Morphine exposure and maternal deprivation during the early postnatal period alter neuromotor development and nerve growth factor levels

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**ABSTRACT**

The objective of this study was to verify whether repeated morphine administration and maternal deprivation in early life alter neurobehavioral development and central nerve growth factor (NGF) levels. A total of 58 male Wistar rat pups were used in our study. From postnatal day 1 (P1), litters were daily deprived of their mother for 3 h; this was continued for the first 10 days of life. Animals were divided into 5 groups: total control (C), did not receive any intervention; saline (S), received saline solution; morphine (M), received morphine; deprived-saline group (DS), were subjected to maternal deprivation and received saline solution; and deprived-morphine (DM), were subjected to maternal deprivation and received morphine. From P8, newborns received subcutaneous (s.c.) injections of morphine or saline (5 \( \mu \)g) once daily for 7 days. Righting reflex, negative geotaxis and gait were chosen as postural parameters to evaluate neuromotor reflexes. In the righting reflex test, a delay in the development of animals was evidenced in the M group. Performance of negative geotaxis was slower in the M and DM groups. In the gait test, all groups showed a daily improvement in performance in terms of locomotion frequency. An increased frequency of rearing was observed in the M, DS, and DM groups from P16 to P20. The DM group presented an increase in NGF levels in the brainstem. An increase in cerebral cortex NGF levels in the M, DS, and DM groups was observed as well. Our results suggest that changes in environmental conditions and the disruption of mother–infant interactions during the neonatal period can produce changes in the neurobiology, physiology, and emotional behavior of rats. This finding has important implications for the maternal-neonate interaction needed for normal brain development in newborns.

**Keywords:** Morphine; Maternal deprivation; Neonate rats; Development; Neurotrophin

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1. Introduction

Morphine treatment in early life is widely used in neonatal pain management. Neonates (birth to 1 month), infants (1 month to 2 years), and children (2–12 years) often experience pain from invasive procedures during intensive care, and during the post-operative period (Pattinson and Fitzgerald, 2004). The use of opioid analgetics, such as morphine, has increased in neonatal intensive care units over the last few decades, as a consequence of changes and advances in the understanding, identification, and treatment of pain in children (El Sayed et al., 2007; Beggs, 2015). However, it has been demonstrated in clinical and pre-clinical studies that prenatal and postnatal exposure to opiates can have devastating effects on the development of fetuses and newborns. It may induce long-term neurobehavioral alterations during postnatal maturation, which become more prominent in adulthood (Kalinichev et al., 2002; Slamberová et al., 2005; Stratmann, 2011). Peripheral, spinal, and supra-spinal afferent pain transmission in neonates undergoes considerable maturation during the early postnatal period. They are able to respond to tissue injury with specific behaviors, and with autonomic, hormonal, and metabolic signs of stress and distress (Nandi and Fitzgerald, 2005). Reflex assessment and motor developmental are important tools to evaluate the neurobehavioral
development of children, as their neurological status can be predicted based on the maturation of motor coordination, and cognitive aspects (Bonnier, 2008).

In the context of postnatal stressor events, it is well established that postnatal maternal deprivation is one of the most potent stressors during development and can cause long-lasting neurobiological effects (Benetti et al., 2009; Fuentes et al., 2014; Gracia-Rubio et al., 2016). Exposure to early life stress such as maternal deprivation can trigger a developmental delay, negatively affecting brain development and increasing the risk of the occurrence of behavioral alterations (Macri et al., 2011; Weiss et al., 2011). This is because early experiences occur during a period of extraordinary brain plasticity, when the brain is maximally capable of being programmed in a durable way (Knudsen, 2004). Previous studies have shown that the immaturity and plasticity of the central nervous system (CNS) of children makes them particularly sensitive to stress, which may cause significant changes in the brain structure and function (Rosenblum et al., 2001; Lupien et al., 2009). In addition, studies using rodents and other mammals have shown that alterations of the infant-mother relationship cause long-term changes in the neurobiology and behavior of the offspring (Renard et al., 2005; Uriarte et al., 2007; Benetti et al., 2009). Furthermore, it has been shown that repeated separation of rat pups from their mothers increases behavioral fearfulness, and the hypothalamus–pituitary–adrenal response to stress. There is evidence that maternal deprivation induces long-lasting changes in reactivity to a novel environment, and alters sensitivity to the effects of morphine on locomotor activity in rats (Kalninchev et al., 2002; Farkas et al., 2009).

