Apomorphine-induced rotations are mainly due to binding of the drug to D_2 receptors that are distributed unequally in both striata. Following apomorphine application, hemi-PD rats and hemi-PD mice rotate to the body side contralateral to the dopamine-depleted hemisphere, which has a higher D_2 receptor concentration than the contralateral one [65,70,75]. Acetylcholine is reduced in BoNT-A-injected striata, and thus, the activation of all medium spiny neurons is reduced. Moreover, striatal receptor concentration measurements investigated in BoNT-A-treated hemi-PD rats speak in favor of a BoNT-A-induced reduction of D_2 receptors in the respective CPu [22,23].

Considering that the majority of striatal D_2 receptors are located on the D_2 receptor bearing medium spiny neuron, an unaltered dopamine concentration in the CPu would result in a movement deficit of the contralateral body side via the indirect basal ganglia loop in BoNT-A-treated striata. However, if the majority of striatal D_2 receptors are located on the presynaptic terminals of the dopamine afferents from the substantia nigra pars compacta, then dopamine release should be increased due to reduced inhibition by D_2 autoreceptors on dopamine terminals [76]. The hypothetically increased striatal dopamine concentration in the BoNT-A-injected CPu would result in an increased movement of the contralateral body side via the indirect basal ganglia loop as suggested by Da Cunha et al. [77]. As all tests for spontaneous motor behavior after striatal BoNT-A revealed an initiation deficit in the contralateral forelimb, the functional significance of D_2 autoreceptors for these non-drug-induced behaviors seemed limited.

Since apomorphine is mainly a D_2 receptor agonist, its application possibly reverses the alterations in basal ganglia circuitry induced by BoNT-A by shifting the dopamine-mediated functional significance from D_2 receptor-bearing medium spiny neuron to D_2 autoreceptor-bearing dopamine terminals, thus resulting in a stronger movement initiation in the contralateral body musculature. If dopamine release by the disinhibited dopamine terminals exceeds the apomorphine-induced disinhibition of the D_2 receptor-bearing medium spiny neuron, then we can assume the occurrence of a mildly increased contralateral forelimb activity via deactivation of the medium spiny neuron of the indirect basal ganglia loop, which would result in the contralateral rotation behavior [66,67].

3.4.2. Amphetamine-Induced Rotations

Mice with intrastriatal injection of 25 or 50 pg BoNT-A show clear anti-clockwise rotations of about 4–10 per min following the application of amphetamine (Figure 2B). In contrast, comparable experiments in rats revealed no significant effect of BoNT-A injections as compared with vehicle injection (Figure 3B). The obvious difference in BoNT-A-inducible amphetamine rotations between mice and rats is not fully understood.

Amphetamine mainly increases the extracellular dopamine concentration by different mechanisms: it competitively inhibits dopamine uptake via dopamine transporter, facilitates the movement of dopamine from the vesicle into the cytoplasm, and promotes DAT-mediated reverse transport of dopamine into the synaptic cleft independent of action potential-induced vesicular release [78]. Additionally, microdialysis studies in rats revealed amphetamine-induced increased extracellular concentrations of glutamate, aspartate, GABA, taurine, glycine, serotonin, acetylcholine, and of different peptides [79–86].

There is a notable species difference regarding amphetamine-induced contralateral rotation behavior in unilaterally BoNT-A-injected animals, since it is present in mice, but not in rats. Three aspects will be discussed: (1) different effects on axon terminals after intrastriatal BoNT-A injections in mice and rats, (2) the concentrations of the most frequent transmitter receptors in the CPu of naïve mice and rats, and (3) the connectome of the basal ganglia of mice and rats.

Hawlitschka et al. [24] showed that striata from mice and rats react differently after BoNT-A injection with respect to the appearance of TH-ir BoNT-A -induced varicosities [24], since they are consistently found in rats, but were never seen in mice [19,24]. BoNT-A-induced varicosities can be interpreted as a sign of structural alterations induced by BoNT-A. The difference in the occurrence of BoNT-A-induced varicosities between mice and rats might be based on the synaptic vesicle glycoprotein C (SV2C) receptor responsible for the internalization of BoNT-A into the neuron [87,88]. Unfortunately, no data exist concerning differences of SV2C affinity or susceptibility between the CPu of rats and mice [87–95]. It cannot be ruled out that the dopaminergic fibers are differently influenced by BoNT-A and thus the balance or interaction of various transmitter systems on the functional outcome underlying the amphetamine-induced rotation behavior of intrastriatally applied BoNT-A may differ [79,96–101]. In contrast to the interspecies differences in BoNT-A-induced varicosities, ChAT-ir BoNT-A-induced varicosities were consistently found in CPu of both rats and mice following intrastriatal BoNT-A.

3.4.3. Receptors and Connectomics of the CPu

To analyze possible interspecies differences of transmitter systems in the CPu of control rats and mice, we compared the multireceptor fingerprints (Figure S1) of Wistar rats (n = 6, data from [23,102,103]) and of C57Bl/6 mice (n = 6; data from [40]). Additionally, the connectome of the mouse basal ganglia generated in a high throughput tract tracing study published as Alan Atlas connectome [104] is compared with rat data (Figure S2). Details to multireceptor fingerprints and connectomes are provided in the Supplement.

4. Conclusions and Future Perspectives

BoNT-A unilaterally injected into the CPu in naïve mice differentially affected various motor behaviors. Lateralized sensorimotor integration, forelimb preference, and forelimb stepping were significantly impaired contralateral to the injected side. Unilateral intrastriatal BoNT-A induced significant contralateral amphetamine-induced rotations from 1 to 9 months post injection, which is completely opposite to the reaction found in rats. The differences in motor behavior induced by unilateral intrastriatal BoNT-A injections between rats and mice is possibly caused by different effects of BoNT-A on TH-ir fibers in rat and mouse striata, interspecies differences in striatal receptor densities, and different connectomes of the basal ganglia between mice and rats.

Local injection of BoNT-A into the striatum of hemi-PD rats is thought to decrease the local release of acetylcholine therein. Hypercholinism of the striatum caused by acetylcholine released from disinhibited tonically active cholinergic interneurons is held responsible for a disturbed basal ganglia circuitry and, consequently, for motor and behavioral dysfunctions [9,46,48,105]. One possible approach to treat PD is the reduction of the hypercholinism using oral anticholinergic drugs [106,107]. Due to the systemic drug application, adverse side effects such as anticholinergic syndrome and dyskinesia are common [13–15,108]. In the experimental hemi-PD rats, BoNT-A injected locally into

the CPu is beneficial for 3 to 6 months with respect to apomorphine-induced rotational behavior [16–21]. Future experiments will cover two topics: First, repeated intrastriatal BoNT-A injections in hemi-PD rats every 6 months should test whether motor behavior could be improved for a longer time period, which would be a prerequisite for the clinical application of BoNT-A [109–112]. Second, extending our studies of intrastriatal BoNT-A injections to naïve mice, BoNT-A will be injected into mice of various parkinsonian models including those with alterations of relevant PD-associated genes to obtain further insights into PD-related etiology [39,40,113–115].

In conclusion, locally applied BoNT-A, or other botulinum neurotoxins, could be useful in treating brain dysfunctions requiring a deactivation of local brain activity [116]. Advantageously, the effect of local BoNT-A is time-limited and reversible. It can be speculated that following prospective experiments in primates botulinum neurotoxins might be applied as an effective and individually-tailored "chemical neurosurgical approach" [116,117].

5. Materials and Methods

5.1. Animals

A total of 46 young adult male C57BL/6 mice (Charles River Wiga, Sulzfeld, Germany) weighing 18–24 g and 28 Wistar rats (strain Crl: WI BR, Charles River Wiga, Sulzfeld, Germany) with a body weight of 280–320 g were used in this study. Animals were housed in standard cages in a temperature-controlled room (22 ± 2 °C) under 12 h light/12 h dark conditions with free access to food and water. All procedures were approved by the State Animal Research Committee of Mecklenburg-Western Pomerania (LALLF M-V/TSD/7221.3-1.1-053/08, LALLF M-V/TSD/7221.3-1.1-003/13 from 26 April 2013 and 13 April 2016).

5.2. BoNT-A application

In the mice, surgery was conducted under aseptic conditions and animals were deeply anesthetized with ketamine (75 mg/kg, bela-pharm Vechta, Germany)/xylazine (5.8 mg/kg, Rompun®, Bayer, Germany), mounted in a mouse adapter (Stoelting, Wood Dale, IL, USA), and fixed in a rat stereotactic apparatus (Kopf, Tujunga, CA, USA). The skull was opened with a dental drill and the mice received an injection of 1 µL BoNT-A solution (Lot No. 13028A1A; List, Campbell, CA, USA, purchased via Quadratech Diagnostics, Surrey, UK) containing a total of 25 pg or 50 pg BoNT-A dissolved in PBS + 0.1% BSA into the right CPu delivered over 4 min using a 26-gauge 5 µL Hamilton syringe, at a rate of 0.25 µL per minute (Figure 5). The sham group received 1 µL BoNT-A vehicle solution. The injection coordinates with reference to bregma were: anterior-posterior = +0.65 mm, lateral = −1.6 mm, and vertical = −3.0 mm from dura, respectively [118] (Figure 5).

Rats were injected either with 1 ng BoNT-A (n = 22) or the vehicle solution (sham, n = 6) into the right striatum under ketamine (50 mg/kg)/xylazine (4 mg/kg) anesthesia. The BoNT-A solution was injected at two sites, each injection consisting of 0.5 ng BoNT-A solved in 1 µL PBS + 0.1% BSA. Sham-injected rats received the vehicle solution. The coordinates according to bregma were: anterior-posterior = +1.3 mm/−0.4 mm, lateral = −2.6 mm/−3.6 mm and ventral = −5.5 mm/−5.5 mm, respectively [119].

