Early life stress causes refractoriness to haloperidol-induced catalepsy

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Running title
Prenatal stress and antipsychotic drug-induced parkinsonism

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List of abbreviations: DA = dopamine; GPi = internal globus pallidus; GPe = external globus pallidus; SNpc = substantia nigra pars compacta; SNpr = substantia nigra pars reticulata; STN = subthalamic nucleus; VA, VL, VM = ventral anterior, ventrolateral, and ventromedial thalamic nuclei; PRS = prenatal restraint stress.
Abstract

The use of classical antipsychotic drugs is limited by the occurrence of extrapyramidal motor symptoms, which are caused by dopamine (DA) receptor blockade in the neostriatum. We examined the impact of early life stress on haloperidol-induced catalepsy using the rat model of prenatal restraint stress (PRS). Adult “PRS rats”, i.e. the offspring of mothers exposed to restraint stress during pregnancy, were resistant to catalepsy induced by haloperidol (0.5-5 mg/kg, i.p.) or raclopride (2 mg/kg, s.c.). Resistance to catalepsy in PRS rats did not depend on reductions in blood or striatal levels, as compared to unstressed control rats. PRS rats also showed a greater behavioral response to the DA receptor agonist, apomorphine, suggesting that PRS causes enduring neuroplastic changes in the basal ganglia motor circuit. To examine the activity of this circuit, we performed a stereological counting of c-Fos+ neurons in the external and internal globus pallidus, subthalamic nucleus, and ventral motor thalamic nuclei. Remarkably, the number of c-Fos+ neurons in ventral motor thalamic nuclei was higher in PRS rats than in unstressed controls, both under basal conditions and in response to single or repeated injections with haloperidol. Ventral motor thalamic nuclei contain exclusively excitatory projection neurons that convey the basal ganglia motor programming to the cerebral cortex. Hence, an increased activity of ventral motor thalamic nuclei nicely explains the refractoriness of PRS rats to haloperidol-induced catalepsy. Our data raise the interesting possibility that early life stress is protective against extrapyramidal motor effects of antipsychotic drugs in the adult life.
Introduction

A common and serious drawback of classical antipsychotics, e.g. haloperidol and phenothiazines, is their association with extrapyramidal motor symptoms (EPSs), such as acute dystonia, akathisia, parkinsonism, and tardive dyskinesias (Haase, 1954; Casey and Keepers, 1988). EPSs are a leading cause of noncompliance with medication (Lieberman et al., 1998), which is associated with relapse of psychotic symptoms and re-hospitalization. Antipsychotic-induced parkinsonism, characterized by bradikynesia, rigidity, and resting tremor, often develops within the first three months of treatment and is managed by lowering the antipsychotic dosage, switching to an “atypical antipsychotic”, or adding an anticholinergic drug. However, not all patients are eligible to treatment with atypical antipsychotics, which may cause weight gain and metabolic syndrome, thereby increasing the risk for cerebrovascular disorders, particularly in patients with Alzheimer’s disease (Newcomer, 2005; Leucht et al., 2009; Lett et al., 2012). Anticholinergic drugs combined with classical antipsychotics can cause cognitive impairment and autonomic adverse effects, which may also limit compliance with medication. If one can predict the occurrence of EPSs in response to classical antipsychotics, then the prophylactic use of anticholinergic drugs might be limited to patients at high risk for pharmacological parkinsonism. Established risk factors for antipsychotic-induced EPSs include age, gender, type and potency of the antipsychotic drug, genetic variants of drug metabolizing enzymes (particularly CYP2D6), dose and duration of treatment, and history of previous EPSs (Keepers et al., 1983; Casey, 1990; Loebel et al., 1992; Robinson et al., 2005). For example, young male patients are particularly vulnerable to acute dystonia, whereas parkinsonism is more prevalent in older-age patients, and akathisia occurs more often in middle-age female patients (Ayd, 1961; Keepers et al., 1983; Casey and Keepers, 1988).
Animal studies have shown that acute stress or stress hormones have a profound influence on haloperidol-induced catalepsy, which models some of the features of antipsychotic-induced parkinsonism (Castagné et al., 2009). In rats, single exposure to a low-intensity stressor enhances haloperidol-induced catalepsy after two weeks, whereas exposure to a high-intensity stressor produces opposite effects (Antelman et al., 1991; 1992). Acute treatment with glucocorticoids attenuates haloperidol-induced catalepsy via a peripheral mechanism mediated by the release of catecholamines from adrenal medulla (Chopde et al., 1995). Whether early life stress influences the vulnerability to antipsychotic-induced EPSs in the adult life is unknown. This question is highly relevant to antipsychotic medication because stressful events occurring early in life, particularly in the prenatal period, are established risk factors for schizophrenia (reviewed by Brody, 1981; Read et al., 2001; Koenig et al., 2002; Howes et al., 2004; Sullivan et al., 2006).

