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One-time Exposure to General Anesthetics Alters Nociceptive Response and Nucleotide Hydrolysis in Infant Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author LFM carried out the design of the study, and performed the experimental assays and statistical analysis; authors AS, JRR, and VSS carried out the experimental assays; authors WC and AMOB participated in the design of the study; author ILST coordinated the study, performed the statistical analysis, and helped to draft the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The objective was to evaluate the single exposure of general anesthetics with or without a surgical procedure at postnatal day 14 (P14) on nociceptive behavioral responses. Furthermore, we evaluated ectonucleotidase activities at P14 and P30.
Place of Study: All experiments were performed at the Animal Experimentation Unit of Hospital de Clínicas de Porto Alegre. The Institutional Committee approved the

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experimental protocol f (GPPG-HCPA protocol No: 08149).
Methodology: Fourteen-day-old male Wistar rats were divided into two experimental designs (ED): the 1st ED – control (C), isoflurane (ISO), isoflurane/surgery (ISO-SUR) and the 2nd ED – control (C), fentanyl/S(+)-ketamine (FK) and fentanyl/S(+)-ketamine/surgery (FK-SUR). Nociceptive responses were evaluated using the formalin and tail-flick tests, and the ectonucleotidase activities were evaluated by spinal cord synaptosome. All assessments were performed at P14 and P30.
Results: The FK and FK-SUR groups displayed an increased latency at P30. For the ectonucleotidase activity analysis, the following results were observed: (a) in the 1st ED, the ISO group displayed a reduction in ATPase and ADPase, and both ISO and ISO-SUR displayed a reduction in AMPase activity at P14; (b) in the 2nd ED, the FK group displayed an increase in AMPase activity at P14 and increased ATPase activity at P30, and both FK and FK-SUR exhibited an increase in AMPase activity at P30.
Conclusion: Our results indicate that single administration of general anesthetics at P14 is able to promote changes in the nociceptive response in the intermediate-term, and in the ectonucleotidase activities in the short- and medium-terms.

Keywords: Anesthesia; surgery; rat pups; formalin test; tail-flick; ectonucleotidases.

1. INTRODUCTION

Millions of young children worldwide receive general anesthesia every year, and accumulating evidence is forcing the anesthesia community to question the safety of this procedure at age extremes. Anesthesia in the neonate has been associated with long-term detrimental side effects [1]. Mellon and colleagues [2] suggest that neurodegeneration is a long-term potential risk after anesthetics administration to neonatal and pediatric patients, and this is related to possible cognitive sequelae. Specific doses of ketamine (20–40 mg/Kg) and midazolam (9 mg/Kg) induce significant neuroapoptosis [3]. Studies are needed to better understand the underlying neuroplasticity changes induced by general anesthesia in the immature nervous system and how this can be related to alterations observed in brain function and behavior.

It is important to highlight that other neurotransmitters, such as ATP, can also lead to apoptosis. ATP stimulates cellular excitability by increasing the release of excitatory amino acids that are involved in the nociceptive response [4,5]. The relationship between the purinergic and nociceptive systems was reviewed by Donnelly-Roberts and Jarvis [6]. ATP hydrolysis produces extracellular ADP, AMP, and adenosine, which exert their activities through specific receptors.

ATP and ADP can be hydrolyzed by members of the ecto-nucleoside triphosphate diphosphohydrolase family (E-NTPDases), and AMP can be hydrolyzed by ecto-5’-nucleotidase to produce adenosine [7]. The E-NTPDases control the availability of ligands for both nucleotide and nucleoside receptors, and the duration of receptor activation [8]. These enzymes may provide a protective function by maintaining extracellular ATP/ADP and adenosine within physiological concentrations [9]. Previous studies illustrated the ontogenetic development of ectonucleotidases in the central nervous system (CNS) [10,11]. In addition, our previous study demonstrated that morphine administration during early life alters E-NTPDase activity and gene expression in the rat spinal cord and cerebral cortex [12].

