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Research Report

Morphine exposure in early life increases nociceptive behavior in a rat formalin tonic pain model in adult life

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ABSTRACT

Considering the importance of a deeper understanding of the effect throughout life of opioid analgesia at birth, our objective was to determine whether morphine administration in early life, once a day for 7 days in 8-day-old rats, alters the nociceptive response over the short (P16), medium (P30), and long term (P60) and to evaluate which system is involved in the altered nociceptive response. The nociceptive responses were assessed by the formalin test, and the behavior analyzed was the total time spent in biting and flicking of the formalin-injected hindpaw, recorded during the first 5 min (phase I) and from 15–30 min (phase II). The morphine group showed no change in nociceptive response at P16, but at P30 and P60, the nociceptive response was increased in phase I, and in both phases, respectively. At P30 and P60, the animals received a non-steroidal anti-inflammatory drug (indomethacin) or NMDA receptor antagonist (ketamine) 30 min before the formalin test. The increase in the nociceptive response was completely reversed by ketamine, and partially by indomethacin. These results indicate that early morphine exposure causes an increase in the nociceptive response in adult life. It is possible that this lower nociception threshold is due to neuroadaptations in nociceptive circuits, such as the glutamatergic system. Thus, this work demonstrates the importance of evaluating clinical consequences related to early opioid administration and suggests a need for a novel design of agents that may counteract opiate-induced neuroplastic changes.

1. Introduction

Opioid analgesics, such as morphine, are the most effective and frequently used substances for the relief of moderate to severe pain. The use of these analgesics has increased in the Neonatal Intensive Care Unit over the last few decades as a consequence of changes and advances in the understanding, identification, and treatment of pain in children (De Lima et al., 1996; El Sayed

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et al., 2007; Suresh and Anand, 2001). In addition, improvements in short- and long-term clinical outcomes of critically ill neonates have necessitated the widespread use of opioid drugs for analgesia and sedation (Suresh and Anand, 2001). However, the consequences for the development of neurophysiological systems remain unknown.

The efficacy of morphine in reducing pain in neonatal animals has already been demonstrated (Nandi and Fitzgerald, 2005; Rozisky et al., 2008). Although descending inhibitory mechanisms are not completely formed until the third week of life (Nandi and Fitzgerald, 2005), morphine and other opioid receptor agonists are effective analgesics during the early neonatal period due to the presence of spinal opioid receptors from birth (Rahman and Dickenson, 1999). In a previous study by our group, using the tail-flick test (a measure of the pain threshold at the spinal level), we observed that animals in the second week of life showed an increased response to repeated morphine administration without developing tolerance. However, at P80 rats showed greater morphine analgesia and a classic tolerance effect. In addition, the animals that received morphine from P8 until P14 displayed a longer duration of morphine analgesia at the same age (P80) (Rozisky et al., 2008). These results indicate that early morphine exposure lead to the development of an altered opioid analgesic response that may be expressed into adulthood. Although such effects are described in the literature, the precise mechanisms that underlie the long-term consequences of chronic opioid treatment in the neonatal period have not been thoroughly investigated, and the use of young animal models to evaluate the long-term effect of morphine on nociceptive systems has not been widely explored.

As described above, previous tests carried out by our group demonstrated an altered nociceptive response in the tail-flick test in animals that received morphine in the second week of life, but it is important to further evaluate the nociception in these animals using other nociceptive tests. To investigate the possible mechanisms underlying this response, we selected one of the most widely used animal models to assess the response generated by injured tissue, which mimics some features of post-injury pain and is thus considered to be more relevant to clinical pain states than phasic pain, bridging the gap between acute and chronic pain (Fig. 1) (Tjølsen et al., 1992).

Considering the relevance of the subject, the aim of this study was to investigate whether repeated morphine exposure during early life alters the neurogenic and inflammatory pain in the short (P16), medium (P30), and long term (P60) using the formalin test, as well as to investigate the possible mechanisms involved in these changes.