The model of prenatal restraint stress (PRS) in rodents recapitulates features of the major psychiatric disorders, such as depression, anxiety, and schizophrenia (Maccari et al., 1995; Morley-Fletcher et al., 2003; 2011; Marrocco et al., 2012; Matrisciano et al., 2012a,b). Adult “PRS rats”, i.e. the offspring of mothers exposed to repeated episodes of restraint stress during pregnancy, show a deregulation of the hypothalamic-pituitary adrenal (HPA) axis, which leads to an increased glucocorticoid secretion in response to stress (Darnaudery and Maccari, 2008).

Recently, we found that PRS rats show a reduced expression/activity of synaptic proteins in the ventral hippocampus (Marrocco et al., 2012), a region that encodes memories related to stress and emotions (Fanselow and Dong, 2010). The evidence that lesions of the ventral hippocampus restrain haloperidol-induced catalepsy (Lipska et al., 1993; 1995), suggested a potential link between early life stress and antipsychotic-induced EPSs.
Here, we report that PRS rats are highly resistant to haloperidol-induced catalepsy as a result of a high constitutive activity of ventral motor thalamic nuclei, which represent the final output station of the basal ganglia motor circuit.

Material and methods

Animals

Nulliparous female Sprague–Dawley rats, weighing approximately 250 g, were purchased from Charles River (France) and housed under standard conditions with a 12 h light/dark cycle. Females were individually housed overnight with a sexually experienced male rat and vaginal smears were examined on the following morning. The day at which the smear was sperm-positive was considered as embryonic day 0.

Prenatal stress procedure

PRS was carried out according to our standard protocol (Maccari et al., 1995; Morley-Fletcher et al., 2003). Briefly, from day 11 of pregnancy until delivery, pregnant females were subjected to restraint stress in a transparent plastic cylinder and exposed to bright light during three daily sessions of 45 minutes. Control pregnant females were left undisturbed in their home cages and weekly handled. After weaning, only male offspring from litter with a balanced sex ratio were used for the experiments. Animals were housed in groups of three and maintained under similar environmental condition. We used male PRS rats and their unstressed controls of 3-4 months of age. All experiments followed the rules of the European Communities Council Directive 86/609/EEC.
Haloperidol induced-catalepsy

Different groups of PRS and control rats were challenged with a single i.p. injection of haloperidol (Haldol®, Janssen-Cilag) at the doses of 0.5, 1, 2 or 5 mg/kg or raclopride (Sigma, France) at the dose of 2 mg/kg, s.c. Haloperidol and raclopride were dissolved in saline. Control groups used for biochemical measurements or cell counting were injected i.p. with saline. Additional groups of control and PRS rats were treated daily with 0.5 mg/kg of haloperidol for 6 days. Catalepsy was assessed as described by Lipska and Weinberger (1993) by an observer who was unaware of the experimental groups. Starting 30 min following haloperidol injection, both front paws were placed on a 12-cm elevated horizontal plastic bar. The time spent by the rat with both paws on the bar was recorded over 3 min at 30, 60, 90, and 120 min following haloperidol injection.

Apomorphine-induced oral stereotypies

Different groups of PRS and control rats were treated s.c. with 0.3 mg/kg of apomorphine hydrochloride (Sigma Aldrich, France; dissolved in saline containing 0.1% ascorbic acid) and placed in a transparent cylinder for observation of stereotypies. Rats were videotaped for 30 min, and the total time spent in sniffing behavior and the number of episodes of oral stereotypies (licking, biting, or gnawing) were recorded by an observer who was unaware of the experimental groups.