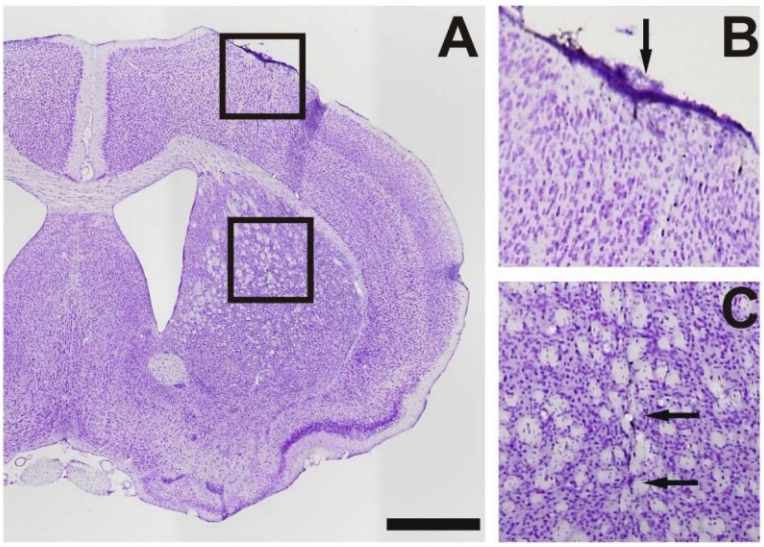

Figure 5. (**A**) A coronal Nissl-stained brain section 30 µm thick of a mouse treated intrastriatally with 25 pg BoNT-A 6 months before sacrifice. (**B**,**C**) Higher magnifications of the boxes in (**A**). In (**B**) the needle tract through the cortex is marked by an arrow, in (**C**) the injection channel in the striatum is indicated by two arrows. Scale bar applies to (**A**): 1 mm.

We founded our experiments on the weight values of BoNT-A, i.e., 25 to 50 pg per mouse CPu and 1 ng per rat CPu. In mice the LD_{50} value after intraperitoneal injection is about 0.25–1.15 ng/kg BoNT-A [120]. Thus, in our experiments a dosage of 50 pg BoNT-A per mouse, injected intrastriatally, is below the classical LD_{50}, and well tolerated. In clinical use the normal amount of BoNT-A (Xeomin, Dysport, Botox and others) is about 200 to 300 units at maximum per patient per session depending on the clinical symptoms and site of application. Remarkably, the clinical efficacy of a "unit" is reported differently by the various manufactures (Botox: 5 ng correspond to 100 units; Dysport: 4.35 ng correspond to 500 units; Xeomin: 0.6 ng correspond to 100 units) [121]. These uncertainties relay on the nonstandardized LD_{50} bioassays required to achieve the median lethal dose = LD_{50} (depending on mouse strain, sex, age, volume and route of injection) [122].

5.3. Body Weight

Body weights were measured before stereotactic surgery and 0.5, 1, 2, 3, 4, 5, 6, 7, and 9 months after injection of BoNT-A or vehicle.

5.4. Behavioral Testing

Three experimental mouse groups were behaviorally tested: (1) animals receiving 25 pg BoNT-A (25 pg BoNT-A group, $n = 15$), (2) animals receiving 50 pg BoNT-A (50 pg BoNT-A group, $n = 20$), and (3) animals receiving vehicle (sham group, $n = 11$). During the experimental period 5 mice died. Smaller groups were evaluated for the stepping test and the corridor task (25 pg, $n = 11$; 50 pg, $n = 12$; sham, $n = 10$). All mice were adapted to the examination room for 1 h before testing. All tests were performed at 4 time points (1, 3, 6, and 9 months) after the injection of BoNT-A or vehicle, and drug-induced rotations were additionally scored 0.5 months after BoNT-A or vehicle, always according to an identical time schedule (Figure 6). In rats drug-induced rotation tests were carried out 1, 2, 3, and 9 months after BoNT ($n = 22$) or vehicle ($n = 6$) injection.

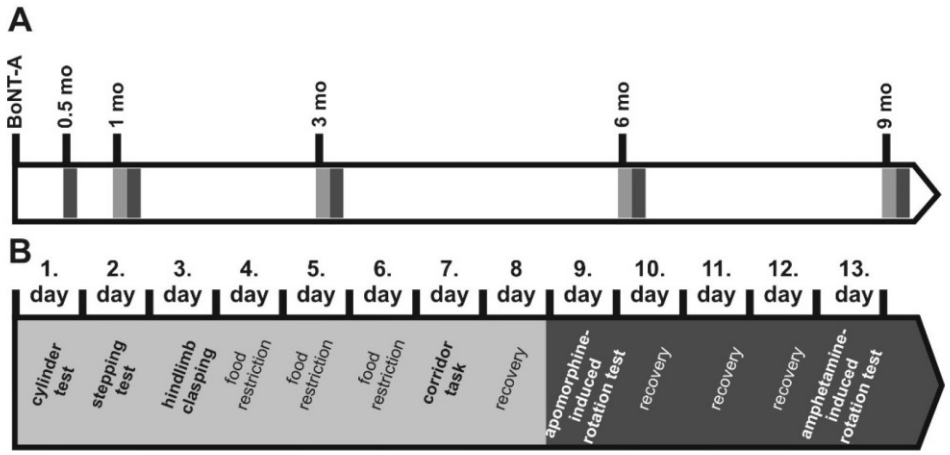

Figure 6. (**A**) Time schedule of the stereotactic application of BoNT-A in mice and behavioral tests. Light grey rectangles symbolize batteries of non-drug-induced behavior tests, dark grey rectangles the subsequently performed apomorphine- and amphetamine-induced rotation tests. (**B**) Detailed visualization of single behavior test batteries. Non-drug-induced tests were performed on the days presented in the light grey part of the time line, drug-induced tests on the days of the dark grey part. The non-drug-induced tests were performed as follows: On the first day the mice underwent the cylinder test until 30 consecutive touches of the glass wall with the forepaws were done. On the second day the stepping test and on the third day the hindlimb clasping were carried out three times per mouse, respectively. On the following 3 days mice underwent food restriction and on the seventh day the corridor task was performed. Following recovery the drug-induced rotation tests were conducted.

5.4.1. Drug-Induced Rotation Tests (Apomorphine, Amphetamine)

Mice rotations were assessed using an automated rotometer system (Rotometer UGO BASILE 43000; Ugo Basile Sri, Gemonio, Varese, Italy). Apomorphine was injected subcutaneously at a dosage of 0.5 mg/kg [123–125] (Teclapharm, Lüneburg, Germany) dissolved in 0.9% sterile saline. Three days later, D-amphetamine sulphate (Sigma Aldrich, München, Germany) was injected intraperitoneally at a dosage of 2.5 mg/kg [124,126,127] (Figure 6). Recording of apomorphine- and amphetamine-induced rotations began 5 min after drug injection and lasted 40 min. Rotations were defined as complete 360° turns and registered as net difference between the two directions per minute [128]. Anti-clockwise rotations were expressed by positive values, rotations in clockwise direction by negative values. Respective rotations in rats were induced by either 0.25 mg/kg apomorphine applied subcutaneously (Teclapharm, Germany) or 2.5 mg d-amphetamine sulfate (intraperitoneal; Sigma Aldrich, München, Germany), both solved in saline. Rotations were measured over 40 min after apomorphine and over 60 min after amphetamine in a self-constructed rotometer according to Ungerstedt and Arbuthnott [128].

5.4.2. Spontaneous Motor Tests

Mice were tested in the cylinder test, stepping test, hindlimb clasping, and corridor task at 1, 3, 6, and 9 months after injection of BoNT-A or vehicle following a constant time table (Figure 6).

Cylinder Test

Forelimb preference was evaluated with the cylinder test as previously described [58,129]. Mice were placed in a glass cylinder (diameter 19 cm, height 20 cm) with mirrors placed behind to allow for a 360° view of every contact with the side of the cylinder. Sessions were taped with a video camera

system (JVC, GZ-MG255E, Yokohama, Japan), and scored later. For each animal thirty consecutive forepaw contacts with the glass cylinder were evaluated by counting the initial contacts of the right or left paw. Then the ratio of left and right forepaw use was calculated. Evaluation of the videotapes was performed by an observer blinded to the animals' identities.

Corridor Task

Lateralized sensorimotor integration and neglect were examined using the corridor task [61]. For our study we used a custom-made 60 cm long, 4 cm wide, and 15 cm high alleyway equipped with 10 pairs of adjacent pots with a diameter of 1 cm, placed at 5 cm intervals and containing 5 sugar pellets (Ain-76A Rodent Tablet 20 mg TestDiet, Richmond, IN, USA). Prior to testing, the mice were food-restricted for three days and maintained at 90% of free-feeding bodyweight during habituation and testing [130]. Animals were adapted to the apparatus for 10 min each on two consecutive days with some scattered sugar pellets along the floor of the corridor and started from different ends of the corridor each day. On the test day, mice were at first positioned in an identical, but empty corridor for 5 min for adaptation and then placed at the end of the testing corridor with bowls containing the pellet. Animals were allowed to move freely along the apparatus for 5 min to retrieve pellets placed on either side of their body. The number of ipsilateral (right side) and contralateral (left side) retrievals made by each mouse was calculated and the data were expressed as a percentage of left and right retrievals of the total number of retrievals. A "retrieval" was defined as a nose poke into a bowl, whether or not pellets were taken, and a new retrieval was counted by investigating a new pot [59,61].