The evaluation of possible physiological effects after general anesthetic exposure is an area of clinical interest. Our previous studies demonstrated that the unique administration of general anesthetics during early life is capable of producing short-, intermediate-, and long-term behavioral alterations [13,14]. Thus, due to the close relationship between purinergic enzymes and the cellular membrane (the membrane may be involved with the mechanism of action of general anesthetics), our present study evaluated the effect of a single exposure to general anesthetic (inhalator and intra-peritoneal) at postnatal day fourteen (P14), with or without performing a surgical procedure in the E-NTPDases and ecto-5'nucleotidase activities and in the nociceptive responses.

2. MATERIALS AND METHODS

2.1 Animals

We used 216 male Wistar rats at 14 days old at the start of the experiment. The rats were housed in cages made of polypropylene material (49 x 34 x 16 cm) with a sawdust floor covering. They were housed with their mothers and maintained on a standard 12-hour dark/light cycle (lights on 7:00 a.m.) at room temperature (22±2ºC). The animals had free access to food and water. Litters were culled to eight pups per dam on P0, and infant rats (P14) were divided into two experimental groups. In the first experimental design, isoflurane was used as a general anesthetic inhalator. The pups were divided into three groups: control (C), isoflurane (ISO), and isoflurane/surgery (ISO-SUR). In the second experimental design, fentanyl/S(+)-ketamine was used as a general anesthetic and rats were divided into three groups: control (C), fentanyl/ S(+)-ketamine (FK), and fentanyl+ S(+)-ketamine/surgery (FK-SUR). The control animals received oxygen and saline in both experimental designs 1 and 2. At 21 days of age, the animals were separated from their mothers. The behavioral tests were performed at P14 (6 hours after the procedure) and P30. The mean weight (g) was 25.45±0.37 for P14 and 67.87±1.51 for P30. The experiment used the number of animals necessary to produce reliable scientific data. To control for the possible effect of outliers, animals that did not present behavioral responses were excluded. It is accepted that rats at P8 have a similar neurological development to that of a human newborn and rats at P21, similar to a 1-year-old child [15]. Additionally, the descending inhibitory system is still in development until P21 [16]. Considering all this information, the animals at P14 were chosen for being in a maturation process of the nociceptive system; however, at P30 these animals already present a mature nociceptive system.

2.2 Pharmacology Treatment and Surgery Procedure

Isoflurane was used as a general inhalatory anesthetic according to Smith and colleagues [17], with some modifications. We used 5% isoflurane for induction and 3% for maintenance, with oxygen (600 mL/min) as the carrier gas.

S(+)-ketamine 20 mg/Kg [intraperitoneal (i.p.)] was used as a general injectable anesthetic. Previous published data has frequently used ketamine in a racemic composition; however, in the present study we used S(+)-ketamine, an enantiomer with more potent effects as an anesthetic and analgesic than the racemic mixture or that of R(-)-ketamine [18]. Published data suggest the use of doses ranging from 10 mg/Kg to 100 mg/Kg [19]. In this study, a dose of 20 mg/Kg was used in the infant rats. For complete anesthesia, it was necessary to include fentanyl (90 µg/Kg; i.p.), an opioid agonist used for analgesia. Fentanyl was injected 10 minutes prior to the use of S(+)-ketamine. We reduced the dose of fentanyl to restore

consciousness more rapidly, despite the published data of Danneman and Mandrell [20] in which 160 µg/Kg of fentanyl was used. Anesthesia was considered to have taken effect when the animal no longer had the ability to retract its foot when pinching stimulated a hind paw.

The Levine method was used for the surgical procedure, as modified by Rice and colleagues [21]. This method normally uses the occlusion of the common carotid artery to produce unilateral brain injury in neonatal rats. For this study, animals at P14 were anesthetized using general anesthetic (isoflurane or fentanyl/ S(+)-ketamine) and exposed to a modified Levine procedure without arterial occlusion. An incision was made to the ventral surface of the neck, parallel and slightly lateral to the trachea. The right common carotid artery was identified, isolated from the nerve and vein, and no occlusion was performed. Animals recovered from anesthesia in an incubator. Following recovery from the procedure, animals were returned to their respective cages to be near their mothers. Control groups (saline and O_2) were given asepsis only in the neck without anesthesia treatment. The anesthesia groups (KF and ISO) were given asepsis in the neck and anesthesia treatment, but no surgery was performed.