2. Results

2.1. *Short-, medium-, and long-term effects of repeated morphine administration on the nociceptive behavior induced by formalin*

After daily morphine exposure, from P8 to P14, the nociceptive behaviors were compared between the control and morphine groups at P16, P30, and P60. The subcutaneous injection of 2% formalin into the plantar region of the hindpaw of animals of all ages and in all groups resulted in behavioral responses, such as biphasic licking, biting, and flicking of the injected paw. At P16, 2 days after the end of repeated morphine exposure, there were no differences between the groups of animals for either phase (phase I: $F=0.69$; phase II: $F=0.05$, Student's t-test, $P>0.05$ for both phases; Fig. 2A). At P30, the morphine group showed a stronger nociceptive response in phase II (phase I: $F=1.16$, Student's t-test, $P>0.05$; phase II: $F=1.21$, Student's t-test, $P<0.05$; Fig. 2B). At P60, the morphine group showed a stronger nociceptive response in both phases of the formalin test (phase I: $F=0.018$; phase II: $F=0.035$, Student's t-test, $P<0.05$ for both phases; Fig. 2C).

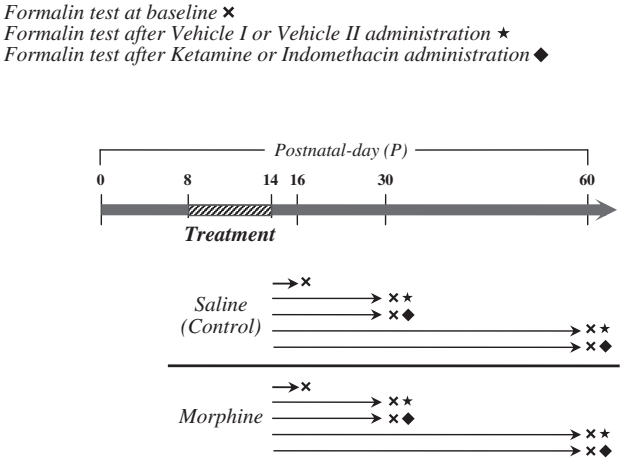

Fig. 1 – Experimental design.

2.2. *Effects of indomethacin administration on behavior in the formalin test at P30 and P60 after repeated morphine administration*

After daily morphine exposure, from P8 to P14, we investigated whether an injection of indomethacin 30 min before the formalin test was able to reverse the increased nociceptive behavior at P30 and P60 in the morphine group compared to the control group. Our results demonstrated that at P30 the control-indomethacin (C-Indomethacin) and morphine-indomethacin (M-indomethacin) animals experienced a decrease in the nociceptive response in both phases of the test when compared to control-vehicle I (C-vehicle I) and morphine-vehicle I (M-vehicle I) (phase I: $F=29.0$, phase II: $F=22.65$, one-way ANOVA, Bonferroni's test, $P<0.05$ for both phases; Fig. 3A). However, the morphine-indomethacin group presented a more intense nociceptive response when compared to control-indomethacin in both phases of the test (one-way ANOVA, Bonferroni's test, $P<0.05$; Fig. 3A). The morphine-vehicle I group showed no difference in the nociceptive response compared to the control-vehicle I group in phase I (one-way ANOVA, $P>0.05$), but it presented a more intense nociceptive response in phase II when compared to all groups (one-way ANOVA/Bonferroni's test $P<0.05$, Fig. 3A).

At P60, we observed a pattern of nociceptive behavior similar to the responses recorded at P30 for all groups in both phases (phase I: $F=6.4$, phase II: $F=12.52$, one-way ANOVA, Bonferroni's test, $P>0.05$, Fig. 3B). However, the morphine-

