Research report

Characterization of liraglutide, a glucagon-like peptide-1 (GLP-1) receptor agonist, in rat partial and full nigral 6-hydroxydopamine lesion models of Parkinson’s disease

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A B S T R A C T

Exendin-4, a glucagon-like peptide-1 (GLP-1) receptor agonist, have been demonstrated to promote neuroprotection in the rat 6-hydroxydopamine (6-OHDA) neurotoxin model of Parkinson’s disease (PD), a neurodegenerative disorder characterized by progressive nigrostriatal dopaminergic neuron loss. In this report, we characterized the effect of a long-acting GLP-1 receptor agonist, liraglutide (500 μg/kg/day, s.c.) in the context of a partial or advanced (full) 6-OHDA induced nigral lesion in the rat. Rats received a low (3 μg, partial lesion) or high (13.5 μg, full lesion) 6-OHDA dose stereotaxically injected into the right medial forebrain bundle (n = 17–20 rats per experimental group). Six weeks after induction of a partial nigral dopaminergic lesion, vehicle or liraglutide was administered for four weeks. In the full lesion model, vehicle dosing or liraglutide treatment was applied for a total of six weeks starting three weeks pre-lesion, or administered for three weeks starting on the lesion day. Quantitative stereology was applied to assess the total number of midbrain tyrosine hydroxylase (TH) positive dopaminergic neurons. As compared to vehicle controls, liraglutide had no effect on the rotational responsiveness to D-amphetamine or apomorphine, respectively. In correspondence, while numbers of TH-positive nigral neurons were significantly reduced in the lesion side (partial lesion E55%; full lesion E90%) liraglutide administration had no influence dopaminergic neuronal loss in either PD model setting. In conclusion, liraglutide showed no neuroprotective effects in the context of moderate or substantial midbrain dopaminergic neuronal loss and associated functional motor deficits in the rat 6-OHDA lesion model of PD.

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1. Introduction

Parkinson’s disease (PD) is a chronic neurodegenerative disorder with cardinal motor symptoms of tremor, rigidity and bradykinesia, largely being attributed to a progressive loss of mesencephalic dopaminergic neurons located in the substantia nigra pars compacta (SNc) (Hirsch et al., 1988, Lees et al., 2009). With L-dopa being the mainstay PD therapy, current pharmacological treatments are merely palliative, show reduced symptom relief during disease progression and is associated with the development of severe motor complications (Fabbrini et al., 2007, Olanow and Schapira, 2013). Consequently, there is an unmet need for more effective therapies with neuroprotective and restorative capabilities.

It has become increasingly clear that glucagon-like peptide-1 (GLP-1) receptor agonists, apart from their prominent effects on glucose homeostasis, may also have important central effects. GLP-1 receptors are widely expressed in the brain (Merchenthaler et al., 1999, Alvarez et al., 2005, Heppner et al., 2015), blood-born GLP-1 receptor agonists can reach the brain, including exendin-4 and liraglutide (Orskov et al., 1996, Kastin and Akerstrom, 2003, Secher et al., 2014), and several studies indicate that GLP-1 receptor agonists could be neuroprotective an various neurodegenerative diseases, including Alzheimer’s and Parkinson’s disease (Bassil et al., 2014, Holscher, 2014, Talbot, 2014). In contrast to the vast number of studies reported in preclinical AD model settings, much less is known about effects of GLP-1 receptor agonists in the context of PD therapy, which warrants more studies on this specific aspect of GLP-1 receptor pharmacology.

To date, preclinical experimental results to suggest disease-modifying effects of GLP-1 receptor agonists in PD management have been derived from studies in rodent dopaminergic
neurotoxin models. Accordingly, short-term treatment with exendin-4 reduces the loss of dopaminergic neurons and alleviates associated rotational behavior in the context of 6-hydroxydopamine (6-OHDA) induced submaximal or complete nigral lesions in rats (Bertilsson et al., 2008, Harkavyi et al., 2008, Abuirmeileh et al., 2012). Similar implications of nigral dopaminergic cell protective properties of exendin-4 have been observed in the murine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model, where exendin-4 treatment abrogated the loss of dopaminergic cell bodies and fibers as well as promoting motor recovery from the insult (Kim et al., 2009, Li et al., 2009). Although little is known about the basis for the restorative dopaminergic effects as well as stimulation of neurogenesis may contribute to the neuronal and neurobehavioral recovery in these neurotoxin models (Bertilsson et al., 2008, Kim et al., 2009, Li et al., 2009).

Interestingly, liraglutide - a once-daily GLP-1 receptor agonist currently used in the management of type 2 diabetes and recently approved also for treatment of obesity (Wadden et al., 2013) - is recently reported to have neuroprotective, anti-apoptotic and neuroproliferative properties other experimental models of neurodegenerative diseases, including Alzheimer’s disease (Parthsarathy and Hölscher, 2013, Hansen et al., 2015), traumatic brain injury (DellaValle et al., 2014, Li et al., 2015) and ischemic stroke (Briyal et al., 2014, Li et al., 2015). These findings may possibly suggest that liraglutide could also have neuroprotective effects in experimental PD settings. We therefore characterized the effect of long-term liraglutide treatment in rat 6-OHDA models of partial and advanced (full) nigral dopaminergic loss, respectively. Because it is suggested that type 2 diabetes poses a risk factor in PD (Santiago and Potashkin, 2013), we also tested the possibility that liraglutide treatment could potentially influence a 6-OHDA induced full nigral lesion in glucose-intolerant diet-induced obese (DIO) rats (Hansen et al., 2012).

2. Results

2.1. Body weight

In the partial lesion model, 6-OHDA application into the MFB resulted in a minor weight drop in both lesion groups (≈ 6%, relative to day –42), and body weight was fully regained one week later (Fig. 2(A)). In accordance with the anorectic and weight-lowering properties of liraglutide and other GLP-1 receptor agonists (Hansen et al., 2012, Hayes et al., 2012, Jelsing et al., 2012), liraglutide administration for four weeks, starting six weeks post-lesion, promoted a final weight loss of approximately 10% (overall p=0.019 vs. vehicle dosing, two-way ANOVA).