2.3 Formalin Test

The formalin test was performed as previously described [22,23] with minor modifications. Twenty-four hours before the test, each animal was placed in the chamber for 10 min to acclimate to the procedure, as the novelty of the apparatus itself can induce antinociception [24]. The animals were injected subcutaneously (s.c.) on the plantar surface of the left hindpaw with 0.17 mL/Kg of 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 Plexiglas box (65 × 25 × 15 cm), and the nociceptive response was recorded for a period of 30 min. This test produces two distinct phases of nociceptive behavior: the early and transient phase (phase I, up to 5 min after the injection) and the late and persistent phase (phase II, 15–30 min after the injection). Phase I has been considered to reflect the direct stimulation of primary afferent fibers, predominantly C-fibers (neurogenic pain) [25], whereas phase II is dependent on peripheral inflammation (inflammatory pain) [23,26,27]. The total time (seconds) spent licking, biting, and flicking of the formalin-injected hindpaw was recorded in phases I and II.

2.4 Tail-flick Test

Rats were tested for antinociception using the tail-flick test [28]. Each animal was placed on the apparatus and its tail was laid across a nichrome wire coil that was then heated using an electric current. The equipment was calibrated to obtain three consecutive baseline tail-flick latencies between 3 s and 5 s. If at any time the animal failed to flick its tail before the temperature reached 75ºC, the tail was removed from the coil to prevent damage to the skin. Three latencies were taken at 3-minute intervals. The animals were exposed to the tail-flick apparatus to acclimate to the procedure 24 h prior to the test session because the novelty of the apparatus can itself induce antinociception [24]. This test was chosen because it has been related to supraspinal activation [29], and can detect systemic analgesia [30].

2.5 Synaptosomal Preparation

After recuperating from the anesthesia, the animals were sacrificed by decapitation, and the spinal cord was rapidly removed, immersed in 10 vol of ice-cold medium consisting of 320 mM sucrose, 0.1 mM EDTA, and 5 mM HEPES (pH 7.5), and gently homogenized using a motor-driven Teflon-glass homogenizer. The synaptosomes were isolated as described previously [31]. Briefly, 0.5 mL of the crude mitochondrial fraction was mixed with 4 mL of 8.5% Percoll solution and layered onto an isosmotic Percoll/sucrose discontinuous gradient (10%/20%). The synaptosomes that banded at the 10%/20% Percoll interface were collected with wide-tip disposable plastic transfer pipettes. The material was prepared fresh daily and maintained at 0–4ºC throughout the preparation procedure.

2.6 Ectonucleotidases Activities

The reaction medium used to assay ATPase and ADPase activities was described previously [32]. The final concentration medium contained 5 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 45 mM Tris–HCl buffer (pH 8.0) in a total volume of 200 μL. The synaptosomal fraction (20 μL) was added to the reaction mixture and pre-incubated for 10 min at 37°C. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1 mM and was stopped by the addition of 200 μL of 10% trichloroacetic acid. The samples were chilled on ice for 10 min, and 100 μL of samples was removed for use in the released inorganic phosphate (Pi) assay [33]. The reaction medium used to assay AMP hydrolysis contained a final concentration of 1.0 mM $MgCl_2$, 100 mM Tris–HCl (pH 7.0), and 150 mM sucrose in a total volume of 200 μL [34]. The synaptosomal fraction (20 μL) was pre-incubated for 10 min at 37°C. The reaction was initiated by the addition of AMP to a final concentration of 1.0 mM and stopped by the addition of 200 μL of 10% trichloroacetic acid; 100 μL samples were used for the released Pi assay [33] The incubation times and protein concentration were determined in pilot studies to ensure the linearity of the reactions. To correct for non-enzymatic hydrolysis of the substrates, controls containing the addition of the enzyme preparation after the addition of trichloroacetic acid were used. All samples were run in duplicate. The protein concentrations were measured by the Coomassie Blue method [35], using bovine serum albumin as the standard. The specific activities are expressed as nmol Pi/min/mg protein.