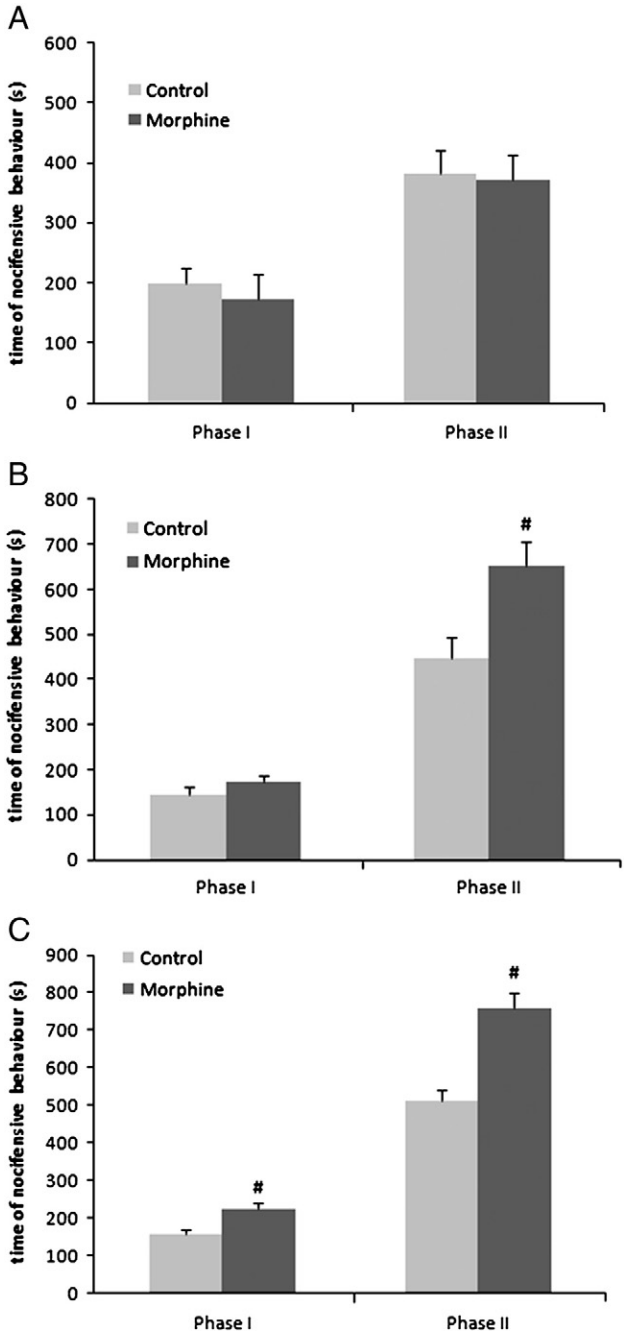

Fig. 2 – Changes in the formalin-induced neurogenic and inflammatory pain after repeated morphine administration in early life: (A) at P16, 2 days after termination of repeated morphine exposure, the animals did not show differences between groups in either phase (Student's t-test, $P>0.05$); (B) at P30, the animals did not show differences between the groups in phase I (Student's t-test, $P>0.05$), but during phase II, they presented a significant difference (Student's t test, $P<0.05$); (C) at P60, there was a significant difference between the animals in both phases of the formalin test (Student's t-test, $P<0.05$, panel C). #Significant difference from control group.

vehicle I group presented a more marked nociceptive response in phases I and II when compared to other groups (one-way ANOVA/Bonferroni's test, $P<0.05$, Fig. 3B).

