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Serotonergic neurons mediate the anxiolytic effect of L-DOPA: Neuronal correlates in the amygdala

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ABSTRACT

Anxiety in Parkinson's disease is a comorbid non-motor symptom that alters the quality of life of patients. Its neuronal substrates and those of L-Dopa treatment are still poorly known. Using different combinations of monoaminergic system lesions in the rat, we addressed the contribution of these systems in the efficacy of L-DOPA on anxiety and on the neuronal activity of basolateral amygdala (BLA), a brain structure involved in anxiety. Anxiety, locomotor activity and motor performance were assessed using the elevated plus maze, the open field and the skinner box, respectively. The neuronal activity of BLA was electrophysiologically recorded and the loss of dopamine, noradrenaline and serotonin neurons was quantified by immunohistochemistry and stereology. Selective bilateral lesion of dopamine neurons, with or without the additional lesions of noradrenaline and/or serotonin neurons, induced anxiety disorder. L-Dopa significantly decreased anxiety in animals with bilateral lesion of dopamine neurons alone or combined with that of noradrenaline neurons. In these two groups, L-DOPA enhanced the firing rate of BLA neurons. However, in animals with combined lesions of dopamine and serotonin neurons or in animals with lesions of the three monoaminergic systems, L-Dopa was no longer able to decrease anxiety behavior or to change the electrophysiological parameters of BLA neurons. Our data provide the first evidence of the key and positive role of the serotonergic system in the combined efficacy of L-Dopa on anxiety and the paralleled BLA neuronal activity, suggesting that the enhancement of the activity of serotonin neurons may boost the anxiolytic action of L-DOPA.

1. Introduction

Parkinson's disease (PD) is characterized by the degeneration of dopamine (DA) neurons (Ehringer and Hornykiewicz, 1960) at the origin of the cardinal motor symptoms of the disease. Moreover, PD is also characterized by the loss of noradrenaline (NA) cells in the locus coeruleus (German et al., 1992) and serotonin (5-HT) neurons in the raphe nucleus (Kish, 2003), which may contribute to the non-motor symptoms (Devos et al., 2010; Zarow et al., 2003) including anxiety. Anxiety is a frequent disorder occurring in approximately 25–50% of PD patients (Dissanayaka et al., 2010; Goetz, 2010; Nègre-Pagès et al., 2010). It can precede the classical motor features of PD by years and contributes to the deterioration of patient's quality of life (Chaudhuri et al., 2006; Reijnders et al., 2009). While some studies suggested the link between anxiety and the degeneration of DA cells (Chaudhuri and Schapira, 2009; Eskow Jaunarajs et al., 2010), others suggested the involvement of NA and/or 5-HT cell loss (Andrade et al., 2004; Graeff,

2002). Using the unilateral 6-OHDA rat model of PD, we provided evidence for the manifestation of anxiety when NA and/or 5-HT depletions were combined with DA depletion, suggesting that PD should be modeled as a monoaminergic pathology (Delaville et al., 2012). In the present study we extended this work by using the bilateral 6-OHDA rat model.

Levodopa (L-Dopa) is the gold standard anti-parkinsonian treatment, which permits the restoration of DAergic transmission to improve the cardinal motor symptoms (Cotzias, 1968; Yahr et al., 1969). However, the impact of L-Dopa on PD non-motor symptoms and its interaction with the monoaminergic systems are not clearly established. In the context of PD, in the absence of DA neurons, 5-HT neurons have been shown at the origin of the diffuse increase in DA neurotransmission induced by L-Dopa (Navailles et al., 2010b). Moreover, another study using metabolic mapping unravelled that non-motor domains, such as the basolateral amygdala (BLA) may play a role in the mechanism of action of L-Dopa (Guigoni et al., 2005). Indeed, BLA receives dense

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innervation from the DAergic, NAergic, and 5-HTergic systems (Asan et al., 2013; Fallon et al., 1978; Smith and Aston-Jones, 2008). However, the efficacy of L-Dopa on anxiety and its effects on the neuronal activity of BLA after monoaminergic system lesions are not determined.

