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THE INVOLVEMENT OF OPIOIDERGIC AND GABAERGIC SYSTEMS AT THE SHORT- AND LONG-LASTING EFFECT OF EARLY SURGICAL PROCEDURE IN RATS' NOCICEPTIVE RESPONSE

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ABSTRACT

Introduction: Neonates and children are often exposed to pain from invasive procedures during intensive care and postoperative period. Evidence suggests that the exposure to acute pain in early life leads to long-term consequences. Opioid analgesics are the most effective and frequently used substances for the relief of moderate to severe pain in adults and children. The aim of the present study was to investigate the impact of surgical procedures in early life and the involvement of opioidergic and GABAergic pathways on the surgery pain modulation through two different experimental designs: A) experimental design 1 evaluated the short (P21) and long-lasting (P45) effect of early surgical procedure on rats' nociceptive response; B) experimental design 2 investigated the effect of opioidergic and GABAergic antagonists on surgery pain modulation.

Methods: Animals were anesthetized with 2% halothane and submitted to Brennan model of incisional pain. Nociceptive response was evaluated through tail-flick latency test (TFL). Animals from experimental design 2 received naloxone or picrotoxin i.p. 30 minutes before surgery.

Results: infant rats (P21) submitted to surgical procedure presented analgesia 30minutes after the surgery. An administration of picrotoxin reverted totally this analgesia at P21/t30 while naloxone was not able to do so. In relation to experimental design 1 animals that had been operated at P21 showed lower nociceptive threshold comparing to the ones that never been operated in life.

Conclusions: Early surgical procedure induced short and long-lasting effect upon rats' nociceptive response. Besides that, an analgesic pattern was presented 30minutes after surgery (at P21), which was only reverted with the administration of picrotoxin. Others studies investigating the impact of surgical procedures in early life and the involvement of pain pathways should be evaluated, due to its importance of being infants and the impact that interventions at this period may cause in these individuals' adult life.

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INTRODUCTION

Neonates, infants and children are often exposed to pain from invasive procedures during intensive care and postoperative period (Pattinson and Fitzgerald, 2004; Ririe *et al*., 2003). Evidence suggests that exposure to acute pain in early life leads to long-term consequences (Sternberg *et al*., 2005; Lidow, 2002). Neonates undergo considerable maturation of peripheral, spinal and supra-spinal afferent pain transmission during the early post-natal period 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). Patients undergoing surgery in early life presented more postoperative pain between 7 and 13 years old, compared with children with the same age without any surgical procedure when neonates (Caumo *et al*., 2000). Another study (Peters *et al*., 2005) demonstrated that three-months-old infants submitted to dermatome surgical procedure and underwent subsequent surgery at three years old, required more intraoperative fentanyl, more postoperative morphine. In addition, they presented higher norepinephrine plasma concentrations, compared to children at the same age who were operated for the first time in life.

Different mechanisms, as sensitization of peripheral nociceptores, can be linked to long-term changes at spinal and supra spinal levels. This phenomenon induces an increase in the sensitivity of dorsal horn nociceptive circuits and/or neuroimmune activation (Schwaller and Fitzgerald, 2014). It is important to note that the descending inhibitory mechanisms are not completely formed until the third week of life (Nandi and Fitzgerald, 2005). Studies have shown that extensive remodeling of opioid receptor expression occurs in the first three postnatal weeks (Beland and Fitzgerald, 2001; Rahman and Dickenson, 1999; Rahman *et al*., 1998). Furthermore, previous study showed that a repeated needle prickling on the right paw at neonatal rats promoted long-lasting changes linked to mechanical hyperalgesia. This increases glial markers density in cortical areas involved in the pain process and pain interpretation (Sanada *et al*., 2014). Our previous study showed increased analgesic response after repeated morphine administration (between eighth (P8) and fourteenth (P14) old days) without developing opioid tolerance (Rozisky *et al*., 2008). Furthermore, in another study, we observed a decrease in the nociceptive response in the first phase of formalin test after unique fentanyl administration in rats at P14 (Medeiros *et al*., 2012). It is interesting to note that the opioid analgesics, such as morphine, are the most effective and frequently used substances for the relief of moderate to severe pain in adults, and children (El Sayed *et al*., 2007; Suresh and Anand, 2001; Lima *et al*., 1996). In this way, it is important to investigate the involvement opioidergic and GABA systems in the surgery pain modulation in the infant age. Considering the exposed above and the theme relevance, we suggest that surgical procedures, pain and pharmacological manipulations at neonatal and infant age can lead to behavioral and biochemical alterations until the adult age. There are a few studies about the experience of pain in neonates and children and there is a limited research in the basic area. Therefore, the aim of the present study was to investigate the impact of surgical procedures in early life and the involvement of opioidergic and GABAergic pathways on the rat surgery pain through two different experimental designs: A) experimental design 1 evaluated the short (P21) and long-lasting (P45) effect of early surgical procedure on rats' nociceptive response; B) experimental design 2 investigated opioidergic and GABAergic modulation in the surgery pain. A) experimental design 1 evaluated the short and long-lasting effect of early surgical procedure at rats' nociceptive response; B) experimental design 2 investigated involvement of opioidergic and GABAergic pathway in the rat surgery pain modulation.