In the full lesion model, vehicle-dosed rats (n=40) showed progressive body weight gain during the pre-surgery period, whereas liraglutide treatment (n=20) in the same period slightly reduced body weight (≈ 5%, overall p=0.047 vs. vehicle controls), see Fig. 2(B). Whereas liraglutide pre-treated rats continued on liraglutide treatment after inducing a full nigral lesion, vehicle pre-dosed rats were allocated to either continued vehicle dosing (n=20) or converted to liraglutide treatment (n=20). The full lesion in normal-weight rats resulted in a similar relative weight drop (≈ 10%, relative to day 0) in all three lesion groups during the first week post-lesion, and a gradual weight regain was noted in rats exposed to vehicle treatment over the following weeks. Similar to the partial lesion model, liraglutide treatment promoted a sustained weight drop in both normal-weight and DIO rats which plateaued during the first treatment week post-lesion in both liraglutide treatment groups, resulting in an end-point weight reduction corresponding to approximately 10%, as compared to vehicle controls.

2.2. Liraglutide shows no ameliorating effect on rotational activity in the partial nigral 6-OHDA lesion model

Rotational response to apomorphine (before start of liraglutide/vehicle administration) and D-amphetamine (before and during liraglutide/vehicle administration), respectively, was quantitatively evaluated in the partial nigral lesion model in order to assess the progress of functional motor deficits. In general, the rats did not elicit responsiveness to apomorphine (< 60 rotations over 15 min), whereas significant D-amphetamine induced rotations (> 10 rotations over 15 min) were apparent in the majority of the rats, being manifest and kept stable from one week post-lesion. Hence, all rats were included in the subsequent treatment period. Vehicle dosed and liraglutide-treated rats were equally sensitive to the D-amphetamine challenge dose during the four week post-lesion period, as

Fig. 1. Outline of experimental settings in 6-OHDA-induced partial or full nigral lesion models for testing effects of liraglutide treatment (500 µg/kg/day, q.d., s.c.), as compared to vehicle dosing (PBS added 0.1% BSA, pH 7.4). In the partial lesion model (upper panel), liraglutide treatment was applied for four weeks starting six weeks post-lesion. Intermittent apomorphine and D-amphetamine rotation tests, respectively, were performed intermittently, as indicated. In the full nigral lesion model (lower panel), liraglutide was administered three weeks pre-lesion and three weeks post-lesion or three weeks post-lesion only. See also Table 1.
determined in three consecutive tests performed after one, three and four weeks of treatment (Table 1). Partially lesioned rats were functionally defined post-hoc by showing a positive D-amphetamine test in the absence of an apomorphine stimulatory profile. When applying these criteria, liraglutide treated rats (n=14) did also not show any ameliorating effect on the rotational response to D-amphetamine, as compared to vehicle controls (n=13, two-way ANOVA, overall p=0.5196), see Fig. 3(A) (Table 2).
respectively. Dosing (n = 13) was commenced 6 weeks post-lesion. Liraglutide did not reduce the rotational sensitivity to D-amphetamine, as compared to vehicle controls. Full 6-OHDA lesion model settings (two-way ANOVA; time effect p < 0.0001). *p < 0.05,**p < 0.01 vs. vehicle controls. Liraglutide administration (pre- and post-treatment, n = 10; post-treatment only, n = 17) in 6-OHDA lesioned DIO rats did not influence the rotational response to apomorphine, as compared to vehicle controls (n = 14).

Table 2.
Liraglutide has no ameliorating effect on o-amprenolime-induced ipsilateral rotations in the 6-OHDA induced partial nigral lesion model of Parkinson’s disease, applied in normal-weight rats. ≥60 contralateral rotations and >10 ipsilateral rotations were considered a positive response in the apomorphine and o-amprenolime test, respectively.

<table>
<thead>
<tr>
<th>Apomorphine test in treatment week</th>
<th>Vehicle (week 1 to 4)</th>
<th>Liraglutide (week 1 to 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean rotations ± S.E.M.</td>
<td>Test responders (≥60 rotations)</td>
<td>Mean rotations ± S.E.M.</td>
</tr>
<tr>
<td>- 5</td>
<td>22 ± 11 (n = 20)</td>
<td>n = 3</td>
</tr>
<tr>
<td>- 3</td>
<td>25 ± 13 (n = 20)</td>
<td>n = 3</td>
</tr>
<tr>
<td>- 1</td>
<td>23 ± 11 (n = 20)</td>
<td>n = 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>D-amphetamine test in treatment week</th>
<th>Vehicle (week 1 to 4)</th>
<th>Liraglutide (week 1 to 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean rotations ± S.E.M.</td>
<td>Test responders (&gt;10 rotations)</td>
<td>Mean rotations ± S.E.M.</td>
</tr>
<tr>
<td>- 5</td>
<td>83 ± 17 (n = 20)</td>
<td>n = 15</td>
</tr>
<tr>
<td>- 3</td>
<td>56 ± 18 (n = 20)</td>
<td>n = 11</td>
</tr>
<tr>
<td>- 1</td>
<td>75 ± 22 (n = 20)</td>
<td>n = 11</td>
</tr>
<tr>
<td>1</td>
<td>87 ± 25 (n = 20)</td>
<td>n = 12</td>
</tr>
<tr>
<td>3</td>
<td>102 ± 29 (n = 20)</td>
<td>n = 12</td>
</tr>
<tr>
<td>4</td>
<td>92 ± 27 (n = 20)</td>
<td>n = 13</td>
</tr>
</tbody>
</table>