Detection and quantification of haloperidol levels

Control and PRS rats under acute treatment with 2 mg/kg of haloperidol were killed by decapitation at 30, 60 or 120 min following drug injection. Rats treated daily with 0.5 mg/kg of haloperidol for 6 days were sacrificed 60 min after the last injection. Brain was rapidly removed
and the neostriatum was dissected, immediately frozen on dry ice, and stored at -80°C. Blood was also collected and immediately centrifuged at 1500 x g for 15 minutes. Aliquots of serum were stored at -80 °C until analysis. Thirty μl of calibration standard samples, rat serum or striatal samples were added to 150 μl of internal standard (IS) working solution (100 μM in acetonitrile). Samples were vortex-mixed for 10 sec and centrifuged at 14000 x g for 5 min. Fifty μl of clean upper layer were diluted in a vial for the autosampler with 150 μl of 0.1% aqueous formic acid and vortexed for 5 sec. Ten μl were finally injected into the chromatographic system.

**LC-MS/MS analysis**

HPLC analysis was performed using an Agilent Liquid Chromatography System series 1100 (Agilent Technologies, USA) which included a binary pump, an autosampler, a solvent degasser, and a column oven. Chromatographic separation was performed on a reversed-phase column (50x2.0 mm, Luna C18, 5 μm, 100 Å pore size, Phenomenex, CA, USA) equipped with a security guard precolumn (Phenomenex, Torrance, CA) containing the same packing material. The column was maintained at room temperature. The mobile phase consisted of a solution of 0,1% aqueous formic acid (elucent A) and 100% acetonitrile (elucent B); elution was performed at flow rate of 300 μl/min, using an elution gradient as follows: initially 1 min isocratic with 10% solvent B and 2 min linear gradient to 100% solvent B followed by an additional period of 1 min in isocratic conditions and 0.5 min linear gradient to the initial conditions; finally, 3.5 min in the initial conditions was sufficient to allow a subsequent analysis. The injection volume was 10 μl, and the total analysis run time was 8 min. The mass spectrometry method was performed on a 3200 triple quadrupole system (Applied Biosystems, Foster City, Ca) equipped with a Turbo Ion Spray source. The detector was set in the positive ion mode. The ion spray voltage was set at 5000 V and the source temperature was 300° C; the collision activation dissociation (CAD) gas
was set at medium value, and nitrogen was used as collision gas. The Q1 and Q3 quadrupoles were tuned for the unit mass resolution. The transition of the precursor ions to the product ions were monitored with a dwell time of 100 ms for each analyte. The instrument was set in the multiple-reaction monitoring (MRM) mode. Mass spectrometer parameters were optimized to maximize sensitivity for each transition of haloperidol and dansilnorvaline (internal standard) as follows:

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precursor ion (m/z)</th>
<th>Fragments (m/z)</th>
<th>CEP (V)</th>
<th>CE (V)</th>
<th>CXP (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>206.2</td>
<td>21.1</td>
<td>34.3</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>Haloperidol</td>
<td>376.6</td>
<td>194.2</td>
<td>21.1</td>
<td>27.5</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>165.1</td>
<td>21.1</td>
<td>29.3</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>DNSnVal</td>
<td>351.1</td>
<td>170</td>
<td>20.3</td>
<td>26</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Data were acquired and processed by Analyst 1.5.1 Software. A calibration curve was established using different concentrations of haloperidol (15.625, 31.25, 62.5, 125, 250, 500, 1000 ng/ml) dissolved in acetonitrile and processed similarly to serum samples.

**Measurements of catecholamine in rat serum samples**

Catecholamine analysis was performed using the Catecholamine Dual Kit (Eureka srl, Chiaravalle, Italy) according to the manufacturer’s instructions. Briefly, plasma samples were centrifuged at 4°C, distributed in aliquots and stored at -80°C until analysis. Chromatographic separation was carried out on a reversed-phase column (150 x 2.0 mm, Luna C18, 5 μm, 100 Å pore size, Phenomenex, Torrance, CA, USA) equipped with a security guard precolumn (Phenomenex) containing the same packing material. The Liquid Chromatography System was
composed by a binary pump (LabFlow 4000, LabService Analytica, Anzola Emilia, Italy), a
dynamic mixer (811C, Gilson, Middleton, WI, USA), an autosampler (Model 231, Gilson), and a
column oven (LabService Analytica). The column was maintained at room temperature. The
injection volume was 100 μl and the total analysis run time was 20 min. Catecholamines were
detected by a fluorescence detector (ProStar, Varian, Walnut Creek, CA, USA; excitation
wavelength = 360 nm; emission wavelength = 490 nm). Data analysis was performed using the
Star Workstation software version 6.20 (Varian).