MATERIAL AND METHODS

Animals

Twenty-one-day-old male and female Wistar rats were housed in groups of five animals per Plexiglas cages (65 cm x 25 cm x 15 cm) with sawdust covering the floor. At this age, rats show neurodevelopment similar to one-year-old child (Fitzgerald and Anand K, 1993) and its physiological state is also immature (Pattinson and Fitzgerald, 2004). All animals were kept on a standard 12-hour light/dark 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 chow *ad libitum*. Experiments and procedures were approved by the Institutional Committee for Animal Care and Use (Ethics Committee of CEUA/HCPA: 11-0173), conformed to the Brazilian law 11794/08 and the Guide Laboratory for the care and use of animals (2011). Animal handling and all experiments were performed in accordance with international guidelines for animal welfare, and measures were taken to minimize animal pain and discomfort. The experiment was performed with the number of animals necessary to produce reliable scientific data.

Drugs and Chemicals

Halothane and Naloxone chlorhydrate (Narcan® 0.4 mg/mL) were purchased from Cristália® (São Paulo-SP). Penicillin procaine (Despacilin® 400.000 UI) and Picrotoxin (powder) were purchased from Bristol-Myers Squibband and Sigma Aldrich, respectively. Naloxone chlorhydrate and picrotoxin were dissolved in NaCl 0.9% (saline) and administered intraperitoneal (i.p) in a volume of 1 mL/kg.

Anesthesia and Surgical Procedure

Animals from surgery group were anesthetized with 2% halothane delivered via a nose cone and submitted to the Brennan model of incisional pain adapted for young rats (Brennan *et al*., 1996). It was made a 0.5-cm longitudinal incision through skin, fascia and muscle of the plantar aspect of rats' right hind paw. Skin was closed using a mattress suture (5.0 nylon, AT 20 mm needle; Med-Goldman®). Liquid nitrofurazone (Furanew Spray®, Vetnil) was applied into the wound site. Animals submitted to the procedure received an intramuscular injection of penicillin (Despacilin® 400.000 UI), aiming to prevent postoperative infection. After surgery, animals were allowed to recover in their cages, and the suture was removed 24 h later. Animals from anesthesia group were only submitted to the administration of halothane for the same time as surgery group, without been operated.

Tail-Flick Test (TFL)

The nociceptive response was evaluated using a tail-flick apparatus and measured as previously described (D'amour and Smith, 1941). This test can estimate spinal and supra spinal activation (Tseng and Tang, 1990) and detect systemic analgesia induced by stress procedures, such as incisional injury (Cepeda *et al*., 2004). Twenty-four hours before the trial, animals were exposed to the apparatus to be familiarized

with the procedure, since the novelty can itself induce antinociception (Netto *et al*., 1987). The light source was focused on a point 2-3 cm rostral at the tail's tip. Light intensity was adjusted to obtain the test's latency baseline of 4 to 5 seconds (0.8 mA). A cut-off time of 10 seconds was used to prevent tissue damage. Basal measurements were taken before any manipulation.

Experimental Designs

Experimental Design 1: Short and long-lasting effect of early surgical procedure on rats' nociceptive response

At P21, male and female rats were divided into control, anesthesia and surgery group. TFL was measured before surgery (baseline- B) and 30 (t30) minutes after procedure (Figure 1).