Stepping Test

Forelimb akinesia was assessed according to Blume et al. [64] and modified by Heuer et al. [65] using an open table (1.5 m in length). Mice were tested three times in one day during the day light cycle. Each trial was recorded on video. As a first step for habituation to the test, mice were allowed to settle at one end of the table for 1–2 s, with all limbs on the table. Secondly, the experimenter gently lifted up the hindlimbs by pulling up on the tail leaving only the forepaws touching the table surface. Then, at a steady pace of 1 m in 3–4 s the experimenter pulled the animal the total test distance of 1 m backwards by the tail. Finally, the numbers of adjusting steps made with the left and right forepaws were counted offline in the videos.

Hindlimb Clasping

Hindlimb clasping is a marker of disease progression in a number of mouse models of neurodegeneration, including certain cerebellar ataxias [131] and parkinsonian mouse models [73,74], and was performed as previously described [72,73]. Outside the cage, each mouse was slowly lifted by the tail for 10 s and then lowered back to the surface. The hindlimbs were observed and the position of the hindlimbs was scored for each trial [72]. A score of 0 indicated that the hindlimbs were consistently splayed outwards and away from the abdomen. A score of 1 indicated that one hindlimb was retracted inwards towards the abdomen more than 50% of the trial period, a score of 2 indicated that both hindlimbs were partially retracted inwards towards the abdomen for at least 50% of the observation period. Mice were tested three times on one day and each trial was recorded on video.

5.5. Receptor Autoradiography and Histology

The autoradiographic procedure was performed according to standard protocols already published [132–136]. Cryosections of the respective brains were stained with cresyl-violet acetate (SIGMA C1791-5G) to verify the injection sites.

5.6. Statistical Analysis

Data of all behavioral tests were subjected to two-way ANOVA with repeated measurements. The Holm–Sidak test was used for post-hoc comparisons. The level of significance was set at $p \leq 0.05$ for all statistical analyses. All statistical tests were done using SigmaPlot 11 Software (Systat Software, Inc., San Jose, CA 95110, USA).

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6651/10/7/295/s1, Figure S1: Multi-receptor fingerprints of the caudate-putamen of mouse (blue) and rat (red), Figure S2: (A) Differential adjacency matrix of the rat and mouse basal ganglia. (B) Differential reciprocity matrix of rat and mouse basal ganglia.

Author Contributions: Conceptualization and Supervision, V.A., A.H., O.S. and A.W.; Investigation, A.H., V.A. and A.W.; Visualization, C.H., O.S. and A.H.; Methodology, K.Z., N.P.-G. and T.M.; Writing-Original Draft Preparation , V.A., C.H., O.S., A.W., A.H., N.P.-G. and K.Z.; Writing-Review & Editing, V.A., C.H., A.W., N.P.-G. and K.Z.; Funding Acquisition, A.H., K.Z.

Funding: Rostock University Medical Center internal funding FORUN 889005 and 889014, European Union's Horizon 2020 Framework Programme for Research and Innovation under Grant Agreement No 720270 (Human Brain Project SGA1) (K.Z.), and Portfolio Theme "Supercomputing and Modeling for the Human Brain" of the Helmholtz Association.

Acknowledgments: We gratefully acknowledge S. Lehmann, F. Winzer, M. Cremer, S. Wilms, S. Buller, J. Bausch, S. Krause and A. Börner for their excellent technical assistance.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

6-OHDA	6-hydroxydopamine
BoNT-A	botulinum neurotoxin-A
ChAT	choline acetyltransferase
CPu	caudate-putamen
D_1	dopamine D_1 receptor
D_2	dopamine D_2 receptor
GABA	α-amino butyric acid
hemi-PD	hemiparkinsonian
ir	immunoreactive
nic	acetylcholine nicotinic $\alpha_4\beta_2$ receptor
PD	Parkinson's disease
SV2C	synaptic vesicle glycoprotein C
TH	tyrosine hydroxylase

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Brain Research xxx (xxxx) xxxx

Contents lists available at ScienceDirect

Brain Research

journal homepage: www.elsevier.com/locate/brainres

Research report

Repeated intrastriatal application of botulinum neurotoxin-A did not influence choline acetyltransferase-immunoreactive interneurons in hemiparkinsonian rat brain – A histological, stereological and correlational analysis

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HIGHLIGHTS

- Repeated intrastriatal injections of 1 ng BoNT-A each were well tolerated.
- The volumes of the injected striata were reduced.
- In injected CPu the number of ChAT-ir interneurons was not significantly changed.
- In injected CPu the density of ChAT-ir BoNT-A-induced varicosities was increased.

ARTICLE INFO

Keywords:
Striatum
6-OHDA
Botulinum neurotoxin-A
Cholinergic interneuron
Botulinum neurotoxin-induced varicosities
Stereology
Sholl analysis
Nucleus of the horizontal limb of the diagonal band
Correlation analysis

ABSTRACT

In Parkinson's disease, dopamine depletion leads to hyperactivity of cholinergic interneurons in the caudate-putamen (CPu). Botulinum neurotoxin-A (BoNT-A) inhibits the release of acetylcholine in the peripheral nervous system and is also thought to act as a local anticholinergic drug when injected intrastriatally. In hemiparkinsonian (hemi-PD) rats, a unilateral intrastriatal injection of 1 ng BoNT-A significantly diminished apomorphine-induced rotation behavior for at least 3 months, the effect fading thereafter. A second intrastriatal BoNT-A application, 6 months after the first one, led to a stronger and longer-lasting, beneficial behavioral reaction.

As a single BoNT-A injection was not cytotoxic in the rat striatum and resembled BoNT-A treatment in clinical practice, here, we investigated the structural outcome of repeated intrastriatal BoNT-A injections with respect to striatal volume, the number of choline acetyltransferase-immunoreactive (ChAT-ir) interneurons and of the length of their dendritic arbors, and the numeric density of ChAT-ir BoNT-A-induced varicosities (BiVs). Repeated unilateral intrastriatal BoNT-A application decreased the volume of the injected CPu, but did not significantly change the number of striatal ChAT-ir interneurons. Also, the total dendrite length of ChAT-ir interneurons after repeated BoNT-A application resembled the values in double vehicle-injected hemi-PD rats. In repeatedly BoNT-A-injected hemi-PD rats, the numeric density of ChAT-ir BiVs in the CPu was increased compared with rats only intrastriatally injected once with BoNT-A. Even repeated BoNT-A injections in rat striata did not cause substantial morphological changes in ChAT-ir neuron, except for the increased numeric density of ChAT-ir BiVs.

Abbreviations: 6-OHDA, 6-hydroxydopamine; Ach, acetylcholine; BiVs, botulinum neurotoxin-A-induced varicosities; BoNT-A, botulinum neurotoxin-A; BW, body weight; ChAT, choline acetyltransferase; CPu, caudate-putamen (striatum); DA, dopamine; HDB, nucleus of the horizontal limb of the diagonal band; hemi-PD, hemiparkinsonian; ir, immunoreactive; MFB, medial forebrain bundle; PD, Parkinson's disease; Rpm, rotations per minute; SNAP-25, synaptosomal-associated protein-25; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase

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https://doi.org/10.1016/j.brainres.2020.146877
Received 6 January 2020; Received in revised form 6 April 2020; Accepted 5 May 2020
0006-8993/

Please cite this article as: Alexander Hawlitschka, et al., Brain Research, https://doi.org/10.1016/j.brainres.2020.146877

1. Introduction

Parkinson's disease (PD) is the most common and complex age-related chronic movement disorder and the second most prevalent neurodegenerative disease after Alzheimer's disease (de Lau and Breteler, 2006; De Rijk et al., 1997; Dorsey et al., 2007; Kalia and Lang, 2015). PD is classically characterized by the progressive loss of about 50–70% of the dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the reduction of dopamine (DA) in the caudate-putamen (CPu, striatum) (Boix et al., 2015; Poirier et al., 2016; Rafael et al., 2016), followed by a profound dysregulation of DA-modulated circuits in the basal ganglia (Albin et al., 1989; DeLong and Wichmann, 2007; Marsden, 1982; Obeso et al., 2008a,b). The resulting deficits cause a reduction of dopaminergic inhibition of tonically active cholinergic interneurons in the CPu (Ding et al., 2006; Oldenburg and Ding, 2011; Pisani et al., 2007; Ztaou et al., 2016); as a result, the concentration of striatal acetylcholine (ACh) increases and contributes to the major motor symptoms of PD such as bradykinesia or akinesia, abnormal gate, rigidity and tremors (Coffield and Yan, 2009; Day et al., 2006; Duvoisin, 1967).

The rationale for using anticholinergic agents in PD is to reduce the excess of ACh in the basal ganglia (Shah, 2017). Current anticholinergic drugs are able to improve tremor and movement symptoms of PD, but after long term intake they evoke unfavorable central and peripheral side effects (Katzenschlager et al., 2003; Müller, 2012; Rizek et al., 2016; Shah, 2017; Singer, 2002). Frequent central side effects are confusion, hallucination, and transient cognitive impairments. Peripheral side effects include nausea, dizziness, dry mouth, constipation, urinary retention, blurred vision and the precipitation of closed angle glaucoma (Clarke, 2002; Fernandez, 2012; Münchau and Bhatia, 2000; Whitney, 2007).