Stereological counts of c-Fos+ cells in brain regions of control and PRS rats

The number of c-Fos+ cells was quantified by stereology in the following brain regions: external
globus pallidus (GPe), internal globus pallidus (GPi), subthalamic nucleus (STN), and ventral
motor thalamic nuclei. Cell counts were carried out by the optical fractionator method using a
light microscope (Zeiss Axio Imager M1 microscope) equipped with a motorized stage, a focus
control system (Zeta axis), and a digital video camera coupled to a desktop computer. The
software Image-Pro Plus 6.2 (Media Cybernetics, Inc., Bethesda, MD) for Windows, equipped
with a Macro, was used for the analysis of digital images. The Macro, the characteristics of
which are published (King et al., 2002), was provided by Immagine and Computer (Bareggio,
Italy). The analysis was performed on 20 μm sections sampled every 240 μm. The regions of
interest were highlighted at low magnification (objective 2.5x Zeiss EC Plan-Neofluar), and
counting was performed at high magnification using an objective with a high numerical aperture
(100 x oil immersion objective, Zeiss EC Plan-Neofluar). We used a grid of dissectors (counting
frame) of 100 x 75 μm, with 1.3 as numerical aperture of the lens. Parameters of the stereological
analysis are described in Table 1. The total number of c-Fos+ cells was computed using the
following formula: \( N = S(n) \times 1/SSF \times 1/ASF \times 1/TSF \), where \( n \) is the total number of cells counted on each dissector; SSF (fraction of sections sampled) the number of regularly spaced sections used for counts divided by the total number of sections across the area of interest; ASF (area sampling frequency) the dissector area divided by the area between dissectors; and TSF (thickness sampling frequency) the dissector thickness divided by the section thickness (17 \( \mu m/20 \mu m \)).

**Statistical analysis**

Behavioral and biochemical data were expressed as the mean ± SEM and analyzed by parametric analysis of variance (ANOVA) followed by Newman-Keul’s post-hoc comparison or by Student’s t test. The level of significance was set at \( p<0.05 \).

**Results**

**PRS rats were highly resistant to haloperidol- and raclopride-induced catalepsy**

Adult PRS rats and their unstressed controls were challenged with different doses of haloperidol (0.5, 1, 2, or 5 mg/kg, i.p.) or with a single dose of raclopride (2 mg/kg, s.c.) for the induction of catalepsy. PRS caused a strong reduction of catalepsy at all times following a single injection of haloperidol at doses of 0.5 mg/kg (ANOVA group effect, \( F_{1,8}=7.13 \) \( p<0.05 \)), 1 mg/kg (ANOVA group effect, \( F_{1,8}=17.54 \) \( p<0.01 \)) or 2 mg/kg (ANOVA group effect, \( F_{1,8}=16.13 \) \( p<0.01 \)). Resistance to catalepsy was also seen in response to high doses of haloperidol (5 mg/kg), but only in the first 60 min after injection (Fig. 1A). The selective D2 receptor antagonist, raclopride, also caused catalepsy but with a delayed onset with respect to haloperidol (catalepsy could be
detected at 60, 90, and 120 min, but not at 30 min following raclopride injection). PRS rats were highly resistant to raclopride-induced catalepsy at 60 and 90 min following drug injection (ANOVA group effect, $F_{(1,10)} = 24.07$ $p<0.01$) (Fig. 1B).

We extended the behavioural analysis to groups of control and PRS rats injected daily with 0.5 mg/kg for 6 days. Catalepsy was measured for 120 min following each single injection. Control rats showed sensitization to haloperidol-induced catalepsy, which reached a plateau as early as the second day of injection. Although sensitization was also seen in PRS rats, the extent of catalepsy was lower than in control rats at all days of treatment (Three-way ANOVA; group-time- and day-effect; $F_{(15,144)} = 3.01; p<0.01$) (Fig. 1C).