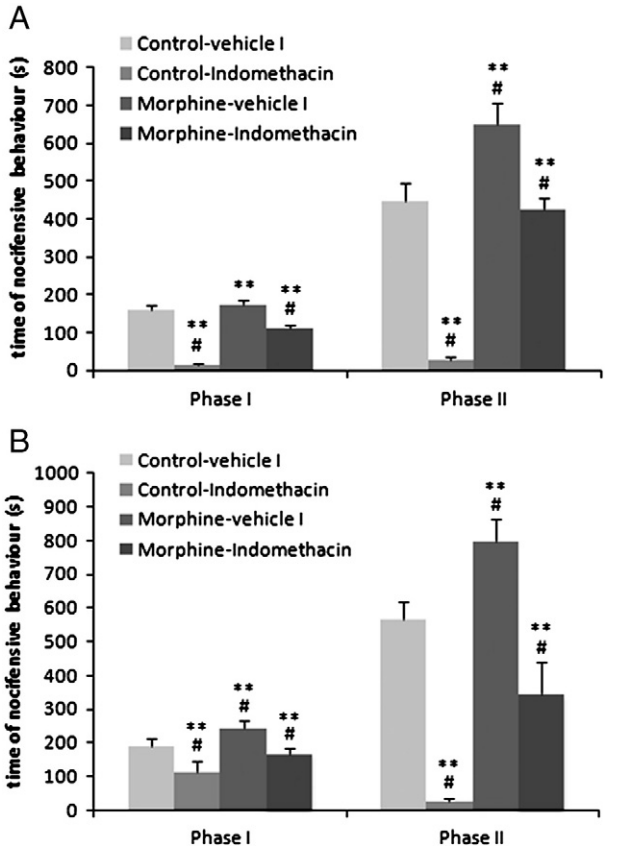

Fig. 3 – Effects of indomethacin administration 30 min before the formalin test at P30 and P60 after repeated morphine administration in early life: (A) at P30 the control-indomethacin and morphine-indomethacin groups were significantly different from all groups in phase I (one-way ANOVA/Bonferroni's test, $P<0.05$); at phase II, all groups were significantly different from each other (one-way ANOVA/Bonferroni's test $P<0.05$). (B) at P60, all groups were significantly different from each other in both phases of the test (one-way ANOVA/Bonferroni's test, $P<0.05$). #Significant difference from control-vehicle I group; **significant difference between groups.

2.3. *Effects of ketamine administration on the formalin test at P30 and P60 after repeated morphine administration*

The administration of ketamine 30 min before the formalin test prevented the higher nociceptive response observed in the morphine group compared to the control group, at P30 and P60. Our results show that at P30, the control-ketamine (C-ketamine) and morphine-ketamine (M-ketamine) groups presented decreased nociceptive responses in both phases of the test when compared to the control-vehicle II (C-vehicle II) and morphine-vehicle II (M-vehicle II) groups (phase I: $F=7.97$, phase II: $F=79.28$, one-way ANOVA, Bonferroni's test, $P<0.05$ for both phases; Fig. 4A). However, the morphine-ketamine group exhibited a less marked nociceptive response when compared to the control-ketamine group in both phases of the test (one-way ANOVA, Bonferroni's test, $P<0.05$; Fig. 4A). The morphine-vehicle II group, in turn, presented a similar nociceptive response to that of the control-vehicle II group in phase I (one-way ANOVA, $P>0.05$), but a higher nociceptive

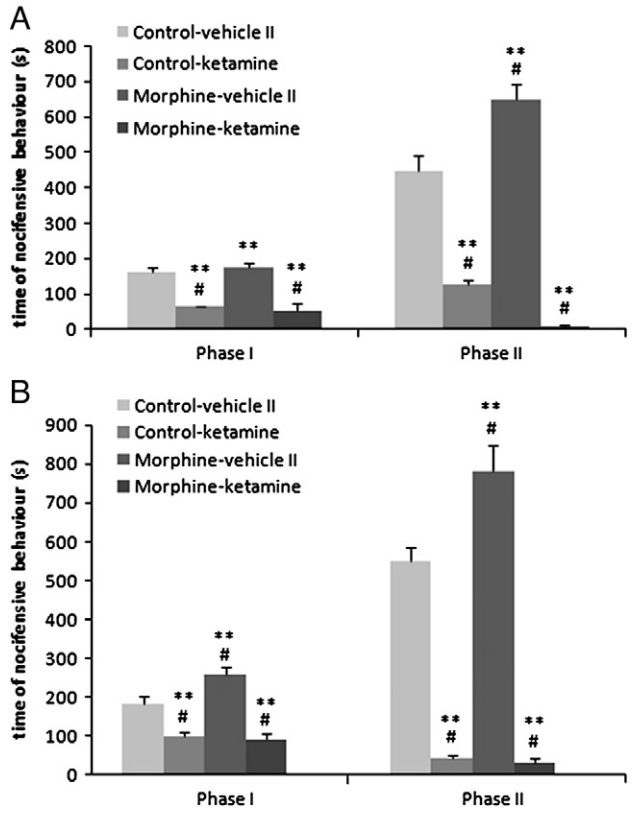

Fig. 4 – Effects of ketamine administration 30 min before the formalin test at P30 and P60 after repeated morphine administration in early life: (A) at P30, the control-ketamine and morphine-ketamine groups were similar, but they were significantly different from other groups (control-vehicle II and morphine-vehicle II) in phase I (one-way ANOVA/ Bonferroni's test, $P<0.05$). At phase II, all groups were significantly different from each other (one-way ANOVA/ Bonferroni's test, $P<0.05$). (B) at P60, all groups were significantly different from each other in both phases of the test (one-way ANOVA/Bonferroni's test, $P<0.05$). #Significant difference from control-vehicle II group; **significant difference between groups.