Botulinum neurotoxin-A (BoNT-A) is a potent neurotoxin, produced by the bacterium *Clostridium botulinum,* which mainly blocks the release of ACh from presynaptic vehicles by specific cleavage of synaptosomal-associated protein-25 (SNAP-25). SNAP-25 is a necessary component of the vesicle fusion apparatus of the cholinergic presynaptic membrane (Caleo et al., 2018; Jankovic, 2017; Kim et al., 2017; Pirazzini et al., 2017). Consequently, peripheral therapeutic BoNT-A applications are used in neurological conditions associated with cholinergic hyperfunction or movement dysfunctions (Jabbari, 2016; Montecucco and Molgó, 2005; Montecucco and Schiavo, 1995; Ney and Joseph, 2007; Safarpour and Jabbari, 2018).

In the mission to avoid unwanted side effects of systematically applied classical anticholinergic drugs, and hypothesizing that BoNT-A inhibits the release of ACh also as a local anticholinergic drug, we injected BoNT-A directly into the CPu in hemiparkinsonian (hemi-PD) rats (Antipova et al., 2019, 2017, 2013; Hawlitschka et al., 2018, 2013; Hawlitschka and Wree, 2018; Holzmann et al., 2012; Wree et al., 2011). One ng of BoNT-A injected intrastriatally into hemi-PD rats significantly annulled apomorphine-induced rotations for a minimum period of 3 months (Antipova et al., 2019, 2017, 2013; Hawlitschka et al., 2018, 2013; Hawlitschka and Wree, 2018; Holzmann et al., 2012; Wree et al., 2011). Rotation behavior gradually increased again during the 6 to 9 months thereafter (Antipova et al., 2017, 2013; Wree et al., 2011).

Additionally, we tested if repeated intrastriatal BoNT-A injections were capable of ameliorating the motor behavior in hemi-PD rats for an even longer time period, comparable with clinical practice used for BoNT-A applications (Fonfria et al., 2018; Gazerani, 2018; Hong et al., 2017; Intiso, 2012; Jankovic, 2018, 2017; Moga et al., 2018; Orsini et al., 2015). For this purpose, 1 month and 7 months following a 6-hydroxydopamine (6-OHDA) lesion, hemi-PD rats were intrastriatally treated with BoNT-A (each dose 1 ng) (Hawlitschka et al., 2018). Repetitive intrastriatal injections of BoNT-A in hemi-PD rats were well tolerated by the animals, and the second BoNT-A application significantly exceeded the behavioral outcome of the first one (Hawlitschka et al., 2018). In earlier experiments, we could also show that a singular intrastriatal BoNT-A injection in hemi-PD and naïve rats led to axonal swellings, so-called botulinum neurotoxin-A-induced varicosities (BiVs), which were immunoreactive (ir) either for choline acetyltransferase (ChAT) or for tyrosine hydroxylase (TH) (Mehlan et al., 2016; Wree et al., 2011). Also, in these rats there was neither a loss of the total number of all neurons nor of ChAT-ir interneurons in the CPu, even one year after unilateral intrastriatal BoNT-A injection (Antipova et al., 2013; Mehlan et al., 2016), speaking in favor of a missing toxic BoNT-A effect (Mehlan et al., 2016).

Here, using quantitative structural parameters, we investigated if repeated intrastriatal application of 1 ng BoNT-A, first 1 month and then 7 months after induction of hemi-PD and behaviorally characterized elsewhere (Antipova et al., 2019; Hawlitschka et al., 2018), had long-term cytotoxic effects on ChAT-ir interneurons and on characteristics of the ChAT-ir BiVs in the CPu. The respective data obtained in repeatedly injected rats are compared with those after singular intrastriatal BoNT-A injection (Mehlan et al., 2016). Additionally, the dendritic arbors of the ChAT-ir neurons of the nucleus of the horizontal limb of the diagonal band (HDB, (Paxinos and Watson, 2015)) were investigated in the same brains, as an area quite distant from the BoNT-A-injected CPu.

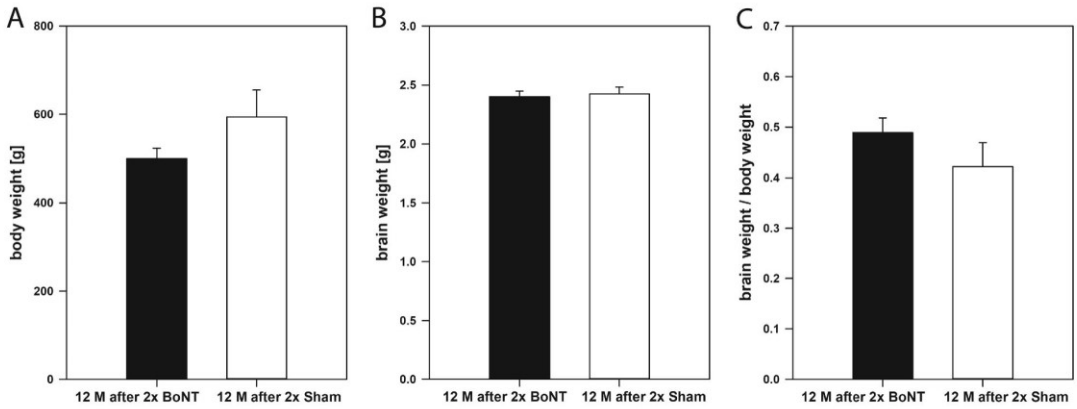

Fig. 1. Body weight (A), brain weight (B), and quotient of brain/body weight (C) of hemi-PD rats evaluated 12 months after the second intrastriatal BoNT-A (12 M after 2x BoNT, n = 8) or vehicle (12 M after 2x sham, n = 4) injections. 2x BoNT-A application neither altered body and brain weight nor the brain/body weight relation compared with 2x vehicle injection. ANOVA results, data are means ± SEM.

2. Results

2.1. Body weight

Both the first and the second intrastriatal BoNT-A injections were well tolerated by rats without any health problems. The body weight of both experimental groups was estimated as an index of possible general adverse effects of BoNT-A application. Rats weighed 290–310 g at the time of the first surgery. Accordingly, 12 months after the second BoNT-A injection hemi-PD rats weighed 500.0 ± 23.8 g (mean ± SEM), and thus did not significantly ($p = 0.111$) differ from the sham group (594.0 ± 61.4 g; Fig. 1A).

2.2. Brain weight

Also the brain weights of BoNT-A- (2.401 ± 0.049 g) (mean ± SEM) and vehicle-injected groups (2.425 ± 0.058 g) 12 months after the second applications were not significantly ($p = 0.769$) different (Fig. 1B). Respectively, the quotients of brain weight/body weight – as a biologically significant marker of neurotoxic damage (Azzaoui et al., 2008; Bailey et al., 2004; Haschek et al., 2013; Nirogi et al., 2014) – of both experimental groups 12 months after the second BoNT-A or vehicle injection did not differ significantly ($p = 0.236$; 2x BoNT: 0.489 ± 0.029; 2x sham: 0.422 ± 0.048) (Fig. 1C).

2.3. CPu volume

Twelve months after the second intrastriatal injection (Fig. 2A-B), the volume of the BoNT-A-injected right CPu (29.45 ± 1.22 mm^3, mean ± SEM; Fig. 2C) was significantly reduced compared with the contralateral non-injected left CPu (36.48 ± 1.29 mm^3; $p < 0.001$). In the sham group the volumes of the injected right (33.43 ± 1.74 mm^3) and non-injected left (37.07 ± 1.44 mm^3) CPu did not differ significantly ($p = 0.152$; Fig. 2C). Moreover, the striatal volumes of the non-injected left hemispheres were not significantly different between both experimental groups ($p = 0.788$; Fig. 2C).

2.4. ChAT-ir neurons

2.4.1. Numeric density of striatal ChAT-ir neurons

The numeric density of ChAT-ir neurons of the right BoNT-A-injected CPu (Fig. 2D) was significantly higher compared with the non-injected left CPu 12 months after the second BoNT-A application (Fig. 2D): right CPu − 703.6 ± 33.2 ChAT-ir neurons per mm^3 (mean ± SEM) versus left CPu − 594.8 ± 26.3 ChAT-ir neurons per mm^3, respectively; $p = 0.021$. In the sham group, the numeric density of ChAT-ir neurons of the injected right (614.5 ± 55.2 neurons per mm^3) and non-injected left (556.9 ± 34.3 neurons per mm^3) CPu did not differ significantly ($p = 0.359$; Fig. 2D). The numeric density of ChAT-ir neurons in the left hemispheres did not differ significantly between both experimental groups ($p = 0.485$; Fig. 2D).