Early reflexes are automatic responses to stimulation, which form and constitute the basis for motor skills. Indeed, impaired motor skill development may be a predictive factor of behavioral modifications in adulthood. In addition, through neonatal reflex evaluation, it is possible to identify the persistence or absence of developmental delay (Heyser, 2004; Berk, 2006). Thus, primitive reflex activity is the first form of integration between human beings and the environment. Additionally, motor development is a product of refining and remodeling of pre-existing patterns, and it is dependent on the interaction of neural maturation and the self-organizing properties of the sensorimotor system (Kamm et al., 1990; Tecklin, 2008). Studies show that there are many factors that may inhibit normal development in humans (Palomo et al., 2003; Connors et al., 2008) and rodents (Kostrewa et al., 2007; Segovia et al., 2008; Brunton and Russell, 2010; Schuch et al., 2016), including visual and hearing impairment and growth disorders. These effects may result from perinatal and postnatal asphyxia, environmental factors, stress, maternal care, and toxic agent’s exposures. In a previous study of our research group, we showed that chronic maternal exposure to caffeine promotes important alterations in neuromotor development (Souza et al., 2015). Therefore, the development of newborn rats during the first three postnatal weeks follows a general pattern of reflex appearance, and maturation of motor skills (Horvath et al., 2013).

It has been shown that several neurochemical changes occur after exposure to opioids, and different paradigms of maternal separation. These include changes in the mesolimbic dopaminergic system and alterations in the neuropeptidergic expression in the brain (Husum et al., 2002; Farkas et al., 2009), besides a decrease in trophic factors and other plasticity markers in the brain (Burton et al., 2007). Long-term changes in neuronal function might be due to modifications in the expression of neurotransphins, such as nerve growth factor (NGF), which have been shown to promote neuronal survival, differentiation, and function during development (Cirulli et al., 2000). Evidences indicate that the effects of maternal deprivation on NGF expression are stronger with longer separations, and it is not restricted to the hippocampal region (Roceri et al., 2004; Cirulli et al., 2007). Our study advances concerns about possible deleterious consequences of environmental stress during the postnatal period. Especially, concern can be focused on neonatal intensive care units because neonates often experience pain from invasive procedures during intensive care and spend their first several days in a critical care where they may be exposed to repeated stressful events. The types of stressors these neonates and infants may suffer are varied; and include prolonged isolation from human contact, and most importantly the absence of maternal care; besides repeated painful procedures (e.g., injection or intubation). Taken together in this study, we investigated whether repeated morphine administration, and maternal deprivation in early life alters neurobehavioral development, as well as its effect on central NGF levels.

2. Material and methods

2.1. Animals

A total of 58 male, Wistar rat pups were utilized. They were kept in home cages made of polypropylene (49 × 34 × 16 cm) with the floor covered in sawdust, with their mothers, maintained on a standard 12 h dark/light cycle (lights on at 7:00 a.m., and lights off at 7:00 p.m.) in a temperature-controlled environment (22 ± 2 °C), and had access to water and food, ad libitum. All experiments in this study were conducted in male rats because the nociceptive process and drug responses are altered by modulations in the hormonal state (Ribeiro et al., 2005). At birth, male litters were standardized according to Silveira et al. (2010, 2011), with minor modifications, containing 8 male pups per dam. Assessing the development of offspring during lactation is the current procedure of adjusting litter in the first days of birth, in order to homogenize nutritional conditions for all pups (Tanaka, 2004). All experiments and procedures were approved by the Institutional Committee for Animal Care and Use (GPPG/HCPA protocol No. 150614), and conducted in compliance with Brazilian laws (Brazil, 2008; MCTI, 2013a; MCTI, 2013b) and the Laboratory Guide for the Care and Use of Animals. The husbandry of animals followed Law No. 11794 (Brazil), which regulates the scientific use of animals. Vigorous attempts were made to minimize animal suffering, and decrease external sources of pain and discomfort, as well as to use the minimum number of animals required to produce reliable scientific data.