**Measurements of haloperidol levels in the serum and striatum of control and PRS rats**

We first examined whether the resistance of PRS to catalepsy was due to changes in the metabolism/distribution volume of haloperidol by measuring drug levels in the serum and striatum of control and PRS rats injected with 2 mg/kg of haloperidol. We found no difference in haloperidol levels between control and PRS rats at 30 or 60 min after injection. In contrast, both serum and striatal levels were markedly reduced in PRS rats at 120 min (ANOVA group effect, serum: $F_{(1,4)} = 13.54$ $p<0.05$; striatum: $F_{(1,4)} = 9.82$ $p<0.05$) (Fig. 2A). Thus, changes in the pharmacokinetics of haloperidol could only explain the resistance to catalepsy at 120 min, but not at 30 or 60 min. Serum haloperidol levels did not differ between control and PRS rats treated daily for 6 days with 0.5 mg/kg of haloperidol (Fig. 2B).

**Serum catecholamine levels in PRS rats**

Chopde et al. (1995) have found that glucocorticoids restrain haloperidol-induced catalepsy in rats by enhancing catecholamine release from the adrenal medulla (Chopde et al., 1995). Hence,
we measured norepinephrine and epinephrine levels in control and PRS rats 1 hour after injection of saline or 2 mg/kg of haloperidol. Serum catecholamine levels did not differ between control and PRS rats, and haloperidol injection reduced both norepinephrine and epinephrine levels to the same extent in the two groups of rats. Norepinephrine levels (pg/ml): unstressed/saline = 581 ± 67; PRS/saline = 495 ± 78; unstressed/haloperidol = 261 ± 33; PRS/haloperidol = 303 ± 58. Adrenaline levels (pg/ml): unstressed/saline = 1835 ± 317; PRS/saline = 1647 ± 218; unstressed/haloperidol = 828 ± 107; PRS/haloperidol = 895 ± 93.

**Increased apomorphine-induced stereotypies in PRS rats**

Knowing that haloperidol-induced catalepsy is the behavioural outcome of dopamine (DA) receptor blockade in the neostriatum, we examined how PRS rats responded to pharmacological activation of DA receptors. Motor stereotypies were recorded in control and PRS rats for 30 min following s.c. injection with 0.3 mg/kg of the DA receptor agonist, apomorphine. In control rats, this dose of apomorphine induced sniffing behavior and virtually no episodes of oral dyskinesias (licking, biting, or gnawing). PRS rats showed an increased number of episodes of oral dyskinesias in response to 0.3 mg/kg of apomorphine (t = 2.56; p<0.05) (Fig. 3). No changes in sniffing behaviour were detected between control and PRS rats treated with 0.3 mg/kg of apomorphine (not shown).

**Functional mapping of the basal ganglia motor circuit: increased activity of ventral motor thalamic neurons in PRS rats**

Haloperidol-induced catalepsy reflects a reduced activity of ventral motor thalamic nuclei that are under the control of the “indirect” and “direct” pathways of the basal ganglia motor circuit. We performed a stereological counting of c-Fos protein-expressing neurons in different stations of
the basal ganglia motor circuit in an attempt to identifying the anatomical site of origin of the resistance to haloperidol-induced catalepsy in PRS rats. In a first set of experiments, we used untreated control and PRS rats to measure the constitutive activity of the circuit. The number of c-Fos\(^+\) neurons did not differ between control and PRS rats in the external globus pallidus and subthalamic nucleus (Fig. 4A). Remarkably, untreated PRS rats showed a two-fold increase in neuronal activity in ventral motor thalamic nuclei (ventral anterior -VA-, ventrolateral -VL-, and ventromedial -VM- nuclei), as compared to untreated control rats (t = 5.37; p<0.01) (Fig. 4A). Untreated PRS rats also showed a mild reduction in the number of c-Fos\(^+\) neurons in the internal globus pallidus (t = 3.05; p<0.05) (Fig. 4A). In a second set of experiments, we restricted the analysis to the VM thalamic nucleus of control and PRS rats receiving a single i.p. injection with saline or haloperidol (2 mg/kg). Animals were killed 1 hour after the injection. Haloperidol injection reduced the number of c-Fos\(^+\) neurons in both groups of rats. However, the number of c-Fos\(^+\) neurons in PRS rats treated with haloperidol was similar to that found in control rats treated with saline (treatment effect, F\(_{1,12}\) = 21.32; p<0.01; group effect, F\(_{1,12}\) = 14.57; p<0.05) (Fig. 4B). Stereological counting of Fos\(^+\) neurons in the VM thalamic nucleus was also carried out in control and PRS rats treated daily for 6 days with 0.5 mg/kg of haloperidol. Fig. 4C shows that the difference between control and PRS rats was maintained following multiple haloperidol injections (t = 2.62; p<0.05). These data suggest that PRS causes a long-lasting increase in the activity of ventral motor thalamic nuclei, thereby protecting against haloperidol-induced catalepsy.
Discussion