The present work aimed to investigate, first, the respective role of combined bilateral lesions of the monoaminergic systems on anxiety behavior; second, whether these monoaminergic lesions can interfere with the effect of L-Dopa on anxiety; third, the changes in the neuronal activity of BLA neurons after the same lesions. The rationale of choosing BLA has been based on the reported data showing that BLA plays a key role in anxiety and fear related behaviors (Herry et al., 2008; Lalumière, 2014).

2. Material and methods

2.1. Animals

Adult male Sprague-Dawley rats (280–380 g) were housed four per cage under artificial conditions of light, temperature and humidity with food and water available *ad libitum*. All experiments were carried out in strict accordance with the Council Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. The protocol was approved by the Ethics local Committee (50120126-A and 50120136-A).

2.2. Drugs

6-Hydroxydopamine (6-OHDA) hydrobromide, L-ascorbic acid, desipramine hydrochloride, 5,7-Dihydroxytryptamine (5,7-DHT) creatinine sulfate salt, Levodopa (L-Dopa) L-3,4-Dihydroxyphenylalanine methyl ester hydrochloride, benserazide-hydrochloride and all anesthesia drugs were purchased from Sigma (Saint-Quentin Fallavier, France).

2.3. Animal groups, surgical procedures and L-Dopa treatment

Animals were divided into five groups: DA-lesioned group ($n = 8$); DA/NA-lesioned group ($n = 9$); DA/5-HT-lesioned group ($n = 12$); DA/NA/5-HT-lesioned group ($n = 9$) and their relative sham groups with 6 rats for each of them. One hour before surgery, rats were injected with Temgesic (0.1 mg/kg, s.c.) for analgesia and rats from the group without NA cell lesion were injected with desipramine (25 mg/kg, i.p.) to protect NA neurons from 6-OHDA toxicity. Rats were anesthetized by ketamine (75 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic frame (Kopf, France), the skull was exposed and the burr holes were made. The toxins or vehicle were slowly injected bilaterally in the medial forebrain bundle (MFB) (in mm: AP 2.8, ML $\pm$ 2, DV -8.4) (Paxinos and Watson, 1996). Vehicle was injected in each MFB in sham animals (0.2% ascorbic acid in 0.9% saline), or 6-OHDA (2 μ l in each MFB, 1.675 μ g/ μ l) in the lesioned-groups and in addition, 5,7-DHT (3 μ l in each MFB, 4 μ g/ μ l) in 5-HT cell lesion groups. After surgery, the rats were given a 3-weeks recovery before behavioral assessments.

L-Dopa (12 mg/kg) and benserazide (15 mg/kg) were dissolved in NaCl 0.9% and administered 40 min before behavioral tests (Navailles et al., 2010b).

2.4. Evaluation of motor performance

The impact of combined monoaminergic lesions on motor behavior was measured using the distance traveled by the animals in the open field and the motor time in a skinner box task as previously described (Zeef et al., 2012; Klinkenberg and Blokland, 2011). Behavioral testing in the open field started by placing the rat in the middle of the arena and locomotor activity was recorded using a camera and Ethovision tracking software (Ethovision®, Noldus Information Technology,

Wageningen, the Netherlands). The distance traveled (cm) by each animal was determined during 10 min in the arena. Motor time was investigated by training and testing the rats in a Skinner box (Med Associates, St. Albans, USA). Animals had to press a lever for five times in order to obtain a 45-mg food reward and a session was terminated after 60 trials or 30 min. Rats were trained everyday and data were collected when the last 3 sessions were stable over time. Motor time was determined as the mean latency between the release of the panel and the lever press as previously described (Cao et al., 2006).

2.5. Evaluation of anxiety

The elevated plus maze (EPM), which is considered as the most common test of anxiety in rodents, was used as previously described (Faggiani et al., 2015; Carobrez and Bertoglio, 2005). Briefly, animals were allowed to explore the maze for 5 min and videotaped. The number of entries and the time spent in the open and closed arms were measured. The first time in the EPM corresponded to the baseline for anxiety according to the different groups. One week later, we performed the same test with L-Dopa treatment.