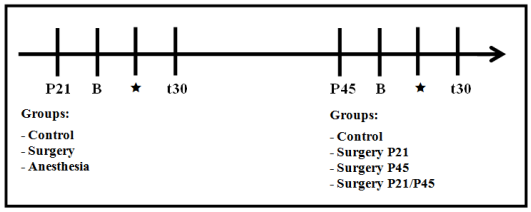


Figure 1. Experimental Design 1: TFL measure in B= baseline (before the surgery) and 30 (t30) minutes after procedure. ★ Surgical procedure

At P45, rats were submitted to a second surgical procedure. Therefore, rats were subdivided into four groups: control (no surgery), surgery P21 (submitted only to the first procedure), surgery P45 (submitted only to the second procedure) and surgery P21/P45 (submitted to both first and second procedures). TFL was measured before surgery (baseline-B) and 30 minutes (t30) after the procedure (Figure 1).

***Experimental Design* 2:** Involvement of opioidergic and GABAergic pathway in the rat surgery pain modulation.

In a new experiment, rats received saline (NaCl 0.9%), opioidergic or GABAergic antagonists i.p. (naloxone or picrotoxin, respectively) 30 min before surgical procedure at P21. Animals from control group did not suffer any manipulation; drug control group (naloxone or picrotoxin) received only drug injection without been exposed to the surgery; control surgery group received saline i.p., before the procedure and drug surgery group (naloxone and picrotoxin) received one of drugs i.p., before been exposed to the procedure. TFL was measured before surgery (baseline- B) and 30 (t30) minutes after procedure (Figure 2).

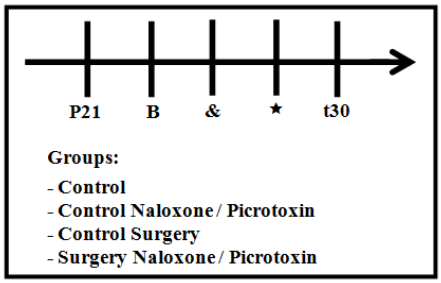


Figure 2. Experimental Design 2: TFL measure in B= baseline (before the surgery) and 30 (t30) minutes after procedure. ★ Surgical procedure. & Saline or drug injection (naloxone or picrotoxin)

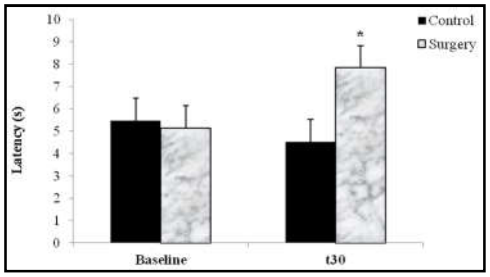
Statistical analysis

A generalized estimating equation (GEE) followed by Bonferroni was performed to analyze the results of nociception. Two-way analysis of variance (ANOVA) followed by Student–Newman–Keuls (SNK) was performed to compare the effect of gender and the nociceptive response. T-test for equality of means was performed to verify difference between control and surgery groups at P45 (baseline). Data were expressed as the mean ± standard error of the mean (SEM) and considered significant at $P \leq 0.05$. SPSS 20.0 for Windows was used for statistical analysis.

RESULTS

Experimental design 1: Short and long-lasting effect of early surgical procedure on rats' nociceptive response

At P21, there was interaction between group*time (Wald $\chi 2 = 26.82$; 6; $P<0.05$, Figure 3). At baseline there was no difference in TFL among groups. However, 30 minutes after the procedure, the surgery group presented an increase in TFL comparing to control group. There was no difference in TFL between control and anesthesia groups at baseline and t30 (data not shown). For this reason, anesthesia group was excluded from this experiment. There was no effect of gender in TFL measurements at P21 (one –way ANOVA, $P>0.05$). Therefore, data from male and female animals were analyzed together.



Data expressed as the mean ± SEM (n = 8 animals/group). *Significant difference between surgery group and control group at t30 (Wald $\chi 2 = 26.82;6$; $P<0.05$).

