

RESEARCH ARTICLE

Pioglitazone abolishes cognition impairments as well as BDNF and neurotensin disturbances in a rat model of autism

Thiago B. Kirsten^{1,2,*}, Renato C. Casarin³, Maria M. Bernardi^{2,3} and Luciano F. Felício¹

ABSTRACT

We have shown that exposure of rats to lipopolysaccharide (LPS) during gestation induces autistic-like behaviors in juvenile offspring and pioglitazone post treatment corrects social and communication deficits. The first objective of the present study was to evaluate the cognition of the rats, because this is also a behavioral sphere committed in autism. Second, biomarkers related to pioglitazone pathways and autism were studied to try to understand their mechanisms. We used our rat model of autism and pioglitazone was administered daily to these young offspring. T-maze spontaneous alternations tests, plasma levels of brain-derived neurotrophic factor (BDNF), beta-endorphin, neurotensin, oxytocin, and substance P were all studied. Exposure of rats to LPS during gestation induced cognitive deficits in the young offspring, elevated BDNF levels and decreased neurotensin levels. Daily postnatal pioglitazone treatment abolished cognition impairments as well as BDNF and neurotensin disturbances. Together with our previous studies, we suggest pioglitazone as a candidate for the treatment of autism, because it improved the responses of the three most typical autistic-like behaviors. BDNF and neurotensin also appeared to be related to the autistic-like behaviors and should be considered for therapeutic purposes.

KEY WORDS: Prenatal, Maternal immune activation, T-maze spontaneous alternations test, LPS, Neuropeptides

INTRODUCTION

Autism spectrum disorder (ASD) is a developmental disorder that is characterized by social, communicative and cognitive deficits and has a higher prevalence in males (DSM-IV, 1994). The incidence of autism is considered high in the population all over the world: about 1 in 59 children have been identified with ASD (Baio et al., 2018). Although it is considered that the etiology of autism is unknown, genetic and environmental factors (such as prenatal infections and maternal dietary disturbs) have been described as autistic triggers (Herbert, 2010; Johnson et al., 2013; Theoharides et al., 2009).

We have shown that exposure of rats to lipopolysaccharide (LPS, gram-negative bacteria endotoxin) during gestation [gestational day

(GD) 9.5] induces socialization and communication deficits as well as repetitive/restricted behavior in juvenile offspring. These behavioral impairments are found just in males and not in the female offspring (Kirsten et al., 2010a, 2012; Kirsten and Bernardi, 2017). Therefore, our model of exposure of rats to LPS during gestation induces autistic-like behaviors in juvenile offspring (Kirsten et al., 2012). We have also shown peripheral interleukin (IL)-1 beta and IL-6 levels increase in adult and young male offspring, respectively (Kirsten et al., 2013, 2018); results that have been described in autistic patients (Li et al., 2009; Jyonouchi et al., 2001; Al-Ayadhi, 2005). Additionally, the mechanisms that are involved in fetal brain disturbances include the production and release of proinflammatory cytokines within the maternal environment (circulation and placenta) (Ashdown et al., 2006; Urakubo et al., 2001; Cai et al., 2000).

There is no effective treatment for ASD, and a small number of drugs are authorized by the US Food and Drug Administration (FDA) (McPheeters et al., 2011). These drugs have limited efficacy and are able to treat only some ASD symptoms and can trigger adverse effects (Wink et al., 2010). Therefore, the present study proposed to use our rat model of autism to find new pharmacological approaches for autism. Pioglitazone was selected as the postnatal treatment to inhibit or relieve the behavioral and immune disturbances induced after the exposure of rats to LPS during gestation. Pioglitazone belongs to the thiazolidinediones class, which act as peroxisome proliferator-activated receptor gamma (PPAR γ) agonists (Gillies and Dunn, 2000; Berger and Wagner, 2002). Pioglitazone is an anti-diabetic drug that is authorized by the FDA to treat type 2 diabetes mellitus (insulin sensitizing effect) (Hanefeld and Belcher, 2001). Besides the anti-diabetic affect, thiazolidinediones has anti-inflammatory properties and has been suggested to treat inflammatory and neurological diseases such as inflammatory bowel disease, psoriasis, atherosclerosis, multiple sclerosis and Alzheimer's (Duval et al., 2002; Katayama et al., 2003; Bongartz et al., 2005; Feinstein, 2003; Landreth, 2006).