2.6. Extracellular single-unit recordings

Extracellular microrecordings in the BLA were performed in rats anesthetized with urethane (1.2 g/kg i.p.) as previously reported (Ni et al., 2001). A single glass micropipette electrode (impedance: 8–12 M Ω) was filled with 4% Pontamine Sky Blue (PSB) in 3 M NaCl and then lowered into the BLA (in mm: AP -0.9 , L 3, D 4.5–7.5) (Paxinos and Watson, 1996). BLA neurons were identified according to their firing activity as previously reported (Pare and Gaudreau, 1996). Extracellular neuronal activity was amplified, bandpass filtered (300–3000 Hz) using a preamplifier (Neurolog, Digitimer, UK) and transferred via a Powerlab interface to a computer with Chart 7 software (AD Instruments, Charlotte, USA). Basal firing was recorded during 20 min to ascertain the stability of the discharge activity. Then, L-Dopa was injected intraperitoneally and the recorded neuron was followed during 2 h. The recording site was marked by an electrophoretic injection of PSB through the micropipette at a negative current of 20 μ A for 7 min (IsoDAM 80, WPI, UK).

2.7. Data analysis

The electrical activity of each neuron was analyzed with a spike discriminator (AD Instruments), and firing parameters (interspike interval 5-ms bin) were calculated using Neuroexplorer program (Alpha Omega, Israel). The firing rate values are the mean $\pm$ SEM (Belujon et al., 2007). Firing patterns were analyzed as previously described (Labarre et al., 2008) and identified as regular, irregular or bursty (Kaneoke and Vitek, 1996).

2.8. Histology and immunohistochemistry

After completion of the experiments, animals were sacrificed by intracardiac perfusion of 4% paraformaldehyde, the brains removed, frozen in isopentane at -45°C and stored at -80°C . Brains were cryostat-cut into 50 μ m coronal sections and acetylcholine esterase staining was used to determine the location of PSB dots as previously described (Chetrit et al., 2009). Only brains with dots in BLA were retained for data analysis.

The loss of DA cells in the SNc/VTA, DA fibers in the striatum, and NA cells in the LC were determined using immunohistochemistry of tyrosine hydroxylase (TH-antibody, Sigma-Aldrich) as previously described (Bouali-Benazzouz et al., 2009). 5-HT cell loss in the dorsal and medial raphe nuclei (DRN and MRN respectively) was determined by immunohistochemistry of Tryptophan hydroxylase (TrH-antibody, Sigma-Aldrich). The number of TH- and TrH-immunoreactive (IR) cells

was obtained using the optical fractionator (Bastide et al., 2014) unbiased stereological method (Leica DM6000B, Mercator Pro, ExploraNova, France). Immuno-labelled cells were counted by a blinded investigator on every 6th section for the SNc, the VTA, the DRN and the MRN and on every 3rd section for the LC previously used with stereological parameters adapted to SNc (Counting frames: $80 \times 80 \mu\text{m}$, Spacing: $240 \times 180 \mu\text{m}$, number of sections: 5), to DRN and MRN (Counting frames: $60 \times 60 \mu\text{m}$, Spacing: $80 \times 120 \mu\text{m}$, number of sections: 4) or to LC (Counting frames: $60 \times 60 \mu\text{m}$, Spacing: $80 \times 100 \mu\text{m}$, number of sections: 3).

2.9. Statistical analysis

Statistical analyses were performed using Sigmaplot (Systat Software) and data were compared using a One-way Anova test. When normality test passed, we used a One-way Anova test followed, when significant, by a student Newman-Keuls multiple post-tests. When normality test failed, we used a Kruskal-Wallis test, when significant, followed by a post-hoc Dunn's method. The effect of L-Dopa in each lesioned group, before and after drug injection, was compared using a Wilcoxon paired *t*-test. Firing rates were compared using a One-way Anova test. The effect of L-Dopa in lesioned-rats, before and after drug injection, was compared using a paired *t*-test. Firing patterns were analyzed as previously described (Boraud et al., 1998; Tai et al., 2003) and their proportions were compared using a χ^2 test.