2.3. Liraglutide shows no ameliorating effect on rotational activity in the full nigral 6-OHDA lesion model

In the full nigral lesion model, apomorphine-induced contralateral rotations were evaluated by the end of each treatment week (n = 17–20 animals per experimental group). Table 3A and 3B shows the temporal development of the rotational responsiveness to apomorphine in normal-weight and DIO rats, respectively. From the second treatment week, vehicle-dosed and liraglutide treated rats showed a robust and stable rotational response in both 6-OHDA model settings (two-way ANOVA; time effect p < 0.0001). In both experiments, the majority of the rats showed a positive
response in the test (≥ 60 rotations over 15 min). For both liraglutide treatment regimens in normal-weight rats, the proportion of rats being apomorphine-responsive was higher in liraglutide-treated rats, as compared to vehicle controls (Table 3A). When excluding overall non-responders (< 60 rotations over 15 min) from the successive apomorphine test results it was apparent that liraglutide treatment, when applied both pre- and post-lesion (n = 15), potentiated rotational response to apomorphine in all three individual post-lesion treatment weeks (two-way ANOVA, treatment effect \( p = 0.002 \)) as compared to vehicle controls (n = 15), see Fig. 3(B). A similar synergistic effect was not indicated when only administering liraglutide in the post-lesion period (n = 13). In contrast, DIO rats showed no significant differential response to liraglutide administration (pre- and post-treatment,

Table 3A.
Liraglutide has no ameliorating effect on apomorphine-induced contralateral rotations in the 6-OHDA induced full nigral lesion model of Parkinson’s disease, applied to normal-weight rats (≥ 60 rotations was considered a positive response to the apomorphine test).

<table>
<thead>
<tr>
<th>Treatment day</th>
<th>Vehicle (day -21 to 21)</th>
<th>Vehicle (day -21 to 1)+ liraglutide (day 0-21)</th>
<th>Liraglutide (day -21 to 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean rotations ± S.E. M.</td>
<td>Test responders (≥ 60 rotations)</td>
<td>Mean rotations ± S.E. M.</td>
</tr>
<tr>
<td>7</td>
<td>26 ± 6 (n=20) n=3</td>
<td>55 ± 12 (n=20) n=10</td>
<td>62 ± 12 (n=20) n=9</td>
</tr>
<tr>
<td>14</td>
<td>106 ± 18 (n=20) n=13</td>
<td>130 ± 19 (n=20) n=15</td>
<td>142 ± 22 (n=20) n=15</td>
</tr>
<tr>
<td>22</td>
<td>102 ± 16 (n=20) n=13</td>
<td>135 ± 18 (n=20) n=17</td>
<td>136 ± 21 (n=20) n=15</td>
</tr>
</tbody>
</table>

Table 3B.
Liraglutide has no ameliorating effect on apomorphine-induced contralateral rotations in the 6-OHDA induced full nigral lesion model of Parkinson’s disease, applied to DIO rats (≥ 60 rotations was considered a positive response to the apomorphine test).

<table>
<thead>
<tr>
<th>Treatment day</th>
<th>Vehicle (day -21 to 21)</th>
<th>Vehicle (day -21 to 1)+ liraglutide (day 0-21)</th>
<th>Liraglutide (day -21 to 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean rotations ± S.E. M.</td>
<td>Test responders (≥ 60 rotations)</td>
<td>Mean rotations ± S.E. M.</td>
</tr>
<tr>
<td>7</td>
<td>39 ± 12 (n=20) n=5</td>
<td>31 ± 10 (n=17) n=4</td>
<td>39 ± 11 (n=20) n=4</td>
</tr>
<tr>
<td>14</td>
<td>101 ± 17 (n=20) n=13</td>
<td>101 ± 20 (n=17) n=10</td>
<td>122 ± 12 (n=20) n=17</td>
</tr>
<tr>
<td>22</td>
<td>102 ± 16 (n=20) n=14</td>
<td>110 ± 23 (n=17) n=10</td>
<td>143 ± 14 (n=20) n=17</td>
</tr>
</tbody>
</table>

Fig. 4. Representative photomicrographs of 6-OHDA induced partial and full nigral lesions in normal-weight rats. Partial lesion model (panels A, B): four weeks of vehicle (A) or liraglutide (B) treatment initiated five weeks post-lesion. Full lesion model (panels C-E): vehicle dosing for three weeks pre-lesion + three weeks post-lesion (C); vehicle dosing for three weeks pre-lesion + liraglutide treatment for three weeks post-lesion (D); liraglutide treatment for three weeks pre-lesion + three weeks post-lesion (E). Scale bar = 200 μm.
A loss of E4560 neurons and corresponding D-amphetamine induced rotations was found in previous stereological studies in the rat (reviewed in by Stark & Pakkenberg (Stark and Pakkenberg, 2004)). Vehicle-dosed controls (n = 14, overall p = 0.4875), when evaluated in the apomorphine test (Fig. 3(C)).

2.4. Liraglutide does not rescue mesencephalic dopaminergic neurons from a 6-OHDA induced partial or full nigral insult

Mesencephalic dopaminergic neurons were determined histologically by conspicuous cytoplasmic TH immunostaining. The total number of TH-positive neurons was subsequently quantified by means of stereology. Representative photomicrographs are shown in Fig. 4.

All rats from the partial lesion study were included in the assessment of TH-positive neuron numbers in the SNc. The total number of TH-positive neurons in the contralateral SNc was equal and highly stable in the vehicle-dosed and liraglutide-treated rats. Hence, the quantitative estimates in both experimental groups were pooled, yielding a mean total of 10,321 ± 527 TH-positive neurons in the unlesioned SNc. The contralateral nigral total TH-positive neuron number was comparable to the baseline level found in previous stereological studies in the rat (reviewed in by Stark & Pakkenberg (Stark and Pakkenberg, 2004)). Vehicle-dosed and liraglutide-treated rats exhibited a similar reduction of total TH-positive neurons in the ipsilateral SNc (4754 ± 667 vs. 4560 ± 562 neurons, p = 0.8247, see Fig. 5(A)), i.e. corresponding to a loss of ≈ 55% TH-positive neurons. The stereological analysis applied to the partial lesion model revealed that the loss of TH-positive neurons displayed a continuum in both experimental groups. A correlation analysis of the number of nigral TH-positive neurons and corresponding α-amphetamine induced rotations was therefore performed. The correlation analysis indicated a strong overall inverse linear relationship of mean nigral TH-positive neuron numbers vs. baseline rotation numbers (n = 26, r² = 0.61, p = 0.0009), see Fig. 5(B). When applying the correlation analysis to the experimental groups separately, this did not change the regression coefficient (vehicle, n = 12: 0.5674, p = 0.0543; liraglutide, n = 14: 0.6689, p = 0.0089). Hence, the rather similar correlation coefficients suggest that the experimental groups were well-balanced in regard to the variability of 6-OHDA neurotoxicity prior to drug treatment start. Also, a correlation analysis applied to the final α-amphetamine rotation test yielded similar results with correlation coefficients being −0.56 (overall, n = 26, p = 0.0025), −0.61 (vehicle controls, n = 12, p = 0.0354), and −0.58 (liraglutide treatment, n = 14, p = 0.0306), see Fig. 5(C).