PRS in rodents causes a series of neuroplastic changes, which might be considered as "maladaptive", and recapitulate some of the features of anxious/depressive disorders and schizophrenia (Maccari *et al.*, 1995; Morley-Fletcher *et al.*, 2003; Marrocco *et al.*, 2012; Matrisciano *et al.*, 2012a,b). Here, we have shown that adult PRS rats are highly resistant to catalepsy induced by haloperidol, one of the most widely used drugs in the treatment of schizophrenia. Catalepsy is a condition characterized by immobility, muscular rigidity, and fixity of posture regardless of external stimuli. Haloperidol-induced catalepsy in rodents mainly results from the blockade of D2 DA receptors in the neostriatum, and is attenuated by antiparkinsonian drugs, such as muscarinic cholinergic or A$_{2A}$ adenosine receptor antagonists, agonists/positive allosteric modulators of type-4 metabotropic glutamate (mGlu4) receptors, and negative allosteric modulators of mGlu5 receptors (Mandhane *et al.*, 1997; Ushijima *et al.*, 1997; Moo-Puc *et al.*, 2003; Ossowska *et al.*, 2002; Niswender *et al.*, 2008; Gonzàlez-Lugo *et al.*, 2010; Goudet *et al.*, 2012; Jones *et al.*, 2012; Xiang *et al.*, 2012; Bennouar *et al.*, 2013). Thus, haloperidol-induced catalepsy in rodents models antipsychotic drug-induced parkinsonism in humans.

An accelerated clearance of haloperidol could explain the resistance of PRS rats to catalepsy only at 120 min, when drug levels were lower in PRS than in control rats. PRS rats already showed a substantial reduction in haloperidol-induced catalepsy at 30 and 60 min, when blood and striatal haloperidol levels were unchanged. We also measured catalepsy in control and PRS rats treated daily for 6 days with 0.5 mg/kg of haloperidol. Control rats showed sensitization to haloperidol-induced catalepsy, in agreement with previous results (Barnes *et al.*, 1990). PRS rats also showed behavioral sensitization, but the severity of catalepsy was always lower than that of the control rats, although blood steady-state levels of haloperidol did not differ between the two groups of
animals. These findings suggest that PRS causes enduring neuroplastic changes within the basal ganglia motor circuit, which alters motor responses involving striatal DA receptors. Accordingly, PRS rats also showed an enhanced behavioral response to apomorphine, a drug that activates DA receptors in the striatum and is currently used in the treatment of Parkinson’s disease. Systemic administration of apomorphine in rats causes a dose-dependent escalation of motor effects, leading to oral dyskinesias at high doses (usually >0.5 mg/kg, s.c.) (Di Chiara and Gessa, 1978; Melzacka et al., 1978). Remarkably, only PRS rats showed oral dyskinesias in response to 0.3 mg/kg of apomorphine.