There are pilot studies showing that pioglitazone treatment associated with other pharmacological and behavioral therapies induce some clinical improvements in autistic patients. For example, daily pioglitazone treatment attenuated irritability, lethargy, stereotypy and hyperactivity in some autistic children, without significant side effects (Boris et al., 2007). Similarly, pioglitazone potentiated the risperidone effects in ASD patients (Ghaleiha et al., 2015).

Using our rat model of autism, we have demonstrated that pioglitazone treatment corrects social and communication deficits as well as elevated plasma IL-6 levels (Kirsten et al., 2018). The first objective of the present study was to evaluate the cognition of the rats, because this is also a behavioral sphere committed in autism. Second, biomarkers related to pioglitazone pathways and ASD were studied to try to understand their mechanisms.

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RESULTS

Fig. 1 shows the effects of prenatal LPS and postnatal pioglitazone exposure on the T-maze spontaneous alternation test of the rats. The performance in the T-maze task was different between groups (KW=13.48, $P=0.0037$). Prenatal LPS exposure decreased T-maze spontaneous alternation in the offspring compared with the control group ($P<0.01$). Although postnatal pioglitazone treatment with 0.25 mg/kg/day did not affect the performance in the T-maze task, postnatal pioglitazone treatment with 1.0 mg/kg/day increased spontaneous alternation in the rats prenatally exposed to LPS (LPS+PI1.0 group versus LPS+DMSO group, $P<0.05$) to the same levels as those in the control group. Thus, prenatal LPS induced cognitive inflexibility, and postnatal pioglitazone treatment abolished these impairments.

Fig. 2 shows the effects of prenatal LPS and postnatal pioglitazone exposure on the total BDNF plasma levels of the rats. The BDNF levels were affected by the treatments [$F(3/28)=5.43$, $P=0.0045$]. Prenatal LPS exposure (LPS+DMSO group) increased the total BDNF levels compared with the control group (SAL+DMSO, $P<0.001$). Both doses (0.25 and 1.0 mg/kg/day) of the post-treatment with pioglitazone decreased BDNF levels in the rats that were prenatally exposed to LPS (versus LPS+DMSO group, $P<0.05$ for both doses) to the same levels as those in the control group.

Fig. 3 shows the effects of prenatal LPS and postnatal pioglitazone exposure on the beta-endorphin, neurotensin, oxytocin and substance P plasma levels of the rats. Both beta-endorphin and neurotensin levels were affected by the treatments [$F(3/28)=3.15$, $P=0.0407$ and $F(3/28)=8.41$, $P=0.0004$, respectively], whereas oxytocin and substance P levels were not affected by the treatments [$F(3/28)=2.34$, $P=0.0944$ and $F(3/28)=2.58$, $P=0.0735$, respectively]. Prenatal LPS exposure (LPS+DMSO group) did not affect beta-endorphin levels compared with the control group (SAL+DMSO). Both pioglitazone doses (LPS+PI0.25 and LPS+PI1.0 groups) increased the beta-endorphin levels compared with the LPS group (versus LPS+DMSO, $P<0.05$ and $P<0.01$, respectively). Prenatal LPS exposure increased neurotensin levels compared with the control group (SAL+DMSO, $P<0.01$). Although postnatal pioglitazone treatment with 0.25 mg/kg/day did