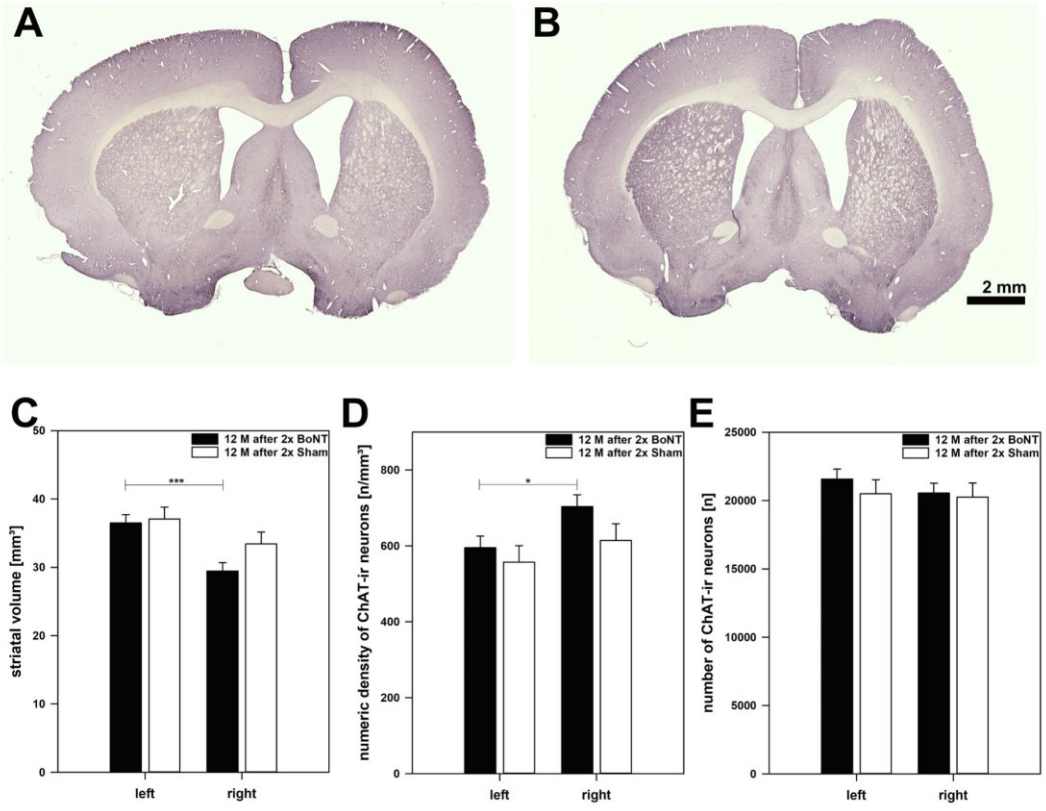

Fig. 2. Frontal sections immunohistochemically stained for ChAT (A-B). Right CPu was injected twice with vehicle (A) or with 1 ng BoNT-A (B). Volume of the left and right CPu (C), numeric density (D), and number of striatal ChAT-ir interneurons of the left and right CPu (E) of hemi-PD rats 12 months after the 2x BoNT ($n = 8$) or 2x sham ($n = 4$) applications. Two-way ANOVA results. Asterisks indicate significant differences compared with the sham group after Holm-Sidak post-hoc test (* $p < 0.05$, *** $p < 0.001$). Data are means ± SEM.

2.4.2. Number of striatal ChAT-ir neurons

The number of ChAT-ir neurons in the BoNT-A-injected right CPu (20546.3 ± 866.6, mean ± SEM) was not significantly different compared with the non-injected left CPu (21573.1 ± 788.2) 12 months after the second intrastriatal BoNT-A application (Fig. 2E). In the vehicle-treated group, the respective numbers were: right CPu – 20257.2 ± 655.23, left CPu – 20496.4 ± 488.4 ChAT-ir neurons. There were no significant interhemispheric differences in the vehicle-injected group (Fig. 2E). Moreover, the counts of ChAT-ir neurons did not differ significantly between both experimental groups ($p = 0.453$; Fig. 2E).

2.4.3. Sholl analysis of striatal ChAT-ir neurons

Twelve months after the second BoNT-A injection in hemi-PD rats (Fig. 3A-D), the total dendrite length of ChAT-ir neurons of the right BoNT-A-injected CPu (132.83 ± 10.87 µm, mean ± SEM) did not significantly differ from the non-injected left hemisphere (117.52 ± 6.15 µm) ($p = 0.475$; Fig. 3E). Correspondingly, the double sham-injected hemi-PD rats showed no significant side differences in dendrite length: right CPu – 89.67 ± 17.31 µm, and left CPu – 95.22 ± 10.96 µm ($p = 0.794$, Fig. 3E). Interestingly, the BoNT-A-injected striata did not differ significantly from the sham-injected group with respect to ChAT-ir neuronś dendrite length ($p = 0.305$).

In contrast, twelve months after the single intrastriatal injection of 1 ng BoNT-A in naïve rats the total dendrite length of ChAT-ir neurons of the ipsilateral CPu was 155.08 ± 20.14 µm, and therewith significantly lower than those found in the non-injected left CPu (206.94 ± 17.64 µm; $p = 0.028$) (Fig. 3E). All in all, the 1x BoNT rats had significantly longer dendrites after 12 months than the hemi-PD rats which had received 2x BoNT-A ($p = 0.002$) or vehicle ($p < 0.001$) (Fig. 3E).

2.4.4. Sholl analysis of ChAT-ir neurons of the nucleus of the horizontal limb of the diagonal band (HDB)

Sholl analysis showed no significant differences in the total dendrite length of ChAT-ir neurons of the HDB of both hemispheres in hemi-PD rats 12 months after the second BoNT-A or vehicle injection into the right CPu: 12 M after 2x BoNT – right HDB: 86.90 ± 8.64 µm, left HDB: 83.33 ± 8.55 µm, $p = 0.767$; 12 M after 2x sham – right HDB: 111.18 ± 0.42 µm, left HDB: 112.53 ± 6.86 µm, $p = 0.911$) (Fig. 3F).

Twelve months after a single intrastriatal BoNT-A injection in naïve rats, the total dendrite length of ChAT-ir neurons of the right and left HDB was rather similar: right – 141.44 ± 12.21 µm, left –

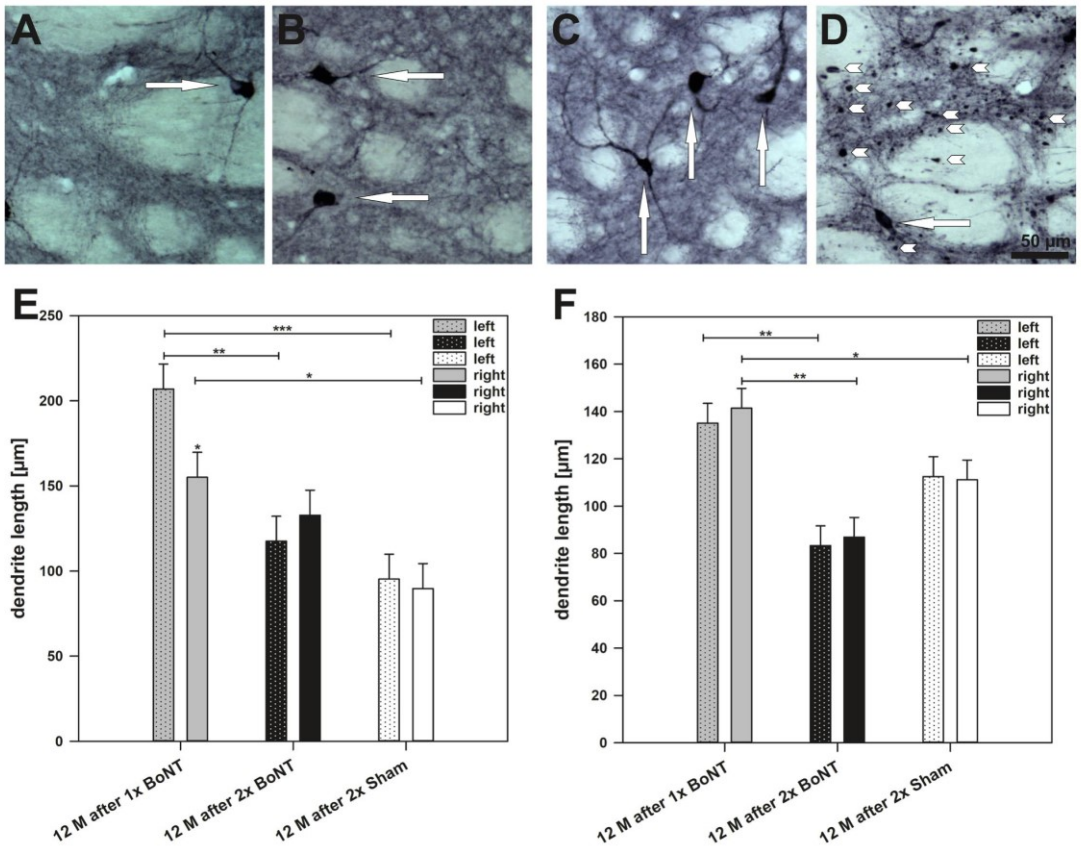

Fig. 3. Sholl analysis of the total dendrite length of ChAT-ir interneurons in the CPu and neurons of the nucleus of the horizontal limb of the diagonal band. Higher magnifications of ChAT staining (A-D) of left non-injected (A) and right sham-injected striata (B) 12 months after 2x vehicle, and of left non-injected (C) and right BoNT-A-injected (D) striata 12 months after 2x BoNT-A application. ChAT-ir interneurons are marked by white arrows, some ChAT-ir BiVs, only occurring in BoNT-A-injected striatum, are indicated by white arrowheads. Sholl analysis Two-way ANOVA results of the total dendrite length of ChAT-ir interneurons in the CPu (E) and neurons of the nucleus of the horizontal limb of the diagonal band (F). In both nuclei, injected right and non-injected left sides were evaluated in 3 rat groups (each $n = 3$): 12 M after 1x BoNT, 12 M after 2x BoNT and 12 M after 2x sham. Asterisks indicate significant post-hoc (Holm-Sidak test) differences between the hemispheres and groups, respectively (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data are means ± SEM.

135.11 ± 8.5 µm (Fig. 3F). Again, rats 12 M after 1x BoNT had significantly longer dendrites than the hemi-PD rats which had received 2x BoNT-A ($p = 0.002$) or 2x vehicle ($p = 0.049$) (Fig. 3F).

2.5. Botulinum neurotoxin-A-induced varicosities (BiVs)

2.5.1. Numeric density of striatal ChAT-ir BiVs

In the right CPu of hemi-PD rats 12 months after the second intrastriatal BoNT-A application we determined 4717.4 ± 562.2 varicosities per mm^2 (mean ± SEM) (Fig. 4A). Twelve months after the single intrastriatal BoNT-A injection in naïve rats only about half as many BiVs per mm^2 were found (1963.8 ± 369.1; $p = 0.02$) (Fig. 4A).