3. Results

For all the parameters that we have evaluated, each lesioned group had its relative sham group with 6 rats each. As the results of the different sham groups were not significantly different (data not shown) we grouped them in a single sham group ($n = 24$).

3.1. Selective bilateral lesions of DA, NA and 5-HT neurons

Bilateral injection of 6-OHDA into the MFB induced DA cell loss in the SNc (Kruskal-Wallis, $F = 76.17$, $p < 0.001$; Fig. 1AB). Compared to sham rats, TH-IR cells decreased by 65% in DA-depleted rats ($p < 0.001$), 69% in DA/NA-depleted rats ($p < 0.001$), 63% in DA/5-HT-depleted rats ($p < 0.001$) and 76% in DA/NA/5-HT-depleted rats ($p < 0.001$). Likewise, it induced DA denervation throughout the striatum (Kruskal-Wallis, $F = 57.51$, $p < 0.001$; Fig. 1CD). The TH-IR density decreased by 73% in DA-depleted rats ($p < 0.001$), 72% in DA/NA-depleted rats ($p < 0.001$), 69% in DA/5-HT-depleted rats ($p < 0.001$) and 87% in DA/NA/5-HT-depleted rats ($p < 0.001$). In addition to the SNc, bilateral injection of 6-OHDA induced moderate but significant DA cell loss in the VTA (Kruskal-Wallis, $F = 35.34$, $p < 0.001$; Fig. 1E). Compared to sham rats, TH-IR cells decreased by 30% in DA-depleted rats ($p < 0.001$), 48% in DA/NA-depleted rats ($p < 0.001$), 33% in DA/5-HT-depleted rats ($p < 0.001$) and 43% in DA/NA/5-HT-depleted rats ($p < 0.001$).

Bilateral injection of 6-OHDA into the MFB of rats without prior desipramine treatment induced NA cell loss in the LC (One-Way Anova, $F = 80.59$, $p < 0.001$; Fig. 1F–H). Compared to sham rats, TH-IR cells decreased by 59% in DA/NA-depleted rats ($p < 0.001$) and 56% in DA/NA/5-HT-depleted rats ($p < 0.001$). However, TH-IR cells in the LC of animals pre-treated with desipramine were not affected.

Bilateral 5,7-DHT injection into the MFB induced specific loss of 5-HT neurons in the DRN (One-Way Anova, $F = 53.49$, $p < 0.001$; Fig. 2A–C) and the MRN (One-Way Anova, $F = 24.15$, $p < 0.001$; Fig. 2ABD). TrH-IR cells decreased by 48% in the DRN and 39% in the MRN of DA/5-HT-depleted rats ($p < 0.01$) and 47% and 44% in DA/NA/5-HT-depleted rats ($p < 0.001$). However, TrH-IR cells in both raphe nuclei of animals who did not receive 5,7-DHT were not affected.

3.2. Motor deficit

In order to assess the consequences of combined monoaminergic lesions on motor component, we measured two parameters: the distance traveled by the animals in the open field and the motor time in the skinner box. Bilateral DA depletion alone or combined with NA and/or 5-HT significantly affected the spontaneous locomotor activity in the open field test (One-way ANOVA, $F = 6.628$, $p < 0.001$; Fig. 3). Indeed, compared to sham rats, bilateral DA depletion significantly decreased the distance of locomotor activity ($p = 0.003$). Furthermore, while additional NA or 5-HT or combined NA and 5-HT depletions significantly decreased the distance traveled by the animals ($p = 0.003$, $p < 0.001$ and $p = 0.003$ respectively compared to sham rats), they did not potentiate the locomotor deficit induced by DA depletion alone ($p > 0.05$ compared to DA-depleted animals).

Bilateral monoamine depletions significantly affected motor time in the skinner box (One-way ANOVA, $F = 3.37$, $p = 0.002$; Fig. 3). While bilateral DA depletion alone did not significantly change the motor time ($p = 0.076$), additional depletion of NA or 5-HT or NA/5-HT increased motor time compared to sham rats ($p = 0.042$, $p = 0.048$ and $p = 0.024$, respectively; Fig. 3).