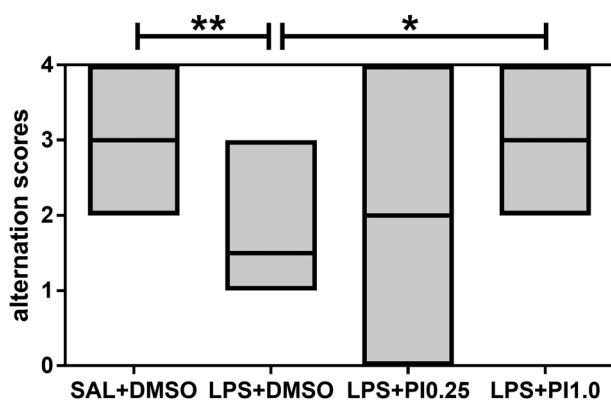


Fig. 1. T-maze. The effects of prenatal LPS (100 µg/kg at GD 9.5) and postnatal pioglitazone [0.25 and 1.0 mg/kg/day between postnatal days (PND) 21 and 29] exposures on the T-maze spontaneous alternation test in juvenile male rat offspring. SAL+DMSO, prenatal saline injection and postnatal daily DMSO injection; LPS+DMSO, prenatal LPS injection and postnatal daily DMSO injection; LPS+PI0.25, prenatal LPS injection and postnatal pioglitazone 0.25 mg/kg/day; LPS+PI1.0, prenatal LPS injection and postnatal pioglitazone 1.0 mg/kg/day ($n=8$ rats/group). * $P<0.05$, ** $P<0.01$ (Kruskal–Wallis test followed by Dunn's test). The data are expressed as the median (minimum and maximum).

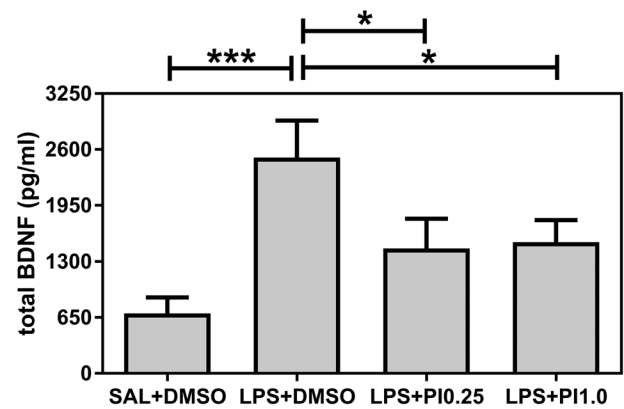


Fig. 2. BDNF. The effects of prenatal LPS (100 µg/kg at GD 9.5) and postnatal pioglitazone (0.25 and 1.0 mg/kg/day between PND 21 and 29) exposures on the total BDNF plasma levels in juvenile male rat offspring. SAL+DMSO, prenatal saline injection and postnatal daily DMSO injection; LPS+DMSO, prenatal LPS injection and postnatal daily DMSO injection; LPS+PI0.25, prenatal LPS injection and postnatal pioglitazone 0.25 mg/kg/day; LPS+PI1.0, prenatal LPS injection and postnatal pioglitazone 1.0 mg/kg/day ($n=8$ rats/group). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (one-way ANOVA followed by the Fisher's LSD test). The data are expressed as the mean±s.e.m.

not affect the neurotensin levels compared with the LPS group it recovered the levels to the same levels as those in the control group. Postnatal pioglitazone treatment with 1.0 mg/kg/day increased the neurotensin levels in the rats prenatally exposed to LPS (LPS+PI1.0 group versus LPS+DMSO group, $P<0.0001$) to the same levels as those in the control group. Thus, prenatal LPS decreased neurotensin levels, and postnatal pioglitazone treatment recovered this disturbance.

DISCUSSION

The most typical symptoms found in ASD are social, communicative and cognitive deficits, such as cognitive inflexibility and repetitive/restricted behavior (DSM-IV, 1994). We have shown that exposure of rats to LPS during gestation impairs communication in terms of ultrasonic vocalization (40 and 50 kHz), socialization (play behavior), induces repetitive/restricted behavior in terms of self-grooming and cognitive inflexibility (T-maze) in male offspring (Kirsten et al., 2010a, 2012, 2015a,b; Kirsten and Bernardi, 2017). Therefore, our model of exposure of rats to LPS during gestation induces autistic-like behaviors in juvenile offspring.