2.2. Maternal deprivation

From postnatal day 1 (P1), litters were deprived of their mother for 3 h daily for the first 10 days of life. Deprivation consisted of removing the mother from the home cage. Two, or at the most, 3 animals per litter were used in each experimental group. Pups were maintained in the original home cage, and grouped in a nest in the presence of maternal odor; they were then transferred to a different room where the temperature was maintained at 30–32 °C to compensate for the mother’s body heat (Renard et al., 2005). We chose this maternal deprivation protocol because it does not require the manipulation of pups (Kosten et al., 2007; Todeschin et al., 2009). Deprivation was carried out between 08:00 a.m. and 11:00 p.m. Non-deprived rats remained undisturbed in the home cage with their mothers. The first bedding was changed only on P11 for both the groups (non-deprived and deprived rats).

2.3. Experimental design

The animals were divided into 5 groups: the total control group (C), which did not receive any intervention; saline group (S), which received saline solution; morphine group (M), which received morphine; deprived saline group (DS), in which pups were subjected to maternal deprivation and received saline solution; and deprived morphine group (DM), in which pups were subjected to maternal deprivation and received morphine. Neurobehavioral testing was performed in a blinded manner, and tests were always performed at the same time, each day (14:00 h). Righting reflex (P8 to P10), negative geotaxis (P8, P10, and P12), and gait (P15 to P20) were chosen to evaluate postural parameters as an index of neuromotor reflexes.
2.4. Pharmacological treatment

Each animal, except for those in the control group, received saline (S and DS groups) or morphine (5 μg s.c. in the mid-scapular area; M and DM groups) starting at P8, which was then continued once daily for 7 days. This dose was chosen on the basis of previous studies by our research group (Rozisky et al., 2008, 2010, 2011, 2012a, 2012b). Rats at P8 were chosen because it is accepted that animals at this age show a stage of neurological development similar to a human newborn (Fitzgerald and Anand, 1993). It is also accepted that they are in a physiologically immature state (Pattinson and Fitzgerald, 2004), since this period is characterized by major developmental changes in the brain and plasticity of the developing pain system (Rabinowicz et al., 1996). Each treatment was administered at the same time each day (11.00 h). One milliliter of morphine sulfate (Dimeflidine, 10 mg/mL, Cristália Ltda., São Paulo, Brazil) was provided by Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil.

2.5. Development of neuromotor reflexes

2.5.1. Righting reflex

The righting reflex test was carried out from P8 to P10. Pups were placed in a supine position, and the latency time to turn over their longitudinal axis to restore a normal prone position was measured. This was considered fully achieved when the pups turned 180° around their longitudinal axis, and their four paws were in contact with the plane surface within the observed 120-s timeframe (Karalis et al., 2011).

2.5.2. Negative geotaxis

The negative geotaxis test consisted of a postural reaction, making the animal face upwards, when placed on a sloped surface facing downwards. The test was carried out at P8, P10, and P12. Rat pups were placed on a 35-cm-long inclined platform (45° slope), facing the downslope. The animals were expected to turn around 180° to face upwards, and climb up the board with their forepaws reaching the upper edge of the board. The results of the test were considered negative if the pups did not succeed in this task within the observed time period of 120-s (Horvath et al., 2013).