2.5.2. Size and size distribution of striatal ChAT-ir BiVs

The mean size, determined as projection area, of ChAT-ir BiVs of the right CPu in hemi-PD rats 12 months after the second intrastriatal BoNT-A injection, was 3.401 ± 0.089 $µm^2$ (Fig. 4B). The same parameter, measured in the CPu 12 months after a single BoNT-A injection in naïve rats, was found to be nearly identical: 3.270 ± 0.220 $µm^2$ ($p = 0.515$; Fig. 4B).

Sorting of striatal BiVs per mm^2 12 M after 1x BoNT-A and 12 M after 2x BoNT-A depending on their expanse revealed that, especially in the four smallest volume categories (0.0 to 3.84 $µm^2$), the rats injected twice with BoNT-A showed significantly more BiVs (Fig. 4C).

2.6. Correlation of volume of CPu, numeric density of ChAT-ir neurons, number of ChAT-ir neurons, and apomorphine-induced rotations

There were neither significant relationships between the apomorphine-induced rotations estimated 12 months after the second BoNT-A injection or vehicle applications in hemi-PD rats, nor between the CPu volumes, numeric density and number of the ChAT-ir neurons of both CPu in individual rats in either experimental group (Fig. S1A-F). Only the volumes of the left and right CPu 12 months after the second BoNT-A ($r(8) = 0.920$; $p = 0.00122$) or vehicle ($r(8) = 0.920$; $p = 0.00122$) injections in hemi-PD rats were significantly correlated (Fig. S1A,B). Also, the numeric density of ChAT-ir neurons of the left and right CPu in hemi-PD rats 12 months after the second vehicle injection showed a significant correlation ($r(4) = 0.952$; $p = 0.0480$; Fig. S1D).

3. Discussion

Single unilateral intrastriatal injection of 1 ng BoNT-A abrogates apomorphine-induced rotations for at least 3 months in hemi-PD rats (Antipova et al., 2019, 2018, 2017, 2013; Hawlitschka et al., 2018, 2013; Hawlitschka and Wree, 2018; Holzmann et al., 2012; Wree et al., 2011). Resembling BoNT-A treatment in clinical practice (Brisinda et al., 2015; De Boulle, 2007; Flynn, 2010; Özcan et al., 2006; Pagan and Harrison, 2012; Schurch, 2006; Sheean, 2006; Vashishta et al., 2013), repetitive intrastriatal BoNT-A applications 1 and 7 months after the 6-OHDA lesion in rats are possible and well tolerated by the animals (Antipova et al., 2019; Hawlitschka et al., 2018). A second BoNT-A injection into CPu had a more intense and longer-lasting effect on behavioral outcomes in hemi-PD rats than the first one (Hawlitschka et al., 2018).

In order to extend our knowledge about the intrastriatal BoNT-A treatment as a possible local anticholinergic therapy in the hemi-PD rat model, here we investigated whether a repeated intrastriatal BoNT-A injection had no or limited effects on the morphology of striatal ChAT-ir interneurons in hemi-PD rats.

3.1. Body weight and brain weight

We used a body and brain weight assessment as an indicator of possible general negative side effects of intrastriatal BoNT-A treatment in hemi-PD rats and estimated both parameters directly before histological analysis.

Neither intrastriatal vehicle nor BoNT-A at the dose of 1 ng injected twice, i.e. 1 and 7 months after 6-OHDA, in hemi-PD rats significantly influenced the body weight of the animals in either experimental group.

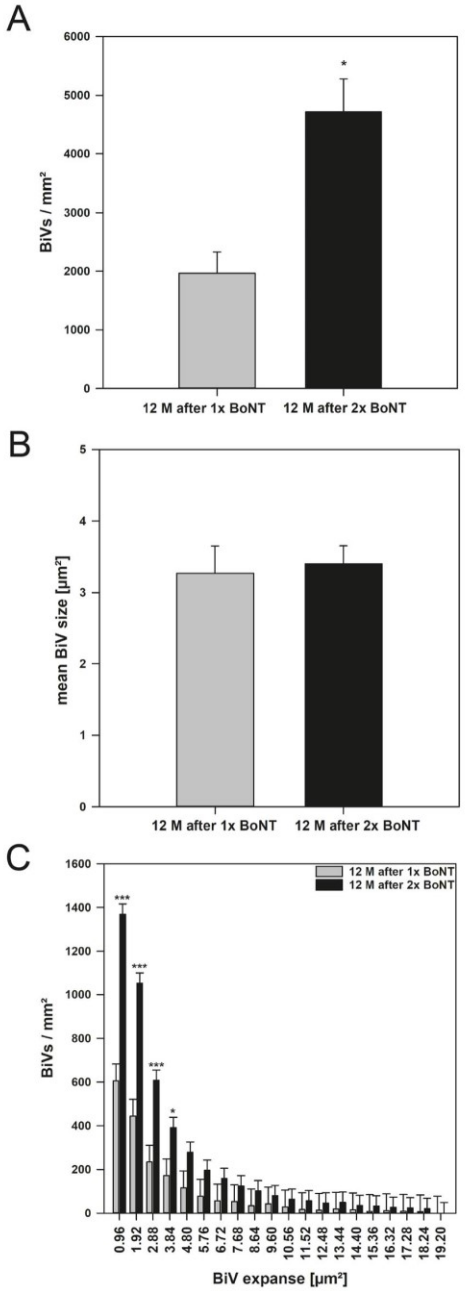

Fig. 4. The numeric density (A), the mean size of the projection area (B) as well as the histogram of the expanse (C) of ChAT-ir BiVs of the BoNT-A-injected striata are depicted from rats 12 M after 1x BoNT ($n = 3$) and 12 M after 2x BoNT ($n = 8$). One-way (A + B) or two-way (C) ANOVA results. Asterisks indicate significant differences between the groups after Holm-Sidak post-hoc test (* $p < 0.05$, *** $p < 0.001$). Data are means ± SEM.

Thus, 12 months after the second application, the body weights of BoNT-A- or vehicle-injected rats did not differ significantly. Also, a repeated intrastriatal BoNT-A injection had no unwanted adverse effects on the health of the animals and was tolerated well. These findings underlined our previous results in hemi-PD rats at different time points after the single intrastriatal BoNT-A application (Hawlitschka et al., 2018; Wedekind et al., 2018) and also 12 months after the second BoNT-A or vehicle application (Hawlitschka et al., 2018).

Likewise, the brain weights were not significantly influenced following the repeated BoNT-A when compared with the respective vehicle applications in hemi-PD rats, performed 1 and 7 months after 6-OHDA lesion. The same held true when comparing the quotient of brain weight to body weight in both experimental groups. These results complement our former findings in C57BL/6 mice following single unilateral 25 pg BoNT-A injection into the right CPu, whereby neither body weight nor brain weight were significantly influenced up to 9 months survival (Antipova et al., 2018; Hawlitschka et al., 2017).

3.2. CPu volume and numeric density of striatal ChAT-ir neurons

Twelve months after the second BoNT-A injection, the volume of the right CPu injected with 1 ng BoNT-A was significantly smaller than the volume of the contralateral non-injected CPu. In contrast, in hemi-PD rats treated twice with vehicle substance in the right striatum, the volumes of the injected CPu did not differ significantly from the contralateral, non-injected CPu.

Interestingly, in naïve rats, once injected with BoNT-A into the right CPu, the volumes of BoNT-A-injected and non-injected contralateral striata were not significantly different 1 month, 6 months and 12 months after injection (Antipova et al., 2013). However, it is as yet not known which neuropil compartment(s) is (are) responsible for the striatal volume reduction after 2x BoNT-A injection.

3.3. Number of striatal ChAT-ir neurons

Twelve months after the second BoNT-A application, the numeric density of ChAT-ir neurons of the right CPu injected with BoNT-A was significantly higher compared with the respective data after vehicle application. However, the striatal volumes showed reciprocal results. Taking both parameters together, the number of striatal ChAT-ir neurons in hemi-PD rats injected twice into the CPu with BoNT-A was not significantly different from rats with repeated vehicle applications. These data corroborate and extend previous results where a constant number of total neurons and of striatal ChAT-ir neurons were found irrespective of a single intrastriatal BoNT-A injection of 1 ng (Antipova et al., 2013; Mehlan et al., 2016). Taken all results together, it can be speculated that the BoNT-A-induced reduction in striatal volume is due to changes of jet unknown components of the neuropil.

3.4. Sholl analysis

Dendrites are specialized compartments that critically influence how neurons receive and process information. Loss of stability leads to defects in dendrite structure and connectivity that contributes to the pathology of psychiatric and neurodegenerative disorders such as schizophrenia, depression as well as to neurodegenerative conditions including PD, Alzheimer's disease, Huntington's disease, stroke and glaucoma (Cochran et al., 2014; Di Polo, 2015; Forman et al., 2004). Sholl analysis (Sholl, 1953) is a widely used method in neurobiology to quantify the complexity of dendritic arbors (Binley et al., 2014) and an invaluable tool for the study of mechanisms of disease, as well as for the quantification of the effects of potential treatments (Rekha et al., 2011).

3.4.1. Sholl analysis of striatal ChAT-ir neurons and of ChAT-ir neurons of the nucleus of the horizontal limb of the diagonal band

We used Sholl analysis (Sholl, 1953) to examine the influence of the repeated intrastriatal BoNT-A or vehicle injection on the complexity of the dendritic arbors of the ChAT-ir neurons in CPu 12 months after the second BoNT-A or vehicle applications in hemi-PD rats. Moreover, we compared our results in hemi-PD rats 12 months after the second BoNT-A or vehicle application with Sholl analysis results in naïve rats 12 months after the single intrastriatal BoNT-A injection.