Considering no effective treatment yet exists for autism, we started to search for new treatments for autistic symptoms using our rat model. We selected pioglitazone because although it is originally an anti-diabetic drug, it also exerts anti-inflammatory effects in several cell types (Berger and Wagner, 2002; Hanefeld and Belcher, 2001). Moreover, there are pilot studies showing that pioglitazone treatment associated with other pharmacological and behavioral therapies induces some clinical improvements in autistic patients (Boris et al., 2007).

We have demonstrated that pioglitazone treatment corrects social and communication deficits (Kirsten et al., 2018). Our present results revealed that postnatal pioglitazone treatment with 1.0 mg/kg/day abolished the decreased T-maze spontaneous alternation induced by prenatal LPS. Together with our previous study (Kirsten et al., 2018), we suggest pioglitazone as a candidate for the treatment of autism, since it improved the responses of the three most typical autistic-like behaviors.

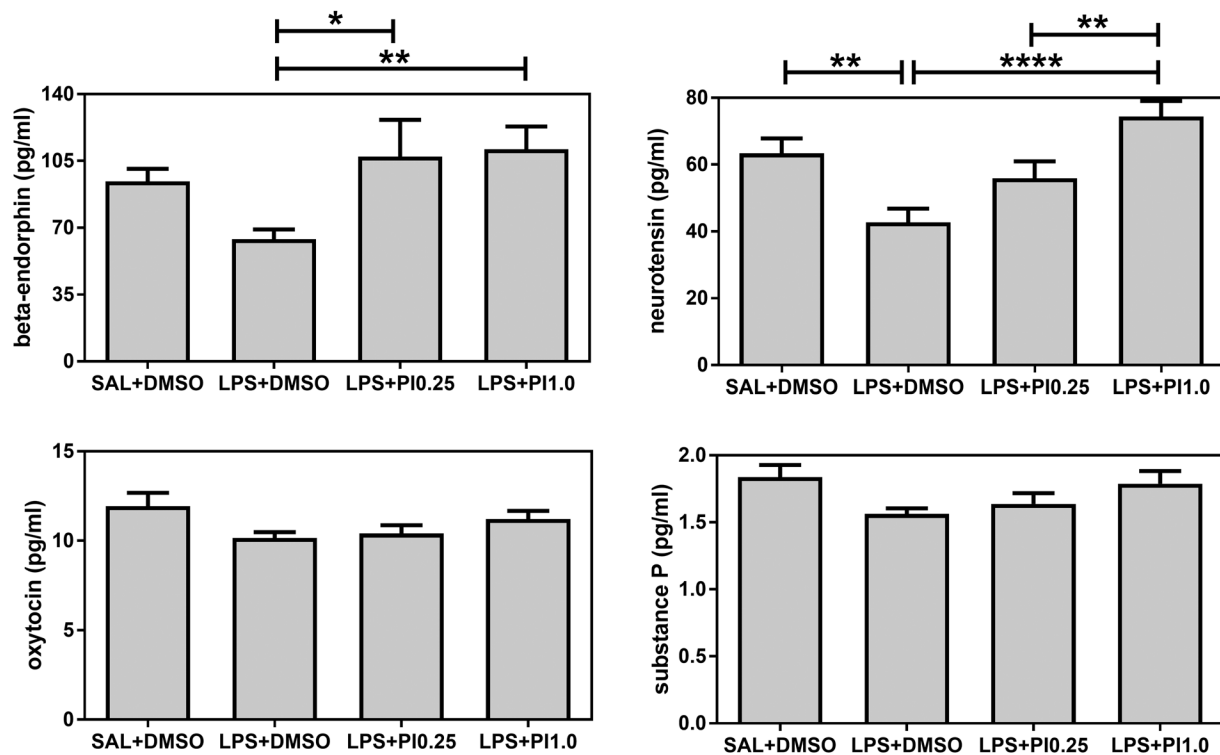


Fig. 3. Neuropeptides. The effects of prenatal LPS (100 µg/kg at GD 9.5) and postnatal pioglitazone (0.25 and 1.0 mg/kg/day between PND 21 and 29) exposures on beta-endorphin, neurotensin, oxytocin and substance P plasma levels in juvenile male rat offspring. SAL+DMSO, prenatal saline injection and postnatal daily DMSO injection; LPS+DMSO, prenatal LPS injection and postnatal daily DMSO injection; LPS+PI0.25, prenatal LPS injection and postnatal pioglitazone 0.25 mg/kg/day; LPS+PI1.0, prenatal LPS injection and postnatal pioglitazone 1.0 mg/kg/day ($n=8$ rats/group). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ (one-way ANOVA followed by the Fisher's LSD test). The data are expressed as the mean±s.e.m.

BDNF is a member of the neurotrophins family and is found in the central nervous system and peripheral blood. It is involved with morphological, developmental and neuronal processes, such as synaptogenesis and synaptic plasticity (Binder and Scharfman, 2004). Several autistic patients present elevated serum (Ricci et al., 2013), plasma (Correia et al., 2010), postmortem brain (Garcia et al., 2012) and neonatal cord blood (Nelson et al., 2001) levels of BDNF. Exposure of rats to LPS during gestation increased BDNF plasma levels. Thus, our rat model of autism also successfully reproduced the BDNF disturbance found in patients.