2.7 Statistical Analysis

The data are expressed as the mean ± standard error of the mean (S.E.M.). A one-way ANOVA was performed followed by a multiple comparisons test (Student-Newman-Keuls method). Differences were considered to be statistically significant if $P<0.05$.

3. RESULTS

The data are presented in two different sections according to the experimental design:

3.1 First Experiment

3.1.1 Effects of isoflurane exposure alone and combined with surgery on nociceptive response at P14 and P30

At P14 and P30, there were no differences in the nociceptive response between the groups (C, ISO, and ISO-SUR) in both phases analyzed using the formalin test (n P14, C=5, ISO=6, ISO+SUR=7 animals; n P30, C=8, ISO= 12, ISO+SUR=10 animals; Table 1).

Table 1. Surgery-associated or unassociated effects of isoflurane on nociceptive behavior in the Formalin test.

Age	Phase	C	ISO	ISO-SUR	F	*P*
P14	1st	135.80±33.27	129.50±22.28	105.86±21.55	0.413	>.05
	2nd	683.20±89.21	630.17±70.71	711.14±76.48	0.291	>.05
P30	1st	111.75±16.33	121.42±21.92	136.50±27.50	0.261	>.05
	2nd	687.50±35.80	667.08±55.96	728.90±56.35	0.371	>.05

The data are reported as the mean ± S.E.M. of time in seconds at each phase. At all phases analyzed in both age groups, no differences were observed (One-way ANOVA, P>.05). C = control group; SUR = animal submitted to anesthesia combined with surgery.

At P14 and P30, there were no differences in the latency between the groups (C, ISO and ISO-SUR) using the tail-flick test (n P14: C=8, ISO=7, ISO+SUR=7 animals, F=3.041; n P30: C=10, ISO =11, ISO+SUR=11 animals, F=0.802; Fig. 1).

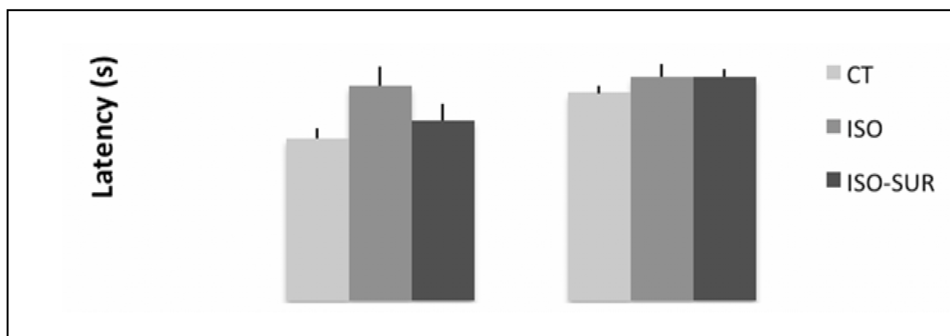

Fig. 1. Surgery-associated or unassociated effects of isoflurane on the behavior evaluated using the tail-flick test.

No differences were observed at either age analyzed (One-way ANOVA, P>.05).
Mean ± S.E.M = Mean values ± Standard error of means.

3.1.2 Effects of isoflurane exposure alone and combined with surgery on ectonucleotidase activities at P14 and P30

At P14, the group that received only isoflurane displayed reduced ATPase and ADPase activities compared to the other groups (Table 2). Both groups that received isoflurane (ISO and ISO-SUR) displayed reduced AMPase activity compared to their respective control (n P14: C=9, ISO=12, ISO+SUR=11 animals; Table 2). At P30, there were no differences observed between the groups (C, ISO, and ISO-SUR) in the enzymatic activities analyzed (*P*>.05; n 30: C= 4, ISO=6, ISO+SUR=6 animals; Table 2).