3.3. Anxiety disorder

Anxiety behavior was assessed using the elevated plus maze and a rat was judged to be anxious when he spent less time in the open arms than in the closed arms compared to sham rats. Bilateral lesion of DA neurons alone or combined with the lesion of NA and/or 5-HT systems significantly affected the number of entries in open arms extremities (One-Way ANOVA, $F = 11.59$, $p < 0.001$; Fig. 4A) and the time spent in open arms (One-Way ANOVA, $F = 10.18$, $p < 0.001$; Fig. 4B) of the elevated plus maze. Bilateral lesion of DA neurons significantly decreased the number of entries in the open-arms extremities by 91% ($p < 0.001$) compared to sham rats. A similar decrease was found by the additional lesion of NA and/or 5-HT systems. Indeed, compared to sham rats, the number of entries decreased by 63% ($p < 0.001$) in rats with DA/NA lesions, by 81% ($p < 0.001$) in rats with DA/5-HT lesions, and by 66% ($p < 0.001$) in rats with DA/NA/5-HT lesions. In the same way, the time spent in the open arms significantly decreased by 66% ($p < 0.001$) in rats with DA lesion, by 55% ($p < 0.01$) in rats with DA/NA lesions, by 75% ($p < 0.001$) in rats with DA/5-HT lesions and by 45% ($p < 0.01$) in rats with DA/NA/5-HT lesions.

In contrast to the open arms, which are important in the evaluation of anxiety, DA lesion alone or combined with the lesion of NA and/or 5-HT did not affect the number of entries in closed arms extremities (One-Way ANOVA, $F = 15.44$, $p > 0.05$; Fig. 5A), but significantly increased the time spent in closed arms (One-Way ANOVA, $F = 21.08$, $p < 0.001$; Fig. 5B).

3.4. L-Dopa and anxiety disorder

Bilateral lesion of 5-HT neurons, but not of NA neurons, interferes with the effectiveness of L-Dopa on anxiety induced by the bilateral depletion of DA (Fig. 4C). L-Dopa increased the number of entries in open arms extremities in rats with bilateral DA lesion (Wilcoxon paired *t*-test, $p = 0.043$, Fig. 4Ca) and in rats with bilateral DA lesion combined with NA lesion (Wilcoxon paired *t*-test, $p = 0.014$, Fig. 4Cb). However, L-Dopa had no effect in rats with DA/5-HT lesion (Wilcoxon paired *t*-test, $p = 0.321$, Fig. 4Cc) as well as in rats with lesions of the three monoaminergic systems (Wilcoxon paired *t*-test, $p = 0.063$, Fig. 4Cd). Similarly, L-Dopa increased the time spent in open arms in rats with bilateral DA lesion alone (Wilcoxon paired *t*-test, $p < 0.01$, Fig. 4Da) and in rats with bilateral DA lesion combined with NA lesion (Wilcoxon paired *t*-test, $p < 0.05$, Fig. 4Db). Interestingly, here again L-Dopa had no effect in rats with DA/5-HT lesion (Wilcoxon paired *t*-test, $p > 0.888$, Fig. 3Dc) and in rats with lesions of the three

Fig. 1. Selective bilateral lesion of dopamine (DA) neurons in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA), fibers in the striatum and selective bilateral lesion of NA neurons in the locus coeruleus (LC). Representative examples of brain slices showing TH immunoreactive (TH-IR) neurons in the SNc and VTA (A), fibers in the striatum (B) and in the LC (F) at three different levels, anterior (a), median (b) and posterior (c) of sham and lesioned rats. Histograms show the number of TH-IR neurons as counted in the SNc (C), the VTA (D), the LC (G) and the optical density (OD) of TH-IR as measured in the striatum (E) of sham rats (n = 24), DA-depleted rats (DA, n = 8), DA and NA-depleted rats (DA NA, n = 9), DA and 5-HT-depleted rats (DA 5-HT, n = 12) and DA, NA and 5-HT-depleted rats (DA NA 5-HT, n = 9). Values are the mean $\pm$ SEM. **p < 0.01; ***p < 0.001 in comparison with sham animals.