Both doses of postnatal pioglitazone treatment (0.25 and 1.0 mg/kg/day) abolished the BDNF disturbance induced by prenatal LPS. Although there are few studies about the relation between pioglitazone and BDNF, it is known that memory impairment, BDNF disturbance and oxidative damage induced by β -amyloid in an animal model of Alzheimer's disease are reversed by chronic administration of pioglitazone (Prakash and Kumar, 2014). Moreover, just like pioglitazone, BDNF has an anti-diabetic effect, ameliorating glucose metabolism and pancreatic dysfunction in type 2 diabetic mice (Yamanaka et al., 2007). Therefore, the BDNF disturbance induced by prenatal LPS was abolished by pioglitazone treatment.

Beta-endorphin, neurotensin, oxytocin and substance P plasma levels were studied because of their relationship with autism. There are reports of increased (Tordjman et al., 2009), decreased (Gillberg, 1992; Weizman et al., 1988) and unaltered (Angelidou et al., 2010) plasma/serum levels of beta-endorphin in autistic patients. Neurotensin levels have been found to be increased in the sera of 3-year old (Angelidou et al., 2010) and 6–12 year-old children with autism (Tsiloni et al., 2014). There are several studies reporting that ASD could be a result of oxytocin system disturbances early in life

(Quattrocki and Friston, 2014). Oxytocin levels have been found to be decreased in the saliva of adolescent boys with autism (Bakker-Huvenaars et al., 2018), as well as in the plasma of Chinese autistic patients (Zhang et al., 2016). However, another group of autistic children and adolescents exhibited elevated serum oxytocin levels (Yang et al., 2017). Finally, substance P is also considered a possible factor in ASD because it is a product of the *Tachykinin 1* gene (*TAC1*), which is located in a candidate region for ASD (Marui et al., 2007). However, both serum (Angelidou et al., 2010) and neonatal blood (Nelson et al., 2001) of children with ASD present similar levels of substance P compared with control subject levels.

The apparent contradictory findings in the literature about the relationship between neuropeptides and autism may be explained by the presumably multifactorial etiopathogenesis of autism. Autism may be a result from a complex interaction between genetic and environmental factors (Posar and Visconti, 2017). Possible triggers include several chromosomal and gene disturbances, air pollutants, pesticides and other endocrine-disrupting chemicals, prenatal infections, mitochondrial dysfunction, electromagnetic pollution, diet modifications and possible epigenetic mechanisms (Posar and Visconti, 2017; Tordjman et al., 2014; Landrigan, 2010; Larsson et al., 2005).

Apparently, prenatal LPS exposure did not affect beta-endorphin, oxytocin and substance P plasma levels. Thus, the induction of autistic-like effects after the exposure of rats to LPS during gestation does not appear to be related to these neuropeptide pathways. However, prenatal LPS decreased neurotensin levels and postnatal pioglitazone treatment with 1.0 mg/kg/day abolished this disturbance.