Table 2. Surgery-associated or unassociated effects of isoflurane on ectonucleotidase activities

Age	Nucleotide hydrolysis	C	ISO	ISO+SUR	F	*P*
P14	ATP	80.05±15.62	47.17±4.73#	75.47±6.45	4.055	< .05
	ADP	20.19±1.76	12.21±1.84#	18.84±1.50	6.338	< .01
	AMP	3.73±0.85	1.72±0.41*	1.63±0.50*	3.908	< .05
P30	ATP	100.12±10.42	100.60±10.81	99.89±8.51	0.001	> .05
	ADP	30.90±9.96	33.11±5.36	25.97±4.05	0.405	> .05
	AMP	7.98±1.67	7.07±1.25	6.93±0.55	0.215	> .05

*The data are reported as the mean ± S.E.M. of nucleotide hydrolysis. #Significant difference from the other groups. *Significant from control group.*

3.2 Second Experiment

3.2.1 Effects of fentanyl/S(+)-ketamine exposure alone and combined with surgery on nociceptive response at P14 and P30

At P14 and P30, there were no differences in the nociceptive behavior between the groups (C, FK, and FK-SUR) during both phases of the formalin test (n P14: C=5, FK=7, FK+SUR=7 animals; n P30: C=7, FK=10, FK+SUR=10 animals; Table 3).

Table 3. Surgery-associated or unassociated effects of fentanyl/S(+)-ketamine on nociceptive behavior in the formalin test.

Age	Phase	C	FK	FK-SUR	F	P
P14	1st	148.20±25.51	186.86±14.06	137.14±20.05	1.958	>.05
	2nd	720.60±75.78	793.14±22.92	755.29±66.32	0.388	>.05
P30	1st	83.43±23.69	100.30±16.19	108.44±11.12	0.515	>.05
	2nd	545.00±107.73	655.10±75.16	668.67±59.12	0.648	>.05

The data are reported as the mean ± S.E.M. of time in seconds at each phase. At all phases analyzed in both age groups, no differences were observed (One-way ANOVA, P>.05). C = control group; SUR = animal submitted to anesthesia combined with surgery.

At P14, there were no differences in the latency between the groups (C, FK, and FK-SUR) in the tail–flick test (F=0.086; n C=7, FK=6, FK+SUR=6 animals; Fig. 2). At P30, both groups that received fentanyl/S(+)-ketamine demonstrated an increased latency compared to the control group (F=7.491; n C=14, FK=11, FK+SUR=13 animals; Fig. 2).

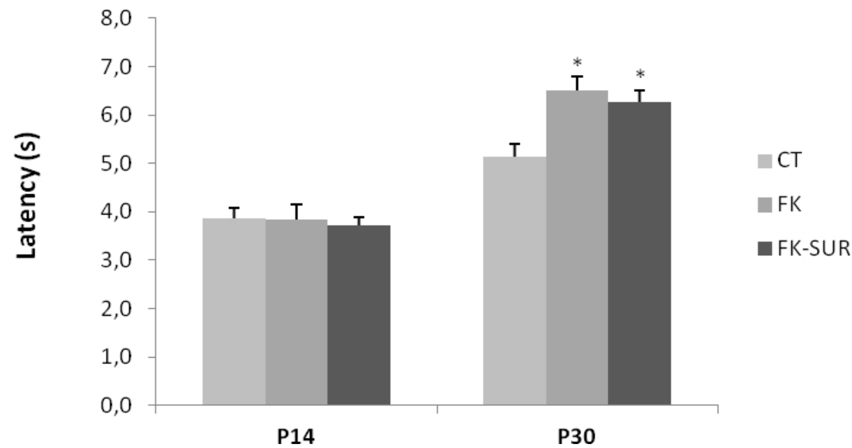

Fig. 2. Surgery-associated or unassociated effects of fentanyl/S(+)-ketamine on the behavior evaluated in tail-flick test.

**Significant from control group.*

Mean ± S.E.M = Mean values ± Standard error of means.

3.2.2 Effects of fentanyl/S(+)-ketamine exposure alone and combined with surgery on ectonucleotidase activities at P14 and P30

At P14, the FK group displayed an increase in AMPase activity compared to the other groups (n=4 animals/group; Table 4). At P30, the FK group exhibited an increase in ATPase activity compared to the other groups (Table 4). Both groups FK and FK-SUR displayed an increase in AMPase activity compared to the control group (n C=6, FK=3, FK+SUR=3 animals; Table 4).