In the full lesion study, only rats showing a stimulatory rotational response in the apomorphine test were included in the histological analysis. Because normal-weight and DIO rats showed a similar marked and robust rotational responsiveness to apomorphine with no ability of liraglutide to reduce the number of rotations, only brains from normal-weight rats were evaluated for TH immunoreactivity. Due to the marked consistency in the behavioral response to apomorphine, eight normal-weight rats per experimental group were randomly selected for subsequent quantitative stereological evaluation. The total number of mesencephalic TH-positive neurons in the contralateral hemisphere was highly stable and did not differ between the experimental groups, applying to both the SNc (p = 0.7475) and VTA (p = 0.6609). The quantitative estimates in all experimental groups were therefore pooled yielding a mean total of 7072 ± 468 (SNc) and 7979 ± 581 (VTA) TH-positive neurons in the contralateral hemisphere. It should be noted that the total number of TH-positive neurons in the contralateral SNc was generally lower in the full

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**Fig. 5.** Stereological quantification of the total loss of TH-positive (TH+) neurons in the partial nigral lesion model, assessed four weeks after lesion induction in normal-weight rats. The dotted horizontal indicates the pooled mean total number of TH-positive neurons in the contralateral SNc (10,321 ± 527 neurons). The ipsilateral loss of TH-positive SNc neurons amounted to approximately 55%, as compared to the contralateral level (***p < 0.001, vs. contralateral level). (B) Correlation of the total number of TH-positive nigral neurons to mean baseline rotation numbers in the α-amphetamine test (threshold > 10 rotations corresponds to a positive response in the test). (C) Correlation of the total number of TH-positive nigral neurons to rotation numbers in the α-amphetamine test after four weeks of vehicle dosing or liraglutide treatment.
lesion model, as compared to contralateral SNC in the partial lesion study, implying that the contralateral SNC was also affected in rats with a full ipsilateral lesion. As compared to the contralateral hemisphere, all experimental groups exhibited a significant reduction in the total number of ipsilateral mesencephalic TH-positive neurons, applying to both the SNC and VTA (see Fig. 6). The total number of ipsilateral TH-positive neurons were 1020 ± 438 (SNC, p < 0.001) and 4285 ± 693 (VTA, p < 0.01), respectively, in vehicle controls. Similarly, rats treated with liraglutide only during the post-lesion period showed a total of 289 ± 79 (SNC, p < 0.001) and 4074 ± 521 (VTA, p < 0.001) TH-positive neurons, whereas rats receiving liraglutide treatment in both the pre- and post-lesion period had a total of 812 ± 394 (SNC, p < 0.001) and 3609 ± 547 (VTA, p < 0.01) TH-positive neurons. The ipsilateral loss of TH-positive neurons in all experimental groups amounted to about 90% in the SNC and 50% in the VTA. The stereological analysis therefore also indicated that liraglutide, irrespective of treatment regimen, did not influence the loss of TH-positive neurons in the full lesion model, as compared to vehicle controls (SNC, p = 0.3242; VTA, p = 0.7144).

3. Discussion

To explore whether GLP-1 receptor stimulation could potentially ameliorate PD pathology, we characterized the effects of chronic liraglutide treatment in conditions of a partial and advanced (full) depletion of nigral dopaminergic neurons, mimicking early or late-stage PD, respectively (Bernheimer et al., 1973, Dauer and Przedborski, 2003). In this study, we employed the 6-OHDA MFB lesion model in the rat, a prototypical in vivo model of PD which is extensively used in the preclinical evaluation of compounds for potential anti-parkinsonian efficacy (Iderberg et al., 2012, Blesa and Przedborski, 2014). The comprehensive stereological analysis indicated that the total number of TH-positive neurons was reduced by approximately 55% in the ipsilateral SNC of partially lesioned rats. There was a significant inverse correlation between the number of remaining TH-immunoreactive cells in the SNC and the number of ipsilateral rotations induced by d-amphetamine. Thus, the rotational responsiveness to a d-amphetamine challenge was proportional to the extent of partial dopaminergic neuronal loss in the SNC, being in accordance with previous studies (Hefti et al., 1980, Carman et al., 1991, Hudson et al., 1993). When applying a higher unilateral 6-OHDA dose, vehicle control rats exhibited significant apomorphine-induced rotations which at study termination corresponded to an almost complete elimination of TH-positive neurons (~90%) and fibers within the ipsilateral SNC. Collectively, this indicates that quantitative assessment d-amphetamine (partial lesion) and apomorphine ('full lesion') induced rotations in this study provides an index with relatively good predictive value of the nigral lesion progression, thereby also being applied to evaluate the pharmacodynamics of liraglutide in 6-OHDA lesioned rats.

D-amphetamine rotational activity in the partially lesioned rats was observed one week after applying 6-OHDA and did not significantly progress further throughout the remainder of study. However, liraglutide did not ameliorate the rotational response to d-amphetamine and ipsilateral total TH-positive neuronal numbers were also equivalent to vehicle control levels. Furthermore, similar correlations between rotation numbers and the total number of TH-positive neurons were observed in the two experimental groups both before and after treatment cessation. Hence, long-term liraglutide treatment, when introduced six weeks after lesion induction, did not influence the progressive nigral dopaminergic cell loss and functional motor outcome in rats with a partial nigral lesion.