Fig. 2. Selective bilateral lesion of 5-HT neurons in the dorsal raphe (DR) and median raphe (MR) nuclei. Representative examples of brain slices showing Tryptophan hydroxylase IR (TrH-IR) cells in the DR and MR nuclei at three different levels of the nucleus, anterior (a), median (b) and posterior (c) of sham (A) and 5-HT DR/MR lesioned rats (B). Histograms show the number of TrH-IR neurons as counted in the DR (C) and MR (D) nuclei. Values are the mean $\pm$ SEM. ***p < 0.001 in comparison with sham animals.

Fig. 3. Effects of combined bilateral monoamine depletions on locomotor activity and motor performance. Histograms showing the distance traveled by the animals in the open field (A) and the motor time as determined in the skinner box (B). Values are the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: non-significant in comparison with sham animals.

monoamine systems (Wilcoxon paired t -test, $p = 0.06$, Fig. 4Dd).

L-Dopa did not affect the number of entries in closed arms extremities (Wilcoxon paired t -test, $p > 0.05$; Fig. 5C), nor the time spent in closed arms (Wilcoxon paired t -test, $p > 0.05$; Fig. 5D) in all the groups of lesioned animals.

3.5. L-Dopa and the neuronal activity of BLA neurons

Bilateral DA lesion alone or combined with NA and/or 5-HT tended to decrease the firing rate of BLA neurons but the difference was not significant (One-way Anova, $F = 0.944$, $p = 0.440$; Fig. 6A). Similarly, the firing pattern was not affected by the bilateral monoamine lesions

compared to sham rats (Chi2 test, $X^2 = 3.95$, $df = 8$, $p > 0.05$; Fig. 6B). Interestingly, L-Dopa significantly increased the firing rate of BLA neurons in rats with bilateral DA depletion alone or combined with NA depletion (paired t -test, $p = 0.029$ and $p = 0.040$ respectively, Fig. 6C–G). However, L-Dopa did not significantly change the firing rate of BLA neurons in rats with combined lesions of DA/5-HT or DA/NA/5-HT cells ($p > 0.05$; Fig. 6G). Concerning the firing pattern, L-Dopa treatment had no effect of BLA neurons in all lesioned groups (Chi2 test, $X^2 = 0$, $df = 1$, $p = 1$, Fig. 6H).

Fig. 4. Effect of monoaminergic system lesions on anxiety behavior and their impact on the efficacy of L-Dopa. Histograms showing the number of entries in open arms extremities (A) and the time spent in open arms (B). C–D: Histograms showing the effects of L-Dopa on the number of entries in open arms extremities (C) and on the time spent in open arms (D). Values are the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: non-significant in comparison with sham animals (AB) or in comparison with lesioned animals without L-Dopa (CD).

Fig. 5. Effect of monoaminergic system lesions on anxiety behavior and their impact on the efficacy of L-Dopa. Histograms showing the number of entries in closed arms extremities (A) and the time spent in closed arms (B). C–D: Histograms showing the effects of L-Dopa on the number of entries in closed arms extremities (C) and on the time spent in closed arms (D). Values are the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: non-significant in comparison with sham animals (AB) or in comparison with lesioned animals without L-Dopa (CD).

4. Discussion

Our results provide evidence for the critical role played by DA cell loss in the manifestation of parkinsonian-like anxiety disorder. Indeed, bilateral selective lesion of DA neurons induced anxiety, which was reversed by L-Dopa. Interestingly, we show that the additional lesion of NA and/or 5-HT neurons did not potentiate the anxiogenic effect of DA cell lesion. While the lesion of NA neurons did not interfere with the beneficial effect of L-Dopa, the lesion of serotonergic neurons was responsible for the loss of effectiveness of L-Dopa on anxiety. Finally, we provide evidence for the parallel of L-Dopa efficacy and the changes in the electrical activity of BLA neurons. The improvement of anxiety induced by L-Dopa was paralleled by an increase in the firing rate of BLA neurons in animals with DA cell loss alone or combined with NA cell loss. However, the absence of efficacy of L-Dopa after lesioning 5-HT neurons was associated with the absence of changes in the electrical activity of BLA neurons.