2.5.3. Gait

The animals were placed in the center of a 45-cm diameter circle on the 15th to 20th day of lactation, always during the same period (Schuch et al., 2016). Each chosen animal was placed individually in the center of the apparatus, and observed for 3 min. The frequency of movement, and rearing (rearing was defined as the moment the rat rose up on its hind legs, ending when one or both front paws touch the floor again) was observed in this test. The record of the frequency of the parameters was made through manual counting.

2.6. Tissue collection

Rats were killed by decapitation at P21 days of age. After the animal’s decapitation, the brainstem and cerebral cortex were removed, stored in independent slots (without buffer solution), and frozen at −80 °C for future analysis.

2.7. Biochemical assays

The brainstem and cerebral cortex of each rat pup was collected and frozen at −80 °C until the time of testing. The cerebral structures were homogenized with a handheld homogenizer with Protease Inhibitor Cocktail (Sigma #P8340) in the proportion of 1:100 in phosphate buffered saline (PBS), at pH 7.2. The homogenate was centrifuged for 5 min at 10,000 rpm. The resultant supernatant was used for the NGF assay. The NGF levels were determined by sandwich enzyme-linked immunosorbent assay (sandwich-ELISA) using monoclonal antibodies specific for each measurement (R&D Systems, Minneapolis, United States). Procedures were performed in accordance with the manufacturer’s protocol. Optical density was measured using an ELISA reader at the wavelength of 450 nm. The data were expressed in pg/mg of protein. Total protein was measured by Bradford’s method using bovine serum albumin as the standard.

2.8. Statistical analysis

Data were expressed as means ± standard error of the mean (SEM). Differences were considered statistically significant if P < 0.05. The SPSS (version 20.0) program was used for statistical analysis. Generalized estimating equation (GEE) followed by Bonferroni was used to analyze repeated-measures data with 1 within-subjects factor (time), and 2 between-subjects factors (group) (Ballinger et al., 2004). In all cases, if a statistically significant interaction was found, additional pairwise comparisons were made. The significance of the effects was determined by the Wald chi-square statistic. The generalized linear model is a more flexible statistical tool than the standard general linear model (GLM) because several types of distributions of the data and different covariance structures of the repeated-measures data could be chosen (Daviu et al., 2014). Furthermore, the data do not necessarily need to present normal distribution for using GEE analysis (Ballinger, 2004). If the database presents missing data, the GEE analysis takes into account unbalanced design, while repeated measures (RM) ANOVA excludes the subject line with missing data (Ma et al., 2012). Biochemical data were presented as means ± standard error of the mean (SEM), and analyzed by one-way ANOVA followed by the Student Newman-Keuls (SNK) test.

3. Results

3.1. Neuromotor development

3.1.1. Righting reflex

In the test of postural reflex, a group effect was observed (Wald χ² = 15.09; 4, P < 0.05). At P8, P9, and P10, there was a significant main effect, suggesting that morphine induces a delay in the development of the righting reflex, determined by an increase in the time needed for the correct response to occur. At P9, the morphine effect was reduced by deprivation, as can be observed in the DM group (Fig. 1).
3.1.2. Negative geotaxis

There was an interaction group × time (Wald $\chi^2 = 122.09; 20, P < 0.05$). The performance of negative geotaxis was slower in M and DM groups, compared to control rats at different time points (Fig. 2).

3.1.3. Gait

In the test of gait, we analyzed two behaviors: frequency and rearing. As the locomotion frequency was altered at different time points, the GEE analysis showed the effect of time (Wald $\chi^2 = 112.52; 5, P < 0.05$). All groups showed an increase in the daily performance of locomotion frequency throughout the observation period (Fig. 3, Panel A). In relation to rearing behavior, the GEE showed interaction between group × time (Wald $\chi^2 = 49.46; 20, P < 0.05$). The animals exposed to morphine treatment and/or maternal deprivation presented an increased frequency of rearing, from P16 to P20. (Fig. 3, Panel B).