These findings contrast previous reports on neuroprotective effects of exendin-4 in similar rat 6-OHDA models of PD. Accordingly, functional recovery in the d-amphetamine test has been observed after three weeks of bi-daily exendin-4 administration, although exendin-4 showed a very modest protective effect on nigral dopaminergic neuron loss after a moderate intra-MFB dose of 6-OHDA (Bertilsson et al., 2008). Because one week of bi-daily exendin-4 administration is reported to promote full

![Fig. 6. Stereological quantification of the total loss of TH-positive (TH+) neurons in the SNC (panel A) and VTA (panel B) in the full nigral lesion model, assessed three weeks after lesion induction in normal-weight rats. The dotted horizontal line indicates the pooled mean total number of TH-positive neurons in the contralateral SNC (7072 ± 3609 neurons) and VTA (7979 ± 547 neurons). The ipsilateral loss of TH-positive SNC and VTA neurons amounted to approximately 90% and 50%, respectively, as compared to the corresponding contralateral level (**p < 0.01, ***p < 0.001, vs. contralateral level).](image-url)
dopaminergic neuronal and motor recovery in rats exhibiting a virtually complete 6-OHDA induced ablation of dopamine neurons in the SNC (Harkavyi et al., 2008, Abuirmeileh et al., 2012), we also characterized the effect of liraglutide in rats with a more advanced SNC lesion following administration of a high dose of 6-OHDA. Similar to the partial lesion model, long-term liraglutide treatment had no ameliorating effect on dopaminergic cell loss in the SNC or VTA, irrespective of liraglutide being administered before and/or after applying an almost full nigral lesion. In addition, we also tested the possibility that similar liraglutide treatment regimens could influence a 6-OHDA induced full nigral lesion in a rat model of diet-induced obesity and glucose-intolerance (Hansen et al., 2012), as type 2 diabetes is proposed to be a risk factor in PD (Santiago and Potashkin, 2013). However, there was also no effect of liraglutide treatment on apomorphine-induced rotations in DIO rats. Although we did not perform quantitative assessment of TH-positive midbrain neurons in the 6-OHDA lesioned DIO rats, the rotational response to apomorphine was as marked as in 6-OHDA lesioned normal-weight rats, indicative of a full nigral lesion being unaffected by liraglutide treatment. Collectively, the experiments point to the lack of neuroprotective effects of liraglutide in the rat G-OHDA model of PD, irrespectively of the variations of the model and treatment settings applied.

It is unclear whether the discrepancy resides from differential 6-OHDA sensitivity or methodologies for quantification of the number of TH-positive neurons. Obviously, differences in drug pharmacokinetics, dosing regimens and histological quantification methods should be considered. A moderately high dose of liraglutide was administered once daily because liraglutide, as compared to exendin-4, displays a relatively longer plasma half-life after subcutaneous dosing in the rat (Parkes et al., 2001, Sturis et al., 2003, Han et al., 2013). Importantly, typical pharmacodynamics of liraglutide was consistently observed in the study, as liraglutide robustly reduced food intake and weight gain in all 6-OHDA lesioned rats. Considering that liraglutide enters the rodent brain after subcutaneous dosing (Secher et al., 2014), the anorectic and body weight lowering properties of liraglutide are dependent on central GLP-1 receptor activation (Secher et al., 2014, Sisley et al., 2014), this would argue for central liraglutide exposure throughout the whole treatment period.

Quantification of dopamine neuron numbers is one of the primary endpoints of PD model characterization. Hence, it is therefore also critical for quantitative assessment of neuroprotection conferred by putative therapeutic drugs. In this regard, stereological analysis is considered the optimal tool for quantitative assessment of morphological changes induced by neurotoxic lesions and treatment interventions, as this method is based on random sampling techniques and generation of unbiased estimates of three-dimensional characteristics (Gundersen et al., 1988, West, 1999). In the present study, we therefore provide stereology-based estimates on the total number of mesencephalic TH-positive neurons in 6-OHDA lesioned rats, whereas previous TH-immunohistochemical results were based on semi-quantitative two-dimensional analyses of relatively few nigral sections per animal (Bertilsson et al., 2008), or simply by qualitative observations only (Harkavyi et al., 2008). It should therefore be emphasized that the comparative basis is limited as a consequence of the few previous reports on GLP-1 receptor agonist treatment in this model.

In the current study as well as in the studies mentioned above, the neurotoxic 6-OHDA lesion was applied into the MFB, i.e. in close proximity to SNCs and VTA dopaminergic neurons. It may therefore be speculated that any potential neuroprotective effect of GLP-1 receptor agonist treatment would involve stimulation of GLP-1 receptor function in mesencephalic dopaminergic neurons. It is currently unknown whether systemically administered GLP-1 receptor agonists may exert direct effects in the dopaminergic SNC, as GLP-1 receptor mRNA and exendin-4 induced c-Fos induction is found in the rat substantia nigra pars reticulata only, a principally GABAergic region (Merchenthaler et al., 1999, Gu et al., 2013). In correspondence, GLP-1 receptor immunoreactivity is confined to only very few nigral fiber-like structures in the non-human primate brain (Heppner et al., 2015). It is therefore conceivable that nigral dopaminergic neurons do not express GLP-1 receptors levels to any relevant functional degree, although this needs to be confirmed in future studies. While the dosing regimen with liraglutide has been shown to lead to measurable levels of liraglutide in several circumventricular organs as well as the hypothalamus, liraglutide does not directly access dopaminergic brain areas at least at the detection limit of current techniques (Secher et al., 2014). However, GLP-1 receptors are expressed at numerous locations in the brain (Merchenthaler et al., 1999) and GLP-1 is a widespread neurotransmitter, so it is entirely possible that central effects can be obtained with peripheral administration of liraglutide. In accordance with this notion, a similar dosing regimen with liraglutide is recently reported to improve memory function and increase hippocampal neuronal numbers in a mouse model of age-related Alzheimer’s disease (Hansen et al., 2015), as well as reducing neurological deficits and tau phosphorylation in a tauopathy mouse model (Hansen et al., 2016).

In contrast to the SNC, GLP-1 receptors in the VTA may likely play a functional role. Accordingly, intra-VTA microinjection of exendin-4 induces GLP-1 receptor-dependent suppression of food-reward associated behavior (Dickson et al., 2012, Mietlicki-Baase et al., 2013). Also, GLP-1 receptor mRNA, radioligand binding, as well as immunoreactive cell bodies and fibers have been detected in the VTA (Gøkø et al., 1995, Merchenthaler et al., 1999, Heppner et al., 2015). On the basis of these reported findings, it may therefore also be speculated that VTA, rather than SNC, dopaminergic neurons were responsive to exendin-4 and GLP-1-induced neuroprotection in a study on 6-OHDA toxicity in rodent mesencephalic primary cell cultures (Li et al., 2009).