The lesion procedures we used caused selective and significant reductions in the number of TH-IR cells in the SNc, VTA and LC and fibers in the striatum. Moreover, the 5,7-DHT caused a reduction of 5-HT neurons in the DRN and MRN. Despite the focus on DA cell loss in PD, it is now admitted that this pathology is a multi-system disorder characterized by other landmarks, such as the loss of NA and 5-HT neurons. Our animals with different combined bilateral monoamine depletions can be considered as models reproducing what happens in human PD patients, useful in studying the impact of combined monoamine lesions

on the efficacy of L-Dopa on anxiety. The EPM test is a well validated and widely used paradigm to evaluate the relative anxiety-related status of rodents (Carobrez and Bertoglio, 2005). The paradigm is based on the conflict between the innate tendencies of the animals to explore novel environments versus their innate tendency to avoid open areas where the thigmotaxis is impossible, and to be afraid of heights (Bourin et al., 2007; File, 2001). Our results showed that bilateral DA cell lesions induced anxiety-like behavior. This anxiogenic effect has not been exacerbated by the additional depletions of NA and/or 5-HT. These results provide evidence that anxiety behavior is a DA-dependent non-motor symptom in agreement with the suggestion of previous clinical studies (Chaudhuri and Schapira, 2009). Our results also showed that the bilateral nature of DA cell loss in the rat was necessary to induce anxiety-like behavior in contrast to a previous study from our team, in which unilateral DA depletion alone was not sufficient to induce anxiety. This behavioral disorder was developed only when the unilateral DA depletion was combined with noradrenergic and/or serotonergic depletions (Delaville et al., 2012). Our results are consistent with previous data showing that anxiety-related behavior in the rat, using the same test, was seen after bilateral partial lesion of SNc DA neurons (Druil et al., 2014; Tadaiesky et al., 2008). Moreover, it is unlikely that anxiety behavior observed in our rats may be due to the motor impairment, as bilateral DA cell loss decreased only slightly locomotor activity and did not significantly affect motor performance in the skinner box, and that additional lesions of NA and/or 5-HT systems were necessary to induce an increase of motor time. The involvement of

Fig. 6. Effect of monoaminergic lesions and of L-Dopa treatment on the spontaneous firing rate and pattern of discharge of BLA neurons.

A–B: The histograms represent the mean $\pm$ SEM of the firing rate (spikes/s, A) and the percentage of distribution of discharge patterns (B) of BLA neurons recorded in the experimental groups: sham rats ($n = 40$ neurons), DA-lesioned rats ($n = 38$ neurons), DA and NA-lesioned rats ($n = 34$ neurons), DA and 5-HT-lesioned rats ($n = 27$ neurons) and DA, NA and 5-HT-lesioned rats ($n = 49$). C–F: Representative examples of cortical EEG recordings and spike trains of irregular and bursty neurons recorded in BLA before (CE) and after (DF) the injection of L-Dopa. G–H: Histograms represent the mean $\pm$ SEM of the firing rate (spikes/s, G) and the percentage of distribution of discharge patterns (H) of BLA neurons recorded before and after L-Dopa treatment. Values are the mean $\pm$ SEM. * $p < 0.05$, ns: non-significant.

NA dysregulation has recently been highlighted in the development of akinesia in PD patients (Albares et al., 2015; Favre et al., 2013).