Figure 3. Effect of the surgical procedure upon nociceptive response at P21

At P45, animals previously submitted to a surgical procedure at P21 presented lower TFL measures at baseline comparing with the ones which never been operated in life (t-test, $F=0.44$, $P<0.05$, Figure 4- Panel A). After 30 minutes (t30) of the second surgical procedure at P45, surgery P21 and surgery P21/P45 groups presented lower nociceptive threshold compared to control and surgery P45 groups (one-way ANOVA $F=14.04$, $P < 0.05$, – Figure 4- Panel B).

Experiment 2: Involvement of opioidergic and GABAergic pathway in the rat surgery pain modulation

At P21, there was interaction between group*time (Wald $\chi 2 = 14.47;3$; $P<0.05$, Figure 5). At baseline there was no difference in TFL among group. After 30 minutes (t30) of the surgical procedure, control surgery group showed higher pain threshold than control and control naloxone groups. This analgesic status 30 minutes after the surgery was also observed in experiment 1 at P21. With the administration of naloxone (an opioidergic antagonist) before the surgical procedure, animals showed a decreased of TFL measures, but not statically different from the others animals.

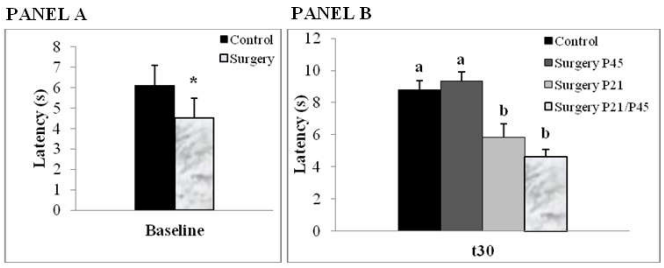


Figure 4. Effect of the second surgical procedure upon nociceptive response at P45. *Panel A: TFL baseline measurements.* Data expressed as the mean ± SEM (n = 9-12 animals/group). *Significant difference between surgery group (that had been operated at P21) and control group (that had never been operated in life) (t-test, F=0.44, P<0.05). Panel B: TFL t30 m*easurements.* Data expressed as the mean ± SEM (n = 3-7 animals/group). Different superscript letters indicate significant difference among groups. Surgery P21 and surgery P21/P45 groups presented lower nociceptive threshold compared to control and surgery P45 groups (one-way ANOVA F=14.04, P < 0.05)

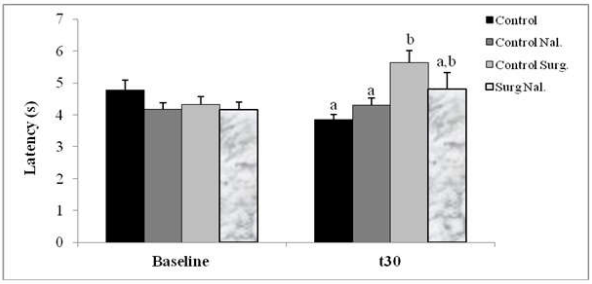


Figure 5. Effect of the previous administration of Nalaxone upon surgical pain modulation at P21: Data expressed as the mean ± SEM (n = 15-36 animals/group). Different superscript letters indicate significant difference among groups. No statically difference was found among groups at baseline measures. At t30, control surgery group presented higher TFL measures than control and control naloxone groups, but not comparing to surgery naloxone group. No statically difference was found among surgery naloxone group and the other groups at t30 (Wald $\chi 2 = 14.47$; 3; P <0.05)

In the experiment using picrotoxin (a GABAergic antagonist), at P21 there was interaction between group*time (Wald $\chi 2$ = 15.3+54;3; P <0.05, Figure 5). At baseline there was no difference in TFL among groups. After 30 minutes (t30) of the surgical procedure, only control surgery group showed higher TFL measures than the other groups. Once more, the analgesic status after the procedure at P21 was presented. However, with the previous administration of picrotoxin to the surgery this analgesic effect was reversed.