There are a few studies about neurotensin and autism, and they report that autism increases neurotensin serum levels in

children with autism (Angelidou et al., 2010; Tsilioni et al., 2014). We would expect that prenatal LPS should increase neurotensin levels because neurotensin may act like a neurotoxic factor and stimulating microglia, which activates the mammalian target of rapamycin (mTOR) signaling kinase and stimulates IL-1 beta gene expression (Patel et al., 2016; Ghanizadeh, 2010). Speaking of which, we have shown that prenatal LPS increases both striatal mTOR (Kirsten et al., 2015a) and serum IL-1 beta (Kirsten et al., 2013) levels in rats.

Neurotensin is widely distributed in the nervous system and peripherally, with neurotransmitter or neuromodulator roles (Vincent et al., 1999), including for cognition (Dobner, 2005). PD149163 administration, which is a neurotensin NTS1-receptor agonist, improves memory performance in Norway rats (Keiser et al., 2014). It is also described that activation of neurotensin receptor 1 has beneficial actions in a mouse model of Alzheimer's disease (Xiao et al., 2014). Considering that neurotensin activation is involved in cognition improvement, it seems reasonable to understand our findings of prenatal LPS reducing neurotensin levels and consequently impairing behavioral parameters of cognition (T-maze).

Moreover, the neurotensin reduction after prenatal LPS may be related to a dopaminergic disturbance. We previously reported that our rat model induces striatal dopaminergic disturbances in the offspring, such as decreased tyrosine hydroxylase expression as well as dopamine and metabolite level reduction (Kirsten et al., 2012, 2010b) as well as dopaminergic hypoactivity in the hypothalamus (Kirsten and Bernardi, 2017). Experimental evidence indicates intimate anatomical and biochemical relationships between the neurotensinergic and the dopaminergic systems (Binder et al., 2001; Rodríguez de Lores Arnaiz and Antonelli, 2016). Neurotensin and dopaminergic neurons are strongly co-located, and its receptors are expressed on both the dopaminergic neurons and on their postsynaptic targets (Muehlmann et al., 2018). Therefore, the neurotensin disturbance induced by prenatal LPS occurred possibly in response to the central dopaminergic impairment.

Future studies focusing on the central nervous system, especially for brain neurotensin levels and NTS1 and NTR3/sortilin receptors would be interesting for a better understanding of the mechanisms involved with ASD and neurotensin, including as a potential therapeutic target for autism.

In conclusion, exposure of rats to LPS during gestation (GD 9.5) induced cognitive inflexibility and elevated BDNF levels in juvenile male rat offspring, which are described as autistic-like symptoms. We also found reduced plasma neurotensin levels after prenatal LPS exposure. Daily postnatal pioglitazone treatment corrected the cognition impairments, as well as BDNF and neurotensin disturbances. Together with our previous studies (Kirsten et al., 2018), we suggested pioglitazone as a candidate for the treatment of autism, because it improved the responses of the three most typical autistic-like behaviors. BDNF and neurotensin also appeared to be related with autistic-like behaviors and should be considered for therapeutic purposes.

MATERIALS AND METHODS

Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the School of Veterinary Medicine, University of São Paulo, Brazil (permit no. 2824/2012). All efforts were made to minimize the suffering, reduce the number of animals used and utilize alternatives to *in vivo* techniques when available. The experiments

were performed in accordance with good laboratory practice protocols and quality assurance methods.

Animals

Nineteen pregnant Wistar rats (*Rattus norvegicus*) of 15–17 weeks of age and weighing 220–275 g were used. The rats' housing, nutritional conditions, determination of GD 0, pregnancy monitoring, weaning and litter sexing during the experiments were the same as previously described by our group (Kirsten et al., 2012, 2018).