The impact of NA and 5-HT cell lesions on the efficacy of L-Dopa on anxiety was investigated in our animal models with different combined

lesions. In PD patients, only few studies focused on the efficacy of L-Dopa on anxiety disorder, with conflicting results (Eskow Jaunarajs et al., 2011). While some studies reported significant improvement of anxiety under L-Dopa (Funkiewiez et al., 2006; Stacy et al., 2010),

others showed no such benefit or reported that L-Dopa exacerbated anxiety disorder (Fetoni et al., 1999; Maricle et al., 1995). In our study, acute administration of L-Dopa significantly improved anxiety behavior in rats with bilateral DA cell lesions. Furthermore, we showed that the same beneficial effect of L-Dopa was observed in animals with additional NA cell lesions, but not in DA-lesioned rats with additional 5-HT cell lesions. These results support the assumption that in the context of PD, the conversion of L-Dopa to DA is widespread and occurs in 5-HT neurons, consistent with biochemical results showing that 5-HT neurons are at the origin of the diffuse increase in DA transmission induced by L-Dopa in 6-OHDA-lesioned rats (Navailles et al., 2010a). Accordingly, we can assume that the loss of 5-HT neurons, even partial (~50%) in our study, in addition to the loss of DA cells, may compromise the release of DA at a sufficient level, resulting in the absence of efficacy of L-Dopa in improving anxiety. Together, our data suggest that in PD patients with an absence or low response to L-Dopa, 5-HT neurons are probably affected by the natural course of the disease.

In order to better understand the pathophysiology of anxiety in the context of PD, we investigated whether the electrical activity of BLA neurons can be influenced by the monoaminergic system lesions and also by the administration of L-Dopa. The rationale of choosing BLA has been based on the reported data showing that BLA plays a key role in anxiety and fear related behaviors (Herry et al., 2008; Lalumiere, 2014). Our results showed that DA depletion induced a tendency to decrease the firing rate without any change of the firing pattern of BLA neurons, which was not potentiated by the additional depletion of NA and/or 5-HT. BLA neurons are known to receive dense dopaminergic innervation and express both D1 and D2 receptors (Scibilia et al., 1992). Moreover, a reduction of TH positive fiber densities has been shown in the BLA of MPTP-treated mice (von Bohlen und Halbach et al., 2005), and that the degeneration of DA neurons in the SNc produces dramatic decreases in DA and its major metabolites in dopaminergic projection areas notably in BLA (Scatton et al., 1983). Altogether, these results suggest that DA plays a role in the modulation of the firing activity of BLA neurons and that the degeneration of DA cells in the context of PD may result in changes in the firing rate, at the origin of the manifestation of anxiety-like disorder. The fact that we found a tendency to decrease the firing rate, in contrast to the significant decrease reported by Chen et al. (2011), can be explained by the differences in the lesioning methods and the anesthetics used. Indeed, we injected 6-OHDA into the MFB, and not into the striatum, to obtain a significant level of DA cell degeneration in the SNc (> 60%) compared to the partial bilateral lesion (~30%) obtained by Chen et al. (2011). Concerning the anesthetic, in our study we used urethane, whereas Chen and Colleagues used chloral hydrate, with the two anesthetics having different mechanisms and impact on neuronal activity (Li et al., 2012).

We then investigated the effect of L-Dopa on the firing activity of BLA neurons. Our results showed that L-Dopa significantly increased the firing rate of BLA neurons in rats with bilateral DA depletion alone or combined with NA depletion to approach the basal level of sham rats. These changes paralleled the improvement of anxiety-like behavior induced by L-Dopa in animals with the same lesions. Our results are consistent with those of a previous study using imaging approaches based on the measure of blood oxygenation level (Tessitore et al., 2002). Their data revealed a robust bilateral amygdala response to sensory information in normal controls that was absent in PD patients during the hypodopaminergic state. DA replacement partially restored this response in PD patients. However, and similar to the absence of changes in behavioral effects, L-Dopa did not change the firing rate of BLA neurons in rats with the additional lesion of 5-HT neurons. Like for anxiety behavior, here again we demonstrate that the combined loss of DA and 5-HT neurons compromised the efficacy of L-Dopa in normalizing the electrical activity of BLA neurons.

Our data provide *in vivo* evidence of the role of L-Dopa in modulating the firing rate of BLA neurons in the context of PD. We also

show that DA replacement restored the abnormal response of the amygdala when the 5-HT neurotransmission is not affected. Furthermore, the loss of 5-HT neurons, which is suggested to result in the absence of DA release, compromised the responses of BLA neurons, and this phenomenon is responsible of the absence of efficacy of L-Dopa on anxiety behavior.

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