The basal ganglia are an interconnected group of subcortical nuclei that control motor behavior. The primary input nucleus of the basal ganglia is the neostriatum (caudate nucleus and putamen), which receives afferent dopaminergic fibers from the substantia nigra pars compacta (SNpc). The primary output nuclei are the substantia nigra pars reticulata (SNpr) and the GPi. Striatal GABAergic projection neurons send signals to these output nuclei both directly and indirectly through the GPe and STN (reviewed by Conn et al., 2005). The GPi and SNpr exert an inhibitory control over ventral motor thalamic nuclei (VA, VL and VM), which, in turn, convey the motor programming to the cerebral cortex. The GPi and SNpr are inhibited by striatal projection neurons of the direct pathway and activated by excitatory neurons of the STN, which is the terminal station of the indirect pathway. The direct pathway is activated by D1 receptors, whereas the indirect pathway is inhibited by D2 receptors (reviewed by Conn et al., 2005). Thus, dopaminergic denervation or D1/D2 receptor blockade increases the activity of GPi/SNpr, with ensuing inhibition of ventral motor thalamic nuclei and reduced motor behavior. We measured the number of c-Fos+ neurons in the GPe, GPi, STN, and ventral motor thalamic nuclei to map the function of the basal ganglia motor circuit in control and PRS rats. The pattern of c-Fos expression provides cellular resolution of neuronal activity because c-Fos is expressed when
neurons fire action potentials (reviewed by Dragunow and Faull, 1989; Kovács, 1998). We were surprised to find that the constitutive neuronal activity in ventral motor thalamic nuclei was two-fold greater in PRS rats than in control rats. Haloperidol treatment reduced neuronal activation in the VM thalamic nucleus to the same extent in PRS and control rats, but the number of c-Fos+ neurons in PRS rats treated with haloperidol was similar to that measured in unstressed rats treated with saline. These findings are highly significant because ventral motor thalamic nuclei contain exclusively excitatory neurons projecting to the cerebral cortex and are devoid of inhibitory GABAergic interneurons (Sawyer et al., 1991). Thus, the increased number of c-Fos+ neurons in ventral motor thalamic nuclei indicates an overflow of the basal ganglia motor programming to the cerebral cortex in PRS rats. This explains the resistance to haloperidol-induced catalepsy and the enhanced motor response to apomorphine we have found in PRS rats, and is in line with the increased motor response of PRS rats to a novel environment and to cocaine or other psychostimulants (Deminière et al., 1992; Henry et al., 1995; Morley-Fletcher et al., 2004; Koenig et al., 2005; Kippin et al., 2008; Thomas et al., 2009; Mairesse et al., 2012; Hausknecht et al., 2013).

The precise mechanism(s) whereby PRS causes an increased activity of ventral motor thalamic nuclei in the adult life is unknown. The possibility that PRS affects the activity of regions that lie upstream of ventral thalamic nuclei seems unlikely because PRS rats showed no changes in c-Fos+ neurons in the GPe and STN, and only a small reduction in the GPi. As a word of caution, however, the GPe, GPi and STN contain interneurons in addition to projection neurons, and the real significance of c-Fos+ neuronal counting in the overall activity of these nuclei is uncertain. Binding studies with the selective D2 receptor ligand, [3H]nemonapride, have shown a small increase in the density of D2 receptors in the medial caudate/putamen, but not in the lateral caudate/putamen of PRS rats (Adrover et al., 2007). We could not find changes in striatal D1 and
D2 receptor levels by immunoblot analysis in PRS rats (Bouwalerh et al., 2012). Whether PRS affects the expression or function of other receptors that regulate the basal ganglia motor circuit, such as A2A adenosine receptors, NMDA receptors, mGlu5 and mGlu4 metabotropic glutamate receptors, α2-adrenergic receptors or muscarinic cholinergic receptors (reviewed by Conn et al., 2005), remains to be determined. Interestingly, lesions of the ventral hippocampus restrain haloperidol-induced catalepsy (Lipska et al., 1993; 1995), and PRS rats are characterized by a selective reduction of glutamate release in the ventral hippocampus (Marrocco et al, 2012). It is possible that PRS primarily causes neuroadaptive changes in the ventral hippocampus, which are transferred to the thalamus via the mammillo-thalamic tract or other connecting pathways.

In conclusion, we have shown that prenatal stress in rats causes a strong refractoriness to haloperidol-induced catalepsy, which is associated with an increased neuronal activity in ventral motor thalamic nuclei. These data raise the interesting possibility that early life stress affects the tolerability profile of classical antipsychotics in humans. The potential relevance of our findings to antipsychotic medication is strengthened by the evidence that resistance to haloperidol-induced catalepsy was maintained in response to multiple injections of the drug. We also wish to highlight that blood steady-state levels of haloperidol in response to repeated injections with 0.5 mg/kg fall within the proposed therapeutic window in humans (between 4 and 55 ng/ml) (reviewed by Van Putten et al., 1991). This dose roughly corresponds to less than 0.1 mg/kg in humans (i.e., less than 7 mg in a non obese schizophrenic patient) using the following dose translation formula between rats and humans: human equivalent dose = rat dose (mg/kg) multiplied by 6 (rat Km factor) divided by 37 (human Km factor) (Reagan-Shaw et al., 2008). Our findings may lay the groundwork for the study of how early life stressful event influence antipsychotic drug-induced parkinsonism in humans.
Authorship Contributions