response in phase II when compared to all groups (one-way ANOVA/Bonferroni's test $P<0.05$, Fig. 4A).

At P60, we observed a pattern of nociceptive response similar to that seen at P30 for all groups in both phases (one-way ANOVA, Bonferroni's test, $P<0.05$, Fig. 4B). However, the morphine-vehicle II group presented a more intense nociceptive response than all other groups in phase I and phase II (phase I: $F=5.63$, phase II: $F=11.92$, one-way ANOVA/Bonferroni's test, $P<0.05$ for both phases, Fig. 4B).

3. Discussion

In this study, we demonstrated that rats that received morphine during the second week of life showed an increase in nociceptive behavior in phase II of the formalin test at P30. This increased response was partially reversed by a non-steroidal anti-inflammatory drug (indomethacin) and completely reversed by an NMDA receptor antagonist (ketamine). Moreover, at P60, the morphine-treated animals showed an increase in the nociceptive response in both phases of the formalin test (representing the neurogenic and inflammatory pain responses), which was also partially reversed by indomethacin and completely reversed by ketamine.

These results indicate that exposure to drugs in early life can have long-lasting implications for the development of the nervous system, such as permanent changes in pharmacological responses and cell signaling (for a review, see Stanwood and Levitt, 2004). Other investigations have uncovered the neurobiological consequences of repeated opioid administration and revealed possible neuroplastic adaptations that likely underlie opioid-induced paradoxical pain (Vanderah et al., 2000, 2001; Gardell et al., 2002). This may be a consequence of the structural and functional immaturity of the neonatal nervous system, and the significant changes in opioid analgesic mechanisms that occur before and after birth (Beland and Fitzgerald, 2001; Marsh et al., 1997; Rahman et al., 1998).

In the formalin test, the rodent hindpaw presents a characteristic biphasic nociceptive response using both weighted pain measures (Dubuisson and Dennis, 1977) and continuous scoring systems (Wheeler-Aceto and Cowan, 1991). The transient early phase (occurring in the first 5–10 min) is interpreted as reflecting direct activation of nociceptive sensory afferents by formalin, while the tonic phase (expressed from 20 to 90 min) is regarded as depending on an ensuing inflammatory response, associated with central sensitization (Tjølsen et al., 1992; Coderre et al., 1993). Formalin can also activate central processes that lead to longer term events (over 3–4 weeks), such as the expression of immediate-early genes and activation of microglia, providing in this context a model of chronic pathological pain (Sawynok and Liu, 2003). Thus, the increase in formalin-induced nociceptive behavior observed in this study suggests a central hyperexcitability of the ascending second-order dorsal horn neurons induced by previous sustained exposure to morphine, and this is a long-term effect. Our results agree with those of Zissen et al. (2006, 2007), who have demonstrated that while infant rats (P5 to P8) are more sensitive to the long-term changes in formalin-induced pain and mechanical thresholds following continuous exposure to morphine, when compared to young rats (P19 to P21), they are also better able to compensate for changes in mechanical thresholds following intermittent administration of morphine, given twice a day for 3 days. It is possible that short bouts of morphine withdrawal-induced excitation may off-set morphine-induced inhibition in infants, but not in young rats, and thus, may better maintain the balance of activity and inactivity during this crucial developmental phase.