Although liraglutide had no influence on the loss of nigral dopaminergic neurons in the 6-OHDA model, liraglutide treatment potentiated apomorphine-induced rotations in normal-weight rats exhibiting a full, but not partial, nigral lesion. Significant apomorphine-induced rotations were also apparent in liraglutide-treated normal-weight rats one week earlier as compared to vehicle controls. This synergistic effect was observed in normal-weight rats treated with liraglutide for three weeks both before and after surgery, but not in rats only receiving liraglutide treatment post-surgery. This effect was not observed in DIO rats, although liraglutide-treated DIO rats showed a slight trend in increased rotational responsiveness in the apomorphine test. We have not characterized this effect further, but it seems less likely that GLP-1 receptor agonists directly modulate striatal dopamine release, as exendin-4 has no effect on extracellular dopamine levels in the rodent nucleus accumbens (Egcioğlu et al., 2013, Mietlicki-Baase et al., 2013). In contrast, subacute exendin-4 treatment is reported to augment l-dopa stimulated striatal dopamine levels and reduce apomorphine rotational responsiveness to l-dopa in rats with a full 6-OHDA nigral lesion (Abuirmeileh et al., 2012), which could potentially suggest therapeutic relevant synergistic effects of exendin-4 and l-dopa co-treatment in PD. In this regard, a recent preliminary open-label proof-of-concept clinical trial (lacking placebo control), however, yielded equivocal results as 24 months of exendin-4 treatment is demonstrated to improve motor function but also increase l-dopa induced dyskinesias scores in patients with advanced PD (Aviles-Olmos et al., 2014).

In conclusion, liraglutide showed no ameliorating effect on dopaminergic neuron loss in the rat 6-OHDA lesion model of PD. It should be noted that liraglutide, but not exendin-4, is recently
demonstrated to prevent nigral dopaminergic neuron loss and improve motor function in the mouse MPTP model (Liu et al., 2015), which contrasts previous findings of neuroprotective and anti-inflammatory effects of exendin-4 in the mouse MPTP model (Kim et al., 2009, Li et al., 2009).

The 6-OHDA model is also characterized by inflammatory activity at the nigral injury site. Accordingly, 6-OHDA neurotoxicity is associated with recruitmentment of pro-inflammatory microglial cells (Walsh et al., 2011, Maia et al., 2012, Espinosa-Oliva et al., 2014), neuroinflammatory and neurodegenerative processes occur concomitantly (Walsh et al., 2011, Maia et al., 2012), and anti-inflammatory drug effects are associated with protection of dopaminergic neurons in the 6-OHDA model (Johnston et al., 2008, Sadeghian et al., 2012, Barnum et al., 2014). In reverse analogy, it is most conceivable that lack of dopaminergic neuronal protection efficacy would parallel a concurrent lack of anti-inflammatory activity of the test compound, as previously also demonstrated by Ambrosi et al. (2010). Although it must be specifically addressed in future studies, the association between neuroprotective and anti-inflammatory drug effects in the 6-OHDA model, therefore argues for that liraglutide did also not exhibit anti-inflammatory effects in the various 6-OHDA model settings employed in the current study.

In conclusion, the discrepancies may potentially suggest PD model-specific differences in treatment effects of GLP-1 receptor agonists. Further experimental studies are therefore needed to address the efficacy of GLP-1 receptor agonist treatment in Parkinson’s disease.

4. Experimental procedures

4.1. Animals

Six-weeks old male Sprague-Dawley rats (175–200 g, n=157) were purchased from Taconic (Ll. Skensved, Denmark). Upon arrival to the animal unit, all rats were uniquely identified with a microchip (Pet ID Microchip, E-vet), implanted under the skin under light CO₂ anaesthesia. The animals were identified using a WS-1 weigh station (MBrose ApS, Faaborg, Denmark) connected to a laptop running a HM02Lab software (Ellegaard Systems, Faaborg, Denmark), which matches chip no. and body weight as well as calculating daily drug dose according to individual daily body weight. Animals were grouped in pairs 5 days after surgery in large polypropylene cages (480 × 265 × 210 mm, floor area of 940 cm²; Scanbur, Karlslunde, Denmark) with stainless steel mesh lids mounted with feeders containing regular chow (Altromin 125, Brogaarden, Hoersholm, Denmark), cages were supplied with Tapvei aspen bedding, Enviro-dri nest material, an aspen stick (Brogaarden, Hoersholm, Denmark) and a hide (Fat Rat Huts; Bio-Serv, Flemington, NJ). The animals were housed in a standard 12-h light/dark cycle (lights on, 6:00 AM; lights off, 6:00 PM) at a room temperature of 20–22 °C and relative humidity of 50–60%. Normal-weight rats had free access to water and chow throughout the study. Diet-induced obese (DIO) rats were established by offering rats a two-choice diet consisting of a standard rodent chow (Altromin #1324, Brogaarden, Denmark) and a Gubra diet, a high palatable high-fat high-sugar diet made up of a paste (1:1:1) of chocolate spread (Nutella, Ferrero, Italy), peanut butter (Skippy, Unilever, USA) and powdered regular rodent chow (Altromin #1324, Brogaarden, Denmark) for 16 weeks (Hansen et al., 2012). When entering the experiment, rats weighed 325 ± 12 g (normal-weight rats), partial 6-OHDA lesion study, six weeks pre-lesion, n=40; 496 ± 5.7 g (normal-weight rats, full 6-OHDA lesion study, three weeks pre-lesion, n=60), and 587 ± 5.7 g (DIO rats, full 6-OHDA lesion study, three weeks pre-lesion, n=57), respectively. Rats in the 6-OHDA full lesion experiments were age-matched. All animal experiments were conducted in accordance with internationally accepted principles for the care and use of laboratory animals, and were approved by the Danish Council for Animal Research (license 2008/561–1565).