In the striatum, the mean total dendrite length of ChAT-ir neurons 12 months after the second BoNT-A injection in hemi-PD rats was not significantly different from that found in the contralateral, non-injected hemisphere. These data also did not significantly differ from those measured in the double vehicle-injected group. Thus, repeated intrastriatal BoNT-A injection has no negative effect on the dendritic arbors of the ChAT-ir neurons in hemi-PD rats. We conclude that the BoNT-A-induced reduction in striatal volume does not result from degenerative changes of cholinergic neuronśdendritic trees.

However, in naïve rats 12 months after the single injection of 1 ng BoNT-A, the total dendrite length of ChAT-ir neurons in the injected right CPu was significantly lower than in the non-injected contralateral CPu. This indeed speaks in favor of a specific BoNT-A effect in the striatum of naïve rats. The local specificity can be deduced from the accompanying results found in the HDB in the same brains, where no BoNT-A-induced changes were seen.

3.4.2. Sholl analysis of neurons of the nucleus of the horizontal limb of the diagonal band

Sholl analysis showed no significant differences in the mean total dendrite length of ChAT-ir neurons of the HDB between either hemisphere in hemi-PD rats 12 months after the second BoNT-A or vehicle injections into the right CPu. Thus, repeated BoNT-A and also repeated vehicle applications did not induce a specific local effect. The same held true for the dendrite length measured in naïve rats 12 months after a single, right-sided intrastriatal BoNT-A injection. Also, a single striatal BoNT-A application did not induce significant changes in the dendrite length of HDB cholinergic neurons. Thus, striatal BoNT-A injection apparently had no non-local effects on cholinergic neurons.

Interestingly, both in the CPu and the HDB, naïve rats 12 M after 1x BoNT had significantly longer dendrites than the hemi-PD rats that had received 2x BoNT-A or 2x vehicle. This phenomenon is probably due to age-related structural alterations in the brain. Hemi-PD rats of the 2x BoNT-A or 2x vehicle groups were, at the end of the experiment, 24 months old, whereas the rats injected only once with BoNT were 15 months old. These results corroborate findings that, when they age, neurons undergo morphological changes such as a reduction in the complexity of dendritic arborization and dendritic length (Dickstein et al., 2007; Matamales et al., 2016; Umegaki et al., 2008).

3.5. Botulinum neurotoxin-A-induced ChAT-ir varicosities (BiVs)

The ChAT-ir BiVs after a single BoNT-A injection into the CPu of naïve and hemi-PD rats were previously described (Hawlitschka et al., 2017; Mehlan et al., 2016; Wree et al., 2011); their appearance was argued to be a neuropathological phenomenon. Here we compared the size and number of ChAT-ir BiVs in hemi-PD rats injected with 1 ng BoNT-A 1 and 7 months after the 6-OHDA surgery with the respective data of ChAT-ir BiVs in naïve rats injected only once with BoNT-A at the dose of 1 ng and a 12 month post-injection survival.

The numeric density of ChAT-ir BiVs measured 12 months after the second BoNT-A injection was about double compared with the numeric density of ChAT-ir BiVs measured 12 months after the single intrastriatal BoNT-A injection. However, the mean BiV size in the striata after repeated or single BoNT-A did not significantly differ. This speaks in favor of a cumulative effect, i. e., the second BoNT-A injection induced BiVs additional to those still existing from the first BoNT-A injection.

Unfortunately, our results showing the increase in BiV density following intrastriatal repetitive BoNT-A application cannot be correlated

to clinical studies. Although BoNT-A-induced remodulation of neuromuscular junctions and cholinergic motoric terminals even after repeated injections in muscles is well documented (Rogozhin et al., 2008), descriptions of effects in the central nervous system following the repetitive respective BoNT-A are still rare (Weise et al., 2019). Most studies concern the clinical use of repetitive multiple injections of the BoNT-A in patients muscles (Brashear et al., 2005; Colosimo et al., 2012; Flynn, 2010; Gordon and Barron, 2006; Şen and Arpaci, 2015). Studies about the long-last effects of BoNT-A mainly describe the persistence and, especially, the increase of duration time of the action of BoNT (Keller et al., 1999; Moritz et al., 2018; Pellett et al., 2015; Whitemarsh et al., 2014) that fits with the prolongation and the increased effectiveness of intrastriatal BoNT-A injections in hemi-PD rats (Hawlitschka et al., 2018).

4. Materials and methods

4.1. Animals

Young adult (2.5 to 3 months old) male Wistar rats, strain Crl:WI BR, weighing 250–290 g were obtained from Charles River Wiga (Sulzfeld, Germany). Rats were housed in standard cages in a temperature-controlled room (22 °C ± 2 °C) under a 12 h light/dark cycle and free access to standard food and water. All procedures used in the present study complied with the guidelines on animal care. The animal experiments were approved by the local State Animal Research Committee of Mecklenburg-Western Pomerania (LALLF M-V 7221.3–1.1–003/13 from 26 April 2013).

4.2. Body weight and brain weight

Body weight and brain weight were measured 12 months after the second BoNT-A or vehicle application.

4.3. 6-OHDA lesion surgery

All surgical manipulations were carried out under aseptic conditions. Rats, weighing 290–310 g at the time of surgery, were deeply anesthetized by intraperitoneal (i. p.) injection of ketamine (50 mg kg^{-1} body weight (BW)) and xylazine (4 mg kg^{-1} BW). Experimental hemi-PD was induced by unilateral injection of 24 µg 6-hydroxydopamine hydrochloride (6-OHDA, Sigma–Aldrich, St. Louis, MO) dissolved in 4 µl 0.1 M citrate buffer over 4 min via a 26-gauge 5 µl Hamilton syringe into the right medial forebrain bundle (MFB) using a stereotactic frame (Stoelting, Wood Dale, IL, USA). Thereafter, the needle was left in place for a further 5 min to avoid reflow. The injection coordinates with reference to bregma were: AP = -2.3 mm, L = 1.5 mm to the right, V = -9.0 mm (Paxinos and Watson, 2015). Successful 6-OHDA lesion was verified by an apomorphine-induced rotation test (Schwarting and Huston, 1996; Ungerstedt and Arbuthnott, 1970) 4 weeks after the 6-OHDA application and morphologically by TH-immunohistochemistry 12 months after the second intrastriatal sham or BoNT-A injection (Fig. 5A-B).

4.4. Injection of BoNT-A into the striatum

Under the same operative conditions intrastriatal application of BoNT-A or vehicle was carried out 6 weeks after the 6-OHDA injection. A solution of either BoNT-A dissolved in phosphate-buffered saline supplemented with 0.1% bovine serum or vehicle (sham BoNT-A) was injected into the right CPu at two sites (Wree et al. 2011; Antipova et al., 2013, 2017, 2018, 2019; Hawlitschka et al. 2018). Thus, rats received 2 × 1 µl BoNT-A solution (Lot No. 13028A1A; List, Campbell, CA; purchased via Quadratech, Surrey, UK) containing a total of 1 ng BoNT-A (n = 10) or vehicle solution (n = 5). The respective coordinates with reference to bregma were: AP = +1.3/-0.4 mm, L = 2.6/3.6 mm to the right, and V = -5.5 mm (Paxinos and Watson, 2015). BoNT-A was handled and stored according to the manufacturer's instructions. Animals received a second BoNT-A or a second vehicle solution 6 months after the first one. Brains were fixed 12 months after the second injections. For comparison to the 12 M after 2x BoNT and 12 M after 2x sham groups, naïve rats (n = 3) from a former experiment (Mehlan et al., 2016) 12 months after the single intrastriatal BoNT-A injection were evaluated.

4.5. Apomorphine-induced rotation test

To verify the success of the 6-OHDA lesion, the apomorphine-induced turning rate was determined 4 weeks after the 6-OHDA lesion. The test provides a sensitive and rapid behavioral correlate of the basal ganglia circuit disturbance caused by the unilateral lesion of the SNpc (Emerich et al., 1996; Nikkhah et al., 1994; Schwarting and Huston, 1996). Apomorphine (0.25 mg × kg^{-1}, Teclapharm, Lüneburg, Germany) dissolved in saline was injected i. p. and animals' turns were registered over 40 min in a self-constructed, automated rotometer modified according to Ungerstedt and Arbuthnott (Ungerstedt and Arbuthnott, 1970), starting 5 min post injection. In right-sided hemi-PD rats the apomorphine-induced, complete, anti-clockwise 360° turns were expressed as positive values. Mean rotation rates of the rats used here, were 5.93 ± 1.04 per min (mean ± SEM) in the rats later injected twice with vehicle, and 5.64 ± 1.05 per min in the rats later injected twice with BoNT-A (Hawlitschka et al. 2018).