Ossipov et al. (2005) showed that opioids can produce hyperalgesia under many circumstances, and that such effects might contribute to the drawbacks of acute and chronic administration of these drugs. Although the mechanisms of this phenomenon have not yet been fully clarified, research has shown that chronic exposure to opioids induces a change in the function of spinal cord neurons that can be manifested as neuronal hyperactivity during opiate withdrawal (Rohde et al., 1997; Vanderah et al., 2001; Gardell et al., 2006). Other studies have reported that repeated opioid exposure can lead

to spinal cord neuroplasticity (for review, see Mao and Mayer, 2001) and these adaptations may involve changes in supraspinal pain modulatory circuits (Ossipov et al., 2005). Consistent with these results, we suggest that exposure to morphine in early life might lead to drug-induced adaptations in the excitatory pain pathways, such as neuroplastic changes at the receptor level and/or in the synthesis of algesic substances (Yaksh et al., 1986), which may produce secondary hyperalgesic effects that increase the intensity of the pain (Celerier et al., 1999; Larcher et al., 1998).

The effect of ketamine seen here may be explained by activation of the glutamatergic system in opioid-mediated hyperalgesia (Sanford and Silverman, 2009). It is well accepted that persistent activation of the NMDA receptor by excitatory amino acids released from primary afferent terminals results in the sensitization of spinal neurons (Baranauskas and Nistri, 1998), and such NMDA receptor-mediated central sensitization is believed to drive enhanced nociception in chronic pain states and opioid-induced abnormal pain (Larcher et al., 1998; Laulin et al., 1999; Mao and Mayer, 2001). The involvement of excitatory neurotransmitters, mainly glutamate, in inflammatory nociception is supported by the increase in levels of these neurotransmitters in the dorsal root ganglion and dorsal horn, elicited by chronic inflammation (Wimalawansa, 1996; Löfgren et al., 1997; Ossipov et al., 2005). In addition, peripheral inflammation is capable of increasing the expression of subunits of the NMDA receptor and enhancing neurotransmitter release in CNS structures related to nociception (Zhuo, 2002; Zhao et al., 2006). Therefore, it is possible that the animals that received morphine in early life presented central sensitization in the medium and long term induced by changes in the glutamatergic system, and this may be responsible, at least in part, for the increase in nociceptive behavior in phase II of the formalin test (which represents the inflammatory pain response) observed in this study. This explanation for the latter result is supported by the fact that an NMDA receptor antagonist (ketamine) completely eliminated the hyperalgesia induced by morphine exposure in early life.

In addition, indomethacin, a nonsteroidal anti-inflammatory drug (NSAID), was unable to completely reverse the hyperalgesia resulting from early morphine treatment. This suggests that there is an inflammatory component involved, but we cannot discard other mechanisms that may contribute to the hyperalgesia observed in this study. Following on from previous studies that found that pre-treatment with an NSAID may increase spinal cord levels of kynurenic acid (an endogenous excitatory amino acid antagonist) (Edwards et al., 2000) and partially inhibit increases in spinal cord levels of *c-fos* (an immediate-early genetic marker of nociceptive activity) stimulated by application of the rat tail ischemia–reperfusion acute model of hyperalgesia (Lin et al., 2000), Grace et al. (2001) reported that subcutaneous injection of NSAIDs completely eliminated the hyperalgesic response elicited in rats by ischemic stimulation of the tail and suppressed the increased prostaglandin formation in the brains of the animals. However, the relief of hyperalgesia was short-lived and corresponded only to the first phase of the (spontaneous) hyperalgesia (Scheuren et al., 1997). In addition, PGE2 has been found in microdialysate of the spinal cord after injection of formalin in the paw of the rat (Malmberg and Yaksh, 1995; Scheuren et al., 1997), and its production was antagonized by systemic injection of paracetamol (Muth-Selbach et al., 1999) or by intrathecal injection of other NSAIDs (Malmberg and Yaksh, 1992). Direct evidence for a spinal antinociceptive action of NSAIDs derives from observations made in patients and animal experiments. It has been reported that intrathecal injection of acetylsalicylic acid, salicylic acid, and indomethacin depressed the nociceptive activity that was evoked in thalamic neurons of rats by electrical stimulation of afferent C-type fibers in the sural nerve (Jurna et al., 1992).