3.2. Biochemical analysis

3.2.1. Brainstem and cerebral cortex NGF levels

The DM group presented an increase in the NGF levels in the brainstem compared to other groups (one-way ANOVA/SNK, $F_{(4,38)} = 4.88; P < 0.05$). Moreover, an increase in the cerebral cortex NGF levels in the M, DS, and DM groups was observed (one-way ANOVA/SNK, $F_{(4,38)} = 40.77; P < 0.05$) (Fig. 4).

4. Discussion

In the present study, we showed that pups that received morphine treatment in early life exhibit a delay in the formation of neonatal reflexes, as evidenced by the inferior performance in the latency of the righting reflex. Notably, we observed a similar effect when measuring negative geotaxis behavior test, which presented a lower performance in the M and DM groups. Sensory and motor abilities are crucial for normal development in humans and animal models (Zafeiriou et al., 1999; Heyser, 2004). An interesting issue is that negative geotaxis in rodents is essential for adaptation to the environment (Golan and Huleihel, 2006), and the absence or persistence of reflex development may interfere with newborn perception and reaction to the external environment (Schuch et al., 2016). We can suggest that repeated treatment with morphine and exposure to early maternal deprivation triggered a developmental delay, negatively affecting brain development. Thus, it led to the increased risk of the occurrence of behavioral alterations, corroborating results of other studies (Kalinichev et al., 2002; Sliberová et al., 2005; Macri et al., 2011; Weiss et al., 2011). The effects of repeated administration of morphine and maternal deprivation are exerted during the early postnatal period, as during this period, the sensory neurological activities and synaptic connections are in the process of being refined and established, respectively (Sale et al., 2007). In addition, the opioid system is under the maturation process during the treatment period, and the administration of this drug produces alterations in neurobehavioral responses (Nandi and Fitzgerald, 2005). Therefore, there are increasing concerns that opiates can induce harmful effects on neurodevelopment (Nandi and Fitzgerald, 2005). As a result, it is important to understand the effects of chronic opiate exposure on the immature neonatal body, and consider that daily observations of reflexes (postural reflex and negative geotaxis) are sensitive indicators of the early developmental stages of the newborn (Gibb and Kolb, 2005).

It has been suggested by Nandi and Fitzgerald (2005) that the development of the opioid system occurs within the first 2 weeks of life. Studies have demonstrated that changing morphine sensitivity in the postnatal period may be a part of a general reorganization in the structure and function of primary afferent synapses, neurotransmitter/receptor expression and function, and excitatory and inhibitory modulation from higher brain centers (Fitzgerald and Howard, 2003; Pattinson and Fitzgerald, 2004). A previous study by our research group showed that newborn rats might be more sensitive to low doses of morphine (Rozisky et al., 2008) because an extensive remodeling of opioid receptor expression takes place in the first 3 postnatal weeks (Beland and Fitzgerald, 2001). The density of binding decreases in the first 3 postnatal weeks, with peak binding at P7, which then falls to adult levels by P21 (Beland and Fitzgerald, 2001). This abundance of $\mu$ORs in early postnatal life could explain why exposure to morphine for 7 days, from P8 to P14, produces analgesia instead of tolerance (Rozisky et al., 2008). Therefore, the greater expression of $\mu$ORs at P7 in comparison to adult rats suggests a more widespread effect of morphine, acting both directly within the spinal cord and indirectly through larger termination profiles of primary afferents (Nandi et al., 2004).

In addition, the data of the present study showed an effect of time on exploratory locomotion activities of the rat offspring. Furthermore, animals exposed to morphine treatment and/or maternal deprivation presented an increase in rearing behaviors from P16 to P20. Exploratory locomotor activity has been investigated frequently in rodents. However, the results are contradictory, some studies finding no effect on locomotor activity, while others showed increase, decrease, or both effects (Gimenez-Llort et al., 2001; Kim et al., 2000; Ellenbroek et al., 2005; Rozisky et al., 2014). We can suggest that the maturation of reflexes in animals is synchronous with the growth and development of the CNS, which is markedly plastic to environmental interventions (Smart and Dobbing, 1971; Souza et al., 2015). Most importantly, ontogenetic studies have shown that amongst opioid receptors, the subtypes $\mu$ and $\kappa$ are present since birth (Skaer, 2006), whereas $\delta$ receptors only appear after the second postnatal week. Moreover, their expression and binding ability undergoes considerable reorganization in the postnatal period (Moreira et al., 2007). In addition, we speculate that exploratory behavior analyzed in gait test is different for each age analyzed.