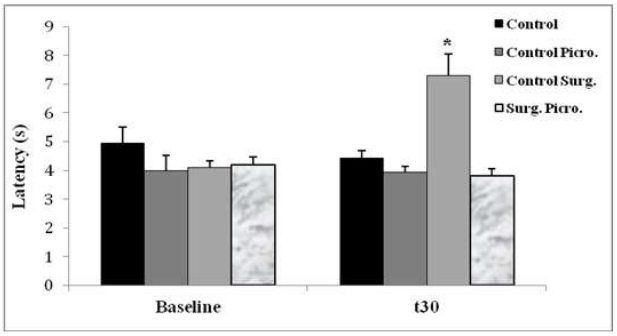


Figure 6. Effect of the previous administration of Picrotoxin upon surgical pain modulation at P21: Data expressed as the mean ± SEM (n = 14-15 animals/group). No statically difference was found among groups at baseline. *Significant difference between control surgery group among the other groups at t30 (Wald $\chi 2 = 15.3+54;3$; P <0.05)

DISCUSSION

In the present study, infant rats (P21) submitted to surgical procedure presented analgesia 30 minutes after surgery. This analgesic pattern at P21/t30 was observed in all the study's experimental designs. At P21/t30, when an opioid antagonist (naloxone) was administered before the surgery, the analgesia was reverted partially, since it was no different from any group. On the other hand, in the same time, the administration of a GABAergic antagonist (picrotoxin) totally reverted the analgesia. In the experimental design 1, the animals were operated at P21, and submitted to a second surgical procedure at P45; these animals presented a lower nociceptive threshold comparing to control animals (never operated), and to animals submitted to a surgical procedure for the first time in life. The lower nociceptive threshold at P45, the baseline and t30 found in animals submitted to a previous surgery at P21, demonstrates that early surgical procedure in life induces short and long lasting effects upon rat's nociceptive response, impacting their quality of life. These effects can be related to long-term potentiation (LTP) induction and/or central sensitization, mechanisms underlying changes of pain pathways (Latremoliere and Woolf, 2009; Sandkühler, 2007). It is important to highlight that animals at P21, like neonates, present immaturity of inhibitory pain pathways in spinal and supra spinal, it can be related to long-term lower nociceptive threshold induced by early surgery.

In the second experimental design, we seek to elucidate the involvement of the opioidergic/GABAergic pathways at this analgesic response at P21/t30. In this way, we showed that the GABAergic system is related to the analgesic pattern at P21/t30; however, the opioidergic system is partially involved in this response. Since halothane increases GABA binding resulting in higher chloride ions input, reducing neuronal excitability (Whalen *et al*., 2016), the analgesic pattern at P21/t30 may be related to the anesthetic residual effect, once the administration of a GABAergic antagonist was able to revert this analgesia. On the other hand, in our experimental design 1 the anesthesia group had nociceptive's thresholds similar to control group design 1, without analgesia at P21/t30, discarding the involvement of the anesthesia's residual effect.
Previous studies showed that stress could alter pain thresholds (da Silva Torres *et al*., 2003; Jayaram *et al*., 1995). Therefore, we can suggest that the analgesic pattern at P21/t30 is related to the stress induced by the surgery; however, we did not measured animals' corticosterone levels to confirm this hypothesis. It is important to notice that the stress induction is mediated by a large number of neurotransmitters and neuropeptides (Butler and Finn, 2009; Vaccarino and Kastin, 2000), and it induces activation of the descending pain modulatory systems including GABA receptors (Millan, 2002). Previous studies have demonstrated surgery-induced-analgesia (da Silva Torres *et al*., 2003; Jayaram *et al*., 1995) similarly to stress-induced-analgesia (SIA) (Martenson *et al*, 2009). Another point that should be considered is the age of the animals, since the balance of excitation and inhibition differs in the neonatal dorsal horn compared with that in the adult (Fitzgerald, 2005). In conclusion, early surgical procedure induces short and long-lasting effect upon rats' nociceptive response. Besides that, an analgesic pattern was presented 30 minutes after surgery (at P21), which was by administration of picrotoxin, a GABAergic antagonist, confirming the involvement of GABAergic system in this phenomenon.

In addition, opioidergic pathway is partially involved in this analgesic response, once the analgesia reversion was not complete. Thus, the impact of interventions at early life may cause long lasting effects in these individuals' life. In this way, it is important the development of more studies to investigate the impact of surgical procedures in early life, and the mechanisms of these nociceptive responses.

Conflict of Interest

There was no financial relationship between any of the authors or any commercial interest in the outcome of this study.

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