4.6. Histochemistry

For histological analysis, rats were killed with an overdose of ketamine/xylazine and transcardially perfused with 50 ml 4 °C cold 0.9% saline followed by 300 ml of 3.7% paraformaldehyde dissolved in phosphate-buffered saline (pH 7.4.). Brains were immediately removed from the skull, postfixed in 3.7% paraformaldehyde overnight at 4 °C, cryoprotected in 20% sucrose in PBS for 1 day at 4 °C and then frozen at −80 °C. Frontal 30 µm thick brain slices were prepared by serial cryocutting (Leica, Wetzlar, Germany). Consecutive sections were stained with cresyl violet according to Nissl (for vital neurons) or immunostained for choline acetyltransferase (ChAT, for cholinergic neurons). A polyclonal goat anti-ChAT affinity purified antibody (Millipore, Schalbach, Germany, 1:200) was applied overnight (4 °C), followed by

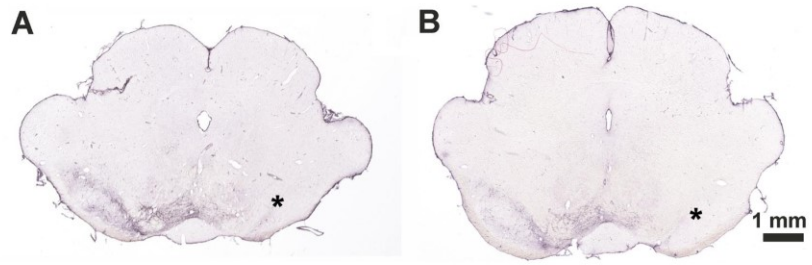

Fig. 5. Frontal sections through the brain stem at the level of the substantia nigra pars compacta, immunohistochemically stained for TH (A-B). Right MFB was injected with 6-OHDA; additionally, rats were injected intrastriatally twice with vehicle (A) or with 1 ng BoNT-A (B). In all hemiparkinsonian rats the SNpc was unilaterally nearly completely destroyed (*).

washing and incubation with rabbit anti-goat IgG overnight at 4 °C (Vector Laboratories, Burlingame, CA, 1:67), and incubation with an avidin–biotin peroxidase complex for 1 h (Vector Laboratories, Burlingame, CA, USA). Color was developed with diaminobenzidine and ammonium nickel sulfate for enhancement.

4.7. Stereological analysis

4.7.1. Striatal volume, numeric density and number of ChAT-ir neurons

The immunohistochemically stained brain slices were analyzed stereologically using the program Stereo Investigator 8.0 (v8.0, MicroBrightFieldBioscience, Vermont, USA). Striatal volumes were calculated using the tracing data from each CPu and the measured section thickness. Regional volumes of all sections of a subject were added to obtain the total volume of the CPu. The numeric density and the number of the ChAT-ir interneurons were calculated by unbiased counting using the optical fractionator method (Gundersen and Jensen, 1987; Hawlitschka et al., 2017; Mehlan et al., 2016).

4.7.2. Sholl analysis of ChAT-ir neurons in the CPu

Dendritic arbors represented in the horizontal, 30 µm thick, ChAT-reacted sections were assessed by dendritic complexity analysis (Sholl, 1953). Both the BoNT-A-injected and the contralateral non-injected CPu were evaluated for comparison. Sholl concentric spheres around the soma separated by a distance of 25 µm were used, and from the drawings of the neuronś dendrites the total dendrite lengths were calculated. In total 5951 CPu neurons were evaluated in rats 12 months after the single intrastriatal BoNT-A injection (n = 3), and in hemi-PD rats 12 months after the second intrastriatal BoNT-A (n = 3) or vehicle (n = 3) injection.

4.7.3. Sholl analysis of neurons of the nucleus of the horizontal limb of the diagonal band

In order to test whether striatal BoNT-A injections have a restricted local effect on the arbors of ChAT-ir interneurons at the injected CPu, we additionally evaluated the arbors of ChAT-ir neurons in the HDB, i.e. a nucleus quite distant from the BoNT-A injection. In the identical brains used for the Sholl analysis in the CPu, Sholl analysis was performed on the ChAT-ir neurons of the HDB in BoNT-A-injected and non-injected hemispheres. A total of 2704 HDB neurons were evaluated in naïve rats (n = 3) 12 months after the single BoNT-A injection into CPu, and in hemi-PD rats 12 months after the second intrastriatal BoNT-A (n = 3) or vehicle (n = 3) injection.

4.7.4. Numeric density and size of ChAT-ir BiVs in the CPu

The numeric density of ChAT-ir BiVs per mm^2 and their size (projection area in μm^2) were calculated in the right (injected) CPu in naïve rats (n = 3) 12 months after the single intrastriatal BoNT-A injection, and in hemi-PD rats 12 months after the second intrastriatal BoNT-A (n = 8) injection; this was done by analysis of micrographs using adaptive segmentation and morphometric quantification of customized scripts (MatLab®, MATLAB 9.5, 41, R2018b, MathWorks, Natick, Massachusetts, USA). Neither the non-injected CPu nor either striatum of the sham rats 12 M after 2x contained BiVs.

4.8. Data analysis

The results were presented as means ± SEM. In all cases, p values ≤ 0.05 were considered significant. All data were subjected to two-way ANOVA. Only size and density of BiVs were subjected to one-way ANOVA. The Holm-Sidak approach was used for post hoc comparisons. To evaluate the strength of the association between the number and numeric density of the ChAT-ir interneurons as well as the volume of CPu and apomorphine-induced rotation data, Pearson Product Moment Correlation tests were done. If residuals (distances of the data points from the regression line) were not normally distributed with a constant variance, Spearman Rank Order Correlation tests were done. All statistical analyses were done using SigmaPlot 14 Software (Systat Software, Inc., San Jose, CA 95110, USA).

5. Conclusions

After repeated unilateral BoNT-A injections at the dose of 1 ng each and applied in a six month interval into the striatum of hemi-PD Wistar rat, there is no statistically significant effect on the number of ChAT-ir interneurons twelve months after the second BoNT-A injection. The volume of the BoNT-A-injected right CPu was significantly reduced compared with the contralateral striatum. Concurrently, the numeric density of ChAT-ir interneurons in the injected CPu was significantly higher than in non-injected left CPu. The total dendrite length of ChAT-ir interneurons in CPu and also in HDB after repeated BoNT-A application resembled the values found in double vehicle-injected hemi-PD rats. To that, the numeric density of ChAT-ir BiVs in the CPu of hemi-PD rats repeatedly injected with BoNT-A was increased in contrast to rats which had been intrastriatally injected with BoNT-A only once. The current results extend the knowledge about the effect of repeated intrastriatal BoNT-A injections in experimental Parkinson research and may suggest the further clinical application of BoNT-A as a local anticholinergic therapeutic agent injected directly into the brain in PD.

Author contributions

A.H., V.A and A.W.: Conceptualization and Supervision; A.H., C.B., V.A. and A.W.: Investigation; O.S. and C.H.: Formal analysis; O.S., C.H. and A.H: Visualization; O.S., A.H., V.A. and A.W.: Methodology; A.H. and A.W.: Funding Acquisition; V.A. and A.W.: Project administration, Writing - Original Draft Preparation; A.H., V.A. and A.W.: Writing-Review & Editing.

Funding

This research was funded by Rostock University Medical Center, Germany, FORUN 889005 and 889014. The study was also supported by the Centre of Transdisciplinary Neuroscience Rostock.

Acknowledgements

The authors thank Susann Lehmann and Frauke Winzer for their excellent technical assistance and Mathias Lietz and Ulf Haase for their affectionate care of the animals.

Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brainres.2020.146877.

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9.3 Vollständiges Publikationsverzeichnis

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***Gleichbeteiligter Erstautor**

Übersichtsartikel

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Posterbeiträge und Fachvorträge

Poster

Hawlitschka A., Haas S., Schmitt O., Wree A.
Proteasome inhibition failed to act as a new animal model of Parkinson' s disease in Wistar rats, but led to changes in tyrosine hydroxylase contents of olfactory bulb and adrenal medulla
(7. Treffen der Deutschen Neurowissenschaftlichen Gesellschaft, 29.03.2007 - 01.04.2007 in Göttingen)

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Proteasome inhibition as a model for Parkinson's Disease?
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Verhaltensänderungen von Ratte nach intrazerebraler Injektion von Botulinumtoxin
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Intrazerebrale Applikation von Botulinumtoxin A im Tierexperiment – neuroanatomische Aspekte
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10. Danksagung

Zuerst möchte ich Herrn Prof. Wree für die Ermöglichung und Unterstützung bei meiner Forschungsarbeit danken.

Herrn Prof. Schmitt, Frau Dr. Antipova, Herrn Dr. Holzmann, Herrn Prof. Witt und Frau Dr. Mann sei für die ausgezeichnete Zusammenarbeit und vor Allem die angenehme Arbeitsatmosphäre gedankt.

Danken möchte ich auch Prof. Dr. Dr. Kipp für seinen positiven Input.

Den von mir im Labor und bei ihren Dissertationen betreuten ehemaligen und noch aktiven Doktoranden und Doktorandinnen Dr. Hans Brosig, Dr. Juliane Mehlan, Dr. Juliane Spiewok, Dr. Sarah Witt, Dr. Franz Diekmann, Johanna Will, Friederike Schümann und Charlotte Berg danke ich für ihren Fleiß im Labor und ihre intellektuell bereichernden Beiträge im Rahmen gemeinsamer Projekte.

Allen Mitarbeitern des Institutes für Anatomie Rostock möchte ich für die guten kollegialen Umgang und die tägliche Zusammenarbeit in Forschung und Lehre danken.

Besonders herzlich bedanke ich mich bei meiner Familie für ihre viele Geduld, Umsicht und Hilfe.

11. Eidesstattliche Erklärung

Hiermit erkläre ich die vorliegende Habilitationsschrift selbständig und ohne unerlaubte fremde Hilfe angefertigt zu haben. Ich habe keine anderen als die im Literaturverzeichnis angeführten Quellen benutzt und sämtliche Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder unveröffentlichten Schriften entnommen wurden, als solche kenntlich gemacht. Ich versichere weiterhin, dass diese Arbeit nicht vorher und auch nicht gleichzeitig bei einer anderen Fakultät als der Universitätsmedizin Rostock zur Eröffnung eines Habilitationsverfahrens eingereicht worden ist.

Rostock, den 19.06.2020 Dr. rer. nat. Alexander Hawlitschka