Moreover, there is evidence that the dopaminergic pathway has a crucial role in the locomotor effects induced by morphine. Further, it is suggested that, in the second postnatal week of life, the dopaminergic system participates in locomotor activity and stereotypic behavior (McDougall et al., 1990), and it modulates locomotor and motivational effects induced by psychostimulants (Pruitt et al., 1995). Morphine stimulates dopaminergic transmission and this effect is considered as the substrate for their motor stimulant effects (Vanderschuren and Kalivas, 2000). Study showed that low doses of opioid, such as morphine, produce two age related effects: behavioral activation, which increases with age, and catalepsy that decreased with age (Katz, 1980). As a result, it could be said that the maturational differences in behavior reflect perhaps a concomitant change in the availability of a certain mediating transmitter, or altered receptor numbers, or sensitivity (Katz,
However, it has been suggested that opiate systems show maturational changes, which last up to the adulthood of the rat (Katz, 1980). Previous studies have shown that rats that experienced daily separations from the mother during the neonatal period, when tested as adults, exhibited elevated locomotor activity in a novel environment (Pryce et al., 2001; Kalinichev et al., 2002). Similar to the effects of maternal deprivation on locomotor responsiveness to novelty, prenatal stress, or prolonged social isolation in rats are associated with enduring locomotor hyperactivity. Evidences suggest that this effect is likely due to hyperactivity of the mesolimbic dopaminergic system (Geyer et al., 1993), or altered dopamine sensitivity in the nucleus accumbens (Henry et al., 1995). Corroborating these data, we can suggest that increased locomotor and exploratory activity observed in deprived animals in the gait test might be indicative of a hyperactive mesolimbic dopamine system (Geyer et al., 1993; Henry et al., 1995).

Interestingly, we observed that NGF levels in the brainstem and cortex increased in the morphine and deprived groups indicated their importance in the development process. Our results can be explained by the fact that the first two weeks after birth is a critical period to development and synaptogenesis in rats. Furthermore, during this period, there is intense stimulation of the outgrowth and maturation of neurons (Hindley et al., 1997; Jane Roskams et al., 1994). However, the limitation of this study involves the lack of assessment of the binding and expression of opioid receptors, and/or intracellular signaling after opioid receptor activation. Furthermore, NGF plays crucial roles in the complex reorganization of the spinal sensory pathways occurring after birth (Juif et al., 2016). It has been shown several neurochemical changes after exposure to opioids and different paradigms of maternal separation, such as alterations in trophic factors and other plasticity markers in the brain (Burton et al., 2007). In fact, it has been demonstrated that rats neurotrophins can be altered following early maternal separation (Cirulli et al., 2000, 2003, 2007). For example, studies in rodents showed that alteration in the modulation of brain-derived neurotrophic factor (BDNF) levels might cause some extent of abnormal morphological and functional development in the CNS (Kuma et al., 1980). However, it has been suggested that opiate systems show maturational changes, which last up to the adulthood of the rat (Katz, 1980). Previous studies have shown that rats that experienced daily separations from the mother during the neonatal period, when tested as adults, exhibited elevated locomotor activity in a novel environment (Pryce et al., 2001; Kalinichev et al., 2002). Similar to the effects of maternal deprivation on locomotor responsiveness to novelty, prenatal stress, or prolonged social isolation in rats are associated with enduring locomotor hyperactivity. Evidences suggest that this effect is likely due to hyperactivity of the mesolimbic dopaminergic system (Geyer et al., 1993), or altered dopamine sensitivity in the nucleus accumbens (Henry et al., 1995). Corroborating these data, we can suggest that increased locomotor and exploratory activity observed in deprived animals in the gait test might be indicative of a hyperactive mesolimbic dopamine system (Geyer et al., 1993; Henry et al., 1995).