*Participated in research design:* Marrocco, Maccari, Morley-Fletcher, Nicoletti

*Conducted experiments:* Marrocco, Mairesse, Battaglia, Bucci, Consolazione, Ravasi, Lionetto, Simmaco

*Data analysis:* Marrocco, Mairesse

*Wrote and contributed to manuscript:* Marrocco, Maccari, Nicoletti
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Footnotes

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Figures legend

**Fig. 1** - Prenatally stressed adult rats are resistant to haloperidol- and raclopride-induced catalepsy.

Measurements of catalepsy in control and PRS rats treated acutely with a single injection of haloperidol (0.5, 1, 2, or 5 mg/kg, i.p.) (A) or raclopride (B), or treated daily for 6 days with 0.5 mg/kg of haloperidol (C). Values with 0.5 mg/kg in (A) are the same as in (C) at the first day of injection. Catalepsy was assessed at 30, 60, 90, and 120 min following each drug injection. Values are means ± S.E.M. of 5-6 animals per group. *p<0.05 vs. the respective controls. Values in (C) *p<0.01 (Three-way ANOVA for repeated measures).

**Fig. 2** - Measurements of haloperidol levels in control and PRS rats.

In (A), rats were treated with 2 mg/kg of haloperidol, and drug levels were measured in the serum and neostriatum after 30, 60, and 120 min. In (B) treated daily for 6 days with 0.5 mg/kg of haloperidol and drug levels were measured in the serum 60 min after the last injection. Drug levels were measured in the same animals used for the assessment of catalepsy. Values are means ± S.E.M. of 5 determinations. *p<0.05 vs. the respective controls (Student’s t test).

**Fig. 3** - Enhanced motor response to apomorphine in prenatally stressed rats

Control and PRS rats received a single s.c. injection of apomorphine at the dose of 0.3 mg/kg. Oral stereotypies (licking, biting, and gnawing) were recorded for 30 min. Values are means ± S.E.M. of 8 determinations. *p<0.05 vs. the respective controls (Student’s t test).
**Fig. 4** - Increased number of c-Fos$^+$ neurons of ventral motor thalamic nuclei in prenatally stressed rats.

Stereological counts of c-Fos$^+$ neurons in the external globus pallidus (GPe), subthalamic nucleus (STN), internal globus pallidus (GPI), and ventral motor thalamic nuclei (VA, VL, and VM) in untreated control and PRS rats are shown in (A), where values are means ± S.E.M. of 4 determinations. *p<0.05 vs. the respective controls (Student’s t test). In (B), control and PRS rats were treated with saline or 2 mg/kg of haloperidol and killed after 1 hour. Values are means ± S.E.M. of 4 determinations. p<0.05 vs. the respective controls (*) or vs. the respective groups treated with saline (#) (Two-way ANOVA for repeated measures + Newman-Keuls). Representative images of c-Fos$^+$ neurons in the VM thalamic nucleus of control and PRS rats treated with haloperidol are shown. The area inside the dotted lines is shown at higher magnification. In (C), c-Fos$^+$ neurons were counted in the VM thalamic nucleus of control and PRS rats treated daily for 6 days with 0.5 mg/kg of haloperidol. Values are means ± S.E.M. of 4 animals per group. *p<0.05 vs. controls.
Figure 2

A

serum
(2 mg/kg – single injection)

Levels of haloperidol (ng/ml)

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B

serum
(0.5 mg/kg – daily injection – 6 days)

Levels of haloperidol (ng/ml)

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Figure 3

0.3 mg/kg apomorphine

Episodes of oral stereotypies

CONT

PRS

*
Figure 4

A

Number of c-Fos+ neurons

GPe  STN  GPI  VM+Vim

CONT  PRS

B

Number of c-Fos+ neurons

saline  2mg/kg halo

2mg/kg haloperidol

C

Number of c-Fos+ neurons

0.5mg/kg/6days halo

VM thalamic nucleus

CONT  PRS