4.2. Compounds

Liraglutide was obtained from Novo Nordisk (Bagsvaerd, Denmark). 6-hydroxydopamine hydrobromide, desipramine hydrochloride, pargyline hydrochloride, L- amphetamine sulphate, and R-(−)-apomorphine hydrochloride hemihydrate were purchased from Sigma-Aldrich (Broendby, Denmark).

4.3. Partial and full 6-OHDA lesions

The individual 6-OHDA model settings are outlined in Table 1 and Fig. 1. Animals were pain-relieved pre-operatively with carprofen (5 mg/ml in isonic NaCl, 0.1 ml/kg, s.c.) immediately prior to surgery, then anesthetized (2.7 ml/kg, s.c.) with a mixture of 1.25 mg/ml midazolam, 2.5 mg/ml fluanisone and 0.079 mg/ml fentanyl (hypnorn/dormicum). A minimum of twenty minutes prior to the 6-OHDA injection, animals were injected with pargyline (5 mg/kg, i.p.) and desipramine (25 mg/kg, i.p.) to prevent injury of midbrain noradrenergic neurons. Animals were placed in a stereotaxic frame and following sinus calibration for the lateral coordinates, 6-OHDA infusions were made into the medial forebrain bundle (MFB) using a 5 μl Hamilton syringe with a 26-gauge needle. All 6-OHDA lesions were performed unilaterally (right hemisphere) with the contralateral (left) hemisphere serving as histological control. The 6-OHDA infusion was applied over two minutes, the needle was then left in place for another 5 min to prevent reflux before being slowly retracted. Fresh 6-OHDA solutions (in saline containing 0.1% ascorbic acid) were made from frozen stock just prior to the infusion and kept on ice during the whole surgery procedure. The stereotaxic coordinates are given below (mm relative to bregma and dural surface), according to the atlas of Paxinos and Watson (1987). Following surgery procedures, animals were pain-relieved with 4 ml isonic NaCl (s.c.) and carprofen (5 mg/ml in isonic NaCl, 0.1 ml/kg, s.c.) once daily for two days. To allow for complete recovery after surgery, rats were single-housed the first week post-surgery then pair-housed throughout the remainder of the study.

The partial nigral lesion model (n=40 rats) was established by infusing 3 μg/2 μl of 6-OHDA into the MFB according to the protocol by Bertilsson et al. (2008) with slight stereotaxic modifications (noose bar –2.7; anterior-posterior –2.8; lateral –1.9; ventral –8.2). To compare with a previous rat study on exendin-4 treatment under equivalent 6-OHDA induced partial nigral lesion conditions (Bertilsson et al., 2008), the 6-OHDA lesion was applied six weeks prior to initiation of liraglutide treatment or vehicle dosing.

To establish the full nigral lesion model in normal-weight (n=60) and DIO rats (n=57), respectively, two successive 6-OHDA infusions were applied into the ascending MFB. Hence, 5–8 min after the first infusion of 6-OHDA (7.5 μg 6-OHDA/2.5 μl, noose bar –2.4; anterior-posterior –4.4; lateral 1.2; ventral 7.8), a second infusion of 6-OHDA was applied (6 μg 6-OHDA/2 μl, noose bar +3.4; anterior-posterior 4.0; lateral 0.8; ventral –8.0).

4.4. Evaluation of rotational activity

Rotational activity in the apomorphine and L- amphetamine test is commonly used to assess the functional consequence of dopaminergic neuronal loss induced by 6-OHDA, which also enables characterization of counteracting effects of a test compound by quantifying the number of amphetamine or apomorphine-
induced rotations. The dopamine receptor agonist apomorphine induces rotations contralateral to the lesioned side due to a compensatory up-regulation of postsynaptic dopamine receptor levels in the denervated ipsilateral striatum (Creese et al., 1977, Hu et al., 1990). In contrast, rats exhibiting an unilateral partial lesion will display ipsiversive rotatory behavioral activity in a p-amphetamine test as a result of imbalanced dopamine release caused by the unilateral loss of DA cells and terminals, and do not show significant asymmetric rotatory response to apomorphine (Carman et al., 1991, Schwarting and Huston, 1996). Rotations were measured using a Rotometric system (AccuScan Instruments, Columbus, OH). Following administration of apomorphine (0.05 mg/kg, sc) or amphetamine (5 mg/kg, sc) animals were placed in plastic bowls (Ø 50 cm). Apomorphine (0.05 mg/kg, 1 ml/kg, sc.) and p-amphetamine rotation protocols were similarly applied to both lesion models, except from the use of different p-amphetamine doses used in the full (5.0 mg/kg, 1 ml/kg, sc.) and the partial (2.5 mg/kg, 1 ml/kg, sc.) lesion study.

In the full nigral lesion study, apomorphine-evoked rotations were assessed on treatment days 7, 14 and 22 (see Fig. 1). Animals received an injection with apomorphine before being placed in the harness and test chamber. After 10 min of habituation time, quantification of the apomorphine response was accomplished by counting the number of turns in a total of 15 min. The number of amphetamine-induced rotations was evaluated 30 min post-administration for a total of 15 min. In the partial nigral lesion study, animals were tested for baseline apomorphine- and amphetamine-induced rotations in week -5, -3 and -1 before dosing. The animals were allowed to fully recover for two days between the apomorphine- and amphetamine-rotation tests. Three days before treatment start, rats were stratified to either vehicle dosing or liraglutide treatment based on the average number of amphetamine-induced turns in the preceding five weeks. When treatment with liraglutide or vehicle was initiated, amphetamine-induced turns were subsequently evaluated by then end of treatment week 1, 3 and 4 (see Fig. 1). A positive drug-induced rotatory response was considered if the animal performed at total of more than 10 ipsilateral rotations (p-amphetamine) or 60 contralateral rotations (apomorphine), respectively, in the 15 min rotation test.