The development of nociceptive pathways is an activity-dependent process (Fitzgerald and Jennings, 1999; Fitzgerald and Beggs, 2001; Beggs et al., 2002), and thus, abnormal activity such as that generated by early opioid exposure may alter normal synaptic development producing changes in somatosensory processing and behavior that would not occur in similarly exposed adults. Our group has demonstrated that neonatal rats may be more sensitive to low doses of morphine because there is extensive re-modeling of opioid receptor expression in the first 3 postnatal weeks (Rahman et al., 1998; Rahman and Dickenson, 1999; Beland and Fitzgerald, 2001). For example, at P14 spinal μ-opioid receptors (μORs) are limited to the dorsal horn, whereas they appear throughout the spinal grey matter at P7, and the density of binding is seen to decrease in the first 3 postnatal weeks, with peak binding at P7 that then falls to the adult level by P21. This abundance of μORs 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). Thus, the greater expression of μORs 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). This, coupled with the over-expression of excitatory amino acid receptors, at the primary afferent-spinal cord synapse, supports a potential role for μORs in the normal maturation of nociceptive circuitry, and hence, disruption of this by exogenous administration of opioid agonists may have detrimental consequences for the maturation of pain circuitry (Thornton and Smith, 1998; Thornton et al., 2000). Activity-dependent processes drive the maturation of nociceptive C-fibers during this period of neurodevelopment (Beggs et al., 2002; Fitzgerald et al., 1994). In adult rats, Aδ and C but not Aβ primary afferent fibers transmit painful stimuli. In contrast, in P7 rats Aβ primary afferents can also transmit such stimuli (Fitzgerald and Jennings, 1999). It has been hypothesized that increased activity in Aβ-fibers early in development may be modulated by sub-threshold C-fiber depolarization that primes the spinal cord for Aβ-fiber input (Dickenson and Rahman, 1999). Functionally, in adult rodents opioid agonists selectively inhibit Aδ- and C-fiber nociceptors but not Aβ-fibers (Dickenson et al., 1987; Rahman and Dickenson, 1999). In contrast, in young rats morphine can inhibit Aβ- and C-fiber-mediated activity in the lumbar spinal cord (Rahman et al., 1998), which parallels expression of μORs in both small (Aδ and C) and large (Aβ) diameter cell bodies in the dorsal root ganglion. Based on the results of our study, we suggest that at P16 the animals do not exhibit increased nociceptive behavior in the formalin test

because repeated exposure to a μOR agonist has influenced the development of C-fibers during maturation. However, we did observe that following the formalin test, the treated animals presented an inflammation-like edema in the formalin-injected hindpaw, which was measured and compared to the volume of the non-injected hindpaw by plethysmometry. It is interesting to note that there were no differences between the volume of formalin-injected hindpaws in the morphine and control groups (data not shown).

Taking into account the importance of a deeper understanding of the effects throughout life of opioid analgesia at birth, and that previous results from our group showed that morphine exposure in early life lead to changes in the analgesic response in adult life (Rozisky et al., 2008), we hypothesized that the use of opioids in early life can induce persistent changes in nociceptive and opioid analgesic responses. We conclude from the present results that the altered nociceptive response induced by repeated morphine exposure can change in an age-dependent manner. In addition, the altered nociceptive response was expressed until adulthood, and this effect was partially reversed by indomethacin and completely reversed by an NMDA receptor antagonist. However, it should be noted that the response is complex and unlikely to be predominantly caused by any single mediator. Taken together, our data indicate that opioids elicit glutamatergic adaptations at the system level. Finally, the behavioral changes seen in response to repeated exposure to morphine during early life illustrate the need to examine nociceptive processing in neonatal patients who have been exposed to therapeutic morphine; moreover, this indicates the importance of evaluating the clinical consequences of long-term opioid administration. These findings 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.