Prenatal treatments

LPS (from *Escherichia coli*; Sigma-Aldrich; serotype 0127: B8) was dissolved in sterile saline (50 µg/ml LPS in a 0.9% NaCl solution) and administered intraperitoneally (i.p.) to pregnant dams at a dose of 100 µg/kg on GD 9.5 ($n=13$). This GD of LPS exposure as well as the dose was chosen based on our previous studies (Kirsten et al., 2010a,b, 2013). Other dams received the vehicle (0.9% sterile saline, SAL) on GD 9.5 ($n=6$). Each dam (LPS and control) received 0.2 ml/100 g of each solution (LPS and vehicle solutions).

Postnatal treatments and groups

The male offspring which were prenatally exposed to LPS (or SAL) also received pioglitazone (Sigma-Aldrich) or its vehicle dimethyl sulfoxide (DMSO, diluted to 1:10 in SAL) daily from PND 21 until 29. Rats received one of two pioglitazone doses: 0.25 and 1.0 mg/kg/day (i.p.) based on the pharmacokinetics of the drug (Actos, Abbott, Rio de Janeiro, Brazil), the autistic children's prescriptions (Boris et al., 2007) and rat studies (Park et al., 2007; Zhang et al., 2011; Alzamendi et al., 2012). The prepubertal period of the daily treatment was also based on an autistic children's study (Boris et al., 2007) and the equivalent age between humans and rats (Quinn, 2005).

Four groups were investigated ($n=8$ rats per group): the SAL+DMSO group (control group), the LPS+DMSO group (LPS group), the LPS+PI0.25 group and the LPS+PI1.0 group. The experimental groups are described in Table 1.

T-maze

One of the most typical symptoms of ASD is cognitive inflexibility (DSM-IV, 1994). To evaluate cognitive inflexibility, we used a T-maze; the T-maze specifications and behavioral protocol that were used during the experiments were the same as previously described by our group (Kirsten et al., 2015a; Cezar et al., 2018). The parameter analyzed was the alternation between the left and right arms after the five sessions. These data were transformed into scores: 0–4, i.e., from no alternations to four alternations.

Plasma evaluations

On PND 36–40, the rats that were previously evaluated for their behavior were decapitated and plasma samples were obtained as previously described by our group (Moraes et al., 2017). BDNF (cat. no. G7610, Promega) analysis was performed using commercial enzyme-linked immunosorbent assay kits. Neuropeptides analysis, which included the analysis of beta-endorphin, neurotensin, oxytocin and substance P, was performed using Luminex/Magpix/Milliplex system (cat. no. RMNPMAG-83K, Millipore).

Statistical analysis

Homogeneity and normality were verified using a Bartlett's test or *F* test. For the T-maze score analysis, a Kruskal–Wallis test was used, followed by a

Table 1. Experimental groups

Group	Prenatal administration (GD 9.5; i.p.)	Postnatal administration (PND 21–29; i.p.)
SAL+DMSO	saline	DMSO
LPS+DMSO	LPS	DMSO
LPS+PI0.25	LPS	pioglitazone 0.25 mg/kg/day
LPS+PI1.0	LPS	pioglitazone 0.25 mg/kg/day

Description of the four experimental groups, including the prenatal administrations on GD 9.5 and the daily postnatal administrations between PND 21 and 29 in juvenile male rat offspring ($n=8$ rats/group).

Dunn's test. One-way analysis of variance (ANOVA) followed by Fisher's LSD multiple comparison tests were used to compare the parametric data between the four groups. The results are expressed as the median (minimum and maximum) or the mean \pm s.e.m. In all cases, the results were considered as statistically significant at $P<0.05$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.B.K., M.M.B., L.F.F.; Methodology: T.B.K., R.C.C.; Validation: T.B.K.; Formal analysis: T.B.K., R.C.C.; Investigation: T.B.K.; Resources: T.B.K., M.M.B.; Data curation: T.B.K.; Writing - original draft: T.B.K.; Writing - review & editing: T.B.K., R.C.C., M.M.B., L.F.F.; Visualization: T.B.K., R.C.C., M.M.B., L.F.F.; Supervision: L.F.F.; Project administration: T.B.K.; Funding acquisition: T.B.K., M.M.B.

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