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2004; Chung et al., 2009). According to Kuma et al. (2004) early maternal deprivation induces decreased of rat hippocampus BDNF expression at 16 days of age. On the other hand, we observed an inverse relation in NGF levels and a possible explanation for these alterations is that, in the developing brain, different regions and cell populations within a particular brain region show a specific course of development and an acquisition of unique structures and functions (Miki et al., 2013). Furthermore, the regions have their own period of brain growth spurts and temporal windows of vulnerability (Miki et al., 2013). Consequently, this may explain the uniform vulnerability of the brain to global insults, including maternal deprivation stress and repeated painful procedures. It is important highlight that such developmental features are highly involved in the differential alterations of neurotransmitter features in various structures of the central nervous system. Importantly, NGF may also play a role in activity-dependent synaptic plasticity, contributing to neural connections during brain development (Cirulli et al., 2000). Moreover, some of the long-term differences in brain functioning, and in behavioral patterns of subjects handled during infancy might be mediated through changes in the expression of neurotrophins such as NGF (Cirulli et al., 2000). In previous studies, using a maternal separation protocol for short (45 min) as well as long periods of time (up to 3 h), the NGF expression has been shown to increase in the dentate gyrus and the hilus of the hippocampus (Cirulli et al., 2000, 2003). Another study has shown that early maternal separation of 3-day-old rat pups for 45 min could result in an elevation of NGF levels in the developing hippocampus (Cirulli et al., 1998). Our results are consistent with these reports despite the differences in deprivation protocols. Furthermore, our findings suggest that maternal deprivation causes an alteration in the levels of neurotrophic factors. Taken together, these results support the results of previous studies and indicate that external factors, such as stressful experiences in the early period of postnatal life, including maternal neglect, can modify the availability of neurotrophic factors in the brain. This indicates that NGF is a potential player in environmentally mediated brain plasticity during development (Cirulli et al., 2000, 2003, 2007).

5. Conclusion
In summary, we showed that an exposure to postnatal stressors, such as repeated morphine administration and maternal deprivation, induces delay in the development of early reflexes. It negatively affects brain development, and increases the risk of the occurrence of behavioral alterations. Therefore, early morphine administration and maternal deprivation can alter, either independently or in conjunction, important phenotypic profiles that can be of high adaptive significance in adulthood, such as habituation and defensive and emotional behaviors. Indeed, experiences during the critical periods of development can affect the formation of neuronal circuits, and exert long-lasting influences on neural function (Nikolaev et al., 2002; Vivinett et al., 2013). Interestingly, our findings may have important implications for the human neonate, and it also highlight the need for further studies involving the design of pharmacological approaches that may counteract opioid-induced neuroadaptations and subsequently prevent abnormal pain states, since morphine exposure and maternal deprivation during the neonatal period can promote behavioral alterations (Rozisky et al., 2008, 2010, 2012a, 2012b, 2014; Rozisky et al., 2016) as well as changes in pain signaling pathways that can be expressed as an increased nociceptive response later in adult life.

Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Contributors
This work was carried out in collaboration between all authors. Authors Carla de Oliveira and Vanessa I. Scarabelot have drawn up the manuscript; participated in the design of the study; statistical analysis and performed the experimental assays. The authors Rafael Vercellino; Lauren NS Adachi; Natalia P Silveira; Lisiane S Silva; Gabriela G Regner; and Isabel Cristina de Macedo; carried out the experimental assays. The author Andressa de Souza; carried out the biochemical assays. The author Wolnei Gaumo; participated in the design of the study; author Iraci LS Torres coordinated the study, performed the statistical analysis, and helped to draft the manuscript. All authors read and approved the final manuscript.

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