4. Experimental procedures

4.1. Animals

Eight-day-old male Wistar rats were divided into two groups: saline-control (C) and morphine-treated (M). Naive animals were housed in home cages made of Plexiglas (65 cm × 25 cm × 15 cm) with sawdust covering the floor. Animals were maintained on a standard 12-h dark/light cycle (lights on between 0700 h and 1900 h) at room temperature (22 ± 2 °C). The animals had free access to food and water. At birth, the litters were standardized to contain up to 8 pups per dam, and the pups remained with their mothers until 21 days of age. Rats at P8 were chosen because it is accepted that animals of this age are at a similar stage of neurological development to that of 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 (Bishop, 1982; Kim et al., 1996; Rabinowicz et al., 1996). Animal handling and all experiments were performed in accordance with international guidelines for animal welfare. The protocol of this experimental study was approved by the Ethics Committee of the institution where the work was conducted.

4.2. Pharmacological treatment

Each animal received saline (control group) or morphine (5 μg s.c. in the mid-scapular area; morphine group) starting at P8, then once a day for 7 days. This dose had been chosen based on a previous study by Rozisky et al. (2008, 2010), and it produced analgesia in all animals submitted to the tail-flick test. All treatments were administered at the same time each day (1100 h). One milliliter of morphine sulphate (Dimorf® 10 mg/ml, obtained from Cristália, Porto Alegre, Rio Grande do Sul, Brazil) was diluted in 9 ml of 0.9% NaCl (saline). The formalin test was performed in 16-, 30-, and 60-day-old rats (Fig. 1). The number of animals used per group was 8 to 15.

At the ages where we observed significant differences in the nociceptive behavior in the formalin test, the control and morphine groups were subdivided into four groups, each one designed to evaluate the effect of i.p. administration of an NMDA receptor antagonist or non-steroidal anti-inflammatory drug (NSAID), applied 30 min before the formalin test: (1) non-steroidal anti-inflammatory drug: 10 mg/kg of indomethacin (Indomethacin®, obtained from Sigma-Aldrich, São Paulo, Brazil) (Bastos et al., 2004) diluted in 1.29% sodium bicarbonate solution (control-indomethacin, morphine-indomethacin); (2) vehicle for indomethacin (vehicle I): 10 mg/kg of 1.29% sodium bicarbonate solution (pH = 7.4) (control-vehicle I, morphine-vehicle I); (3) NMDA receptor antagonist: 30 mg/kg of ketamine (Cetamine®, obtained from Hospital de Clínicas de Porto Alegre, Brazil) (Campos et al., 2006) diluted in 0.9% saline (control-ketamine, morphine-ketamine); (4) vehicle for ketamine (vehicle II): 30 mg/kg of saline (control-vehicle II, morphine-vehicle II) (Fig. 1). The number of animals used per group was 6 to 9.

4.3. Formalin test

The formalin test was performed as previously described (Tjølsen et al., 1992; Tai et al., 2006) with minor modifications. Twenty-four hours before the test, each animal was placed in the chamber for 10 min to familiarize them with the procedure, since the novelty of the apparatus itself can induce antinociception (Netto et al., 2004). The animals were injected s.c. on the plantar surface of the left hindpaw with 0.17 ml/kg of a 2% formalin solution (Formaldehyde P.A.®, obtained from Sigma-Aldrich, São Paulo, Brazil) diluted in 0.9% NaCl (saline). Each animal was observed in a varnished wood cage, measuring 60 × 40 × 50 cm, with the inside lined with glass, and the nociceptive response was recorded for a period of 30 min. This test produces two distinct phases of nociceptive behavior: an early, transient phase (phase I; up to 5 min after the injection) and a late, persistent phase (phase II; 15–30 min after the injection). Phase I has been considered to reflect direct stimulation of primary afferent fibers, predominantly C-fibers (neurogenic pain) (Martindale et al., 2001), whereas phase II is dependent on peripheral inflammation (inflammatory pain) (Dubuisson and Dennis, 1977; Shibata et al., 1998; Tjølsen et al., 1992). The total time (seconds) spent in licking, biting, and

flicking of the formalin-injected hindpaw was recorded in phases I and II. The test was performed once only in each rat.

4.4. *Statistical analysis*

Data were expressed as means ± standard error of the mean (SEM). Depending on the experiment, Student's *t*-test or one-way ANOVA was performed, followed by a multiple comparisons test (Bonferroni's test) when indicated. Differences were considered statistically significant if $P<0.05$.

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