4.5. Drug treatment

The liraglutide dosing regimens are outlined in Fig. 1. A fixed dose of liraglutide (500 μg/kg/day, 1 ml/kg, q.d.) or phosphate-buffered saline (PBS, pH 7.4) vehicle added 0.1% bovine serum albumin (BSA, Roche Diagnostics, Mannheim, Germany) was used in all dosing regimens. For all liraglutide-treated rats, a dose-scalation scheme, starting from an initial daily dose of 50 μg/kg, was implemented to reduce anticipated initial side-effects of liraglutide, including taste aversion and pica behavior, as GLP-1 receptor induced discomfort in rodents is transient and typically only observed within the first 2–3 days of dosing (Kanoski et al., 2012). Dose escalation was continued through daily increments until reaching the target dose of 500 μg/kg/day on treatment day 5. Body weight was recorded throughout the study. The daily dose of liraglutide is comparable to doses previously characterized in other rodent models of neurodegenerative diseases (Xiong et al., 2013, DellaValle et al., 2014, Hansen et al., 2015), as well as preclinical models of diabetes and obesity (Larsen et al., 2008, Jelsing et al., 2012, Hansen et al., 2015). In the partial nigral lesion model, liraglutide treatment (n=20) or vehicle dosing (n=20) was applied for four weeks, starting six weeks post-lesion induction. Two different dosing regimens were applied to the 6-OHDA induced full lesion model and one dosing regimen was characterized in the 6-OHDA induced partial lesion model. When applied to the full nigral lesion model in normal weight and DIO rats, liraglutide (normal weight rats, n=20; DIO rats, n=20) or vehicle (normal weight rats, n=40; DIO rats, n=37) was administered for three weeks starting on the 6-OHDA lesion day, or for a total of six weeks starting three weeks before the 6-OHDA lesion was applied on day 0. All vehicle controls, received vehicle-treatment for 21 days prior to lesion whereupon rats in this cohort were randomized (according to body weight on day –1) to either continued vehicle dosing (n=20) or liraglutide treatment (n=20), respectively, for three weeks after the full nigral lesion was applied.

4.6. Tissue sampling

Two days after the last rotation trial, animals were terminated using CO2/O2 anaesthesia followed by decapitation, the brains were collected and immersion fixed in 4% paraformaldehyde for a minimum of 48 h. Samples were stored at −4 °C until further processing. The brains were weighed and cut in two halves at the level of the optic chiasm. The caudal part containing the midbrain was paraffin-infiltrated overnight and embedded in paraffin block. Five µm sections containing the entire substantia nigra and ventral tegmental area (Paxinos and Watson, 1987) were cut on a microtome (Microm HM340E, ThermoScientific) in the coronal plane in a systematic uniform random sampling fashion allowing for subsequent stereological determination of total number of TH-positive neurons. Sections were collected in pairs so that two neighbouring sections were on the same slide to allow for a subsequent physical dissector stereological neuron number counting. The sections were collected directly onto flex glass (DAKO, Glostrup, Denmark) using a predetermined sampling fraction (full lesion model, every 30th section; partial lesion model, every 50th section) and a random number within the section sampling interval. In the event of poor section quality the adjacent section was sampled instead. The sections were dried overnight at 37–40 °C, and deparaffinized in series of toluene and ethanol before immunostaining.

4.7. Quantitative assessment of midbrain tyrosine-positive neuronal numbers

In brief, the deparaffinized sections were subjected to antigen retrieval by treating the sections in a Tris-EGTA buffer (10 mM, pH 9.0, 90 °C) for 15 min and rinsed in Tris-buffered saline (TBS) before staining. Sections were then stained using a Link 48 autostainer (DAKO, Glostrup, Denmark). Endogenous peroxidase activity was blocked for 10 min in 1% H2O2 in TBS added 0.25% BSA and Tween-20 (pH 7.6). Blockade of non-specific binding was obtained by incubating the sections for 20 min in 5% normal swine serum in TBS +1% BSA +0.25% Tween-20 (TBS + BSA + Tween-20, pH 7.6) followed by incubation for 30 min with a primary anti-TH antibody (1:16,000, Sigma-Aldrich (Broendby, Denmark) diluted in TBS + BSA + Tween-20. After a brief rinse in TBS + BSA + Tween-20, sections were incubated for 30 min in Envision Polymer antimouse secondary antibody (DAKO, Glostrup, Denmark), then rinsed in buffer before being subjected to 3,3′-diaminobenzidine as chromagen (Dako, Glostrup, Denmark). Development was stopped after 10 min with water and sections were counterstained with Mayer’s haematoxylin (Dako, Glostrup, Denmark). Following mounting on cover glass with Pertex, slides were allowed to dry overnight and scanned on a digital slide scanner (Aperio ScanScope AT, Leica Biosystems, Ballerup, Denmark).

Counting of the total TH neuron number in the SNC and VTA in both hemispheres was performed using the physical dissector, using a NewCast Autodisector system (Visiopharm, Denmark) which allows automated alignment using virtual (digital) slides. The region of interest (ROI) was defined on each section using ROI
delineation; sections were aligned and an optimized sampling protocol was applied. The dissector counting frame and sampling frequency was adjusted allowing for an average of 150 neurons to be identified in around 100 dissectors using the principle by Gundersen and Jensen (1987). The individual reference volume of the SNc or VTA was determined using the upper right corner of the dissector as counting point, i.e. all dissectors with the upper right corner hitting the ROI were counted. The total number of neurons in each subdivision (N) were estimated by using the following equation: \( N = \Sigma x (ssf^{-1} \times asf^{-1} \times hsf^{-1}) \), where \( \Sigma \) = number of TH-positive cells counted; ssf = section sampling fraction; asf = area sampling fraction (area of counting frame divided by area of each x,y movement); hsf = height sampling fraction (height of dissector divided by tissue Section, 5 \( \mu m \)).

4.8. Data analysis

All data were fed into Excel spread sheets and subsequently subjected to relevant statistical analyses using GraphPad Prism (GraphPad Software, La Jolla, CA) or SigmaStat (Systat Software, San Jose, CA), where applicable. Results are presented as mean ± standard error of the mean (S.E.M.). Body weight, food intake, water intake and rotational behavior were analyzed using a two-way repeated measurement analysis of variance (ANOVA). Stereological data were evaluated statistically by an unpaired t-test (paired lesion model), or a one-way ANOVA followed by Dunnett’s post-hoc test (full lesion model). Spearman’s correlation test was applied to evaluate the relationship between individual neuron number and corresponding Amphetamine-induced rotations. A p-value less than 0.05 was considered statistically significant.

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