Table 4. Surgery-associated or unassociated effects of fentanyl/S(+)-ketamine on ectonucleotidase activities

Age	Nucleotide hydrolysis	C	FK	FK+SUR	F	*P*
P14	ATP	103.34±16.00	93.10±11.69	97.78±7.22	0.178	> .05
	ADP	19.94±1.66	21.52±2.40	26.57±4.42	1.230	> .05
	AMP	2.18±0.38	3.57±0.56#	1.32±0.18	8.210	< .05
P30	ATP	99.96±4.78	146.18±8.92#	104.96±1.83	17.189	< .01
	ADP	42.50±3.40	50.81±4.60	47.58±3.39	1.245	> .05
	AMP	7.63±1.05	14.97±1.71*	12.71±0.79*	10.244	< .01

*The data are reported as the mean ± S.E.M. of nucleotide hydrolysis. #Significant difference from the other groups. *Significant from control group.*

3.3 Comparison of the effects upon ectonucleotidases between the two anesthetics protocols

At P14, the ISO group presented a decreased ATPase activity in relation to other groups, and decreased ADPase activity in relation to FK and FK-SUR groups. The ISO, ISO-SUR, and FK groups presented decreased AMPase activity in relation to FK group (data not shown).

At P30, the FK-SUR presented increased AMPase activity in relation to ISO and ISO-SUR (data not shown).

4. DISCUSSION AND CONCLUSION

This study demonstrated that a single administration of fentanyl combined with S(+)-ketamine at P14 (KF and KF-SUR groups) causes alterations in the nociceptive response (analgesic behavior) at the intermediate timepoint (P30), but neither group displayed differences in the neurogenic and inflammatory response. On the other hand, the animals exposed to isoflurane (ISO and ISO-SUR) at P14 did not show any alteration in the nociceptive response. Thus, these results demonstrate that isoflurane may be used in animal models to evaluate nociception, because it presented no alteration in the nociceptive behavior in the short- and medium-term since the association of fentanyl and S(+)-ketamine was able to sensitize this pathway.

Differences between formalin and tail-flick tests need to be considered. In the tail-flick, the nociceptive response is related to the reflex of the spinal cord [36,37] but the response remains under control of supraspinal structures [38]; it involves phasic pain, with short duration stimulus, and the pain threshold is measured and involves the thermal stimulation of Aδ fibers [39]. In contrast, the formalin test involves tonic pain, a long-term stimulus that triggers the nociceptive response, which involves the stimulation of C-type fibers [39]. The Aδ fibers are present at birth (P0) while the C fibers are in process of maturation during the first three weeks of life [40]. During this time period, there is an increase of C-type fibers in the spinal cord and the corresponding decrease in Aδ fiber type does not occur immediately. Therefore, both fibers occupy the same space within the spinal cord [40]. Interestingly, our previous study [14] showed that when we used fentanyl or S(+)-ketamine alone at P14, no differences in the tail-flick latency test were observed, regardless of the age analyzed (P14 or P30). In the present study, the fentanyl/S-(+)-ketamine administered at P14 was capable of promoting an analgesic response at P30. Thus, we suggest this unique administration during the maturation stage (at P14) can promote an adaptive process in the nociceptive response mainly involving Aδ fibers.

Additionally, the effect observed at P30 may be due to the infant's immature nervous system, which is highly sensitive to the depressant effects of anesthetics [41]. General anesthetics are able to interact with a variety of neuronal systems, including the GABAergic, glycinergic, cholinergic, and glutamatergic systems [42,43]. The NMDA receptors play an important role in the development and plasticity of connections in the immature CNS [44]. The affinity and density of NMDA receptors are similar in both neonates and adults; however, their distribution differs. In adults, these receptors are restricted to the gelatinous substance, while in neonates they are distributed throughout the marrow and achieve the adult distribution only at P28 [45]. During the postnatal period, until the third week of life, there is a reorganization of the subunits of the NMDA receptors, and also a relocating of the spinal cord [46]. We cannot exclude a possible adaptive process in the transmission and/or modulation of the glutamatergic system in the analgesia observed on the 16th day post drug administration taking into account the maturation process.

It is interesting to note that S(+)-ketamine was combined with fentanyl, an opioid agonist. Recently, our group showed an interaction between opioid agonist administration and NMDA receptors. In that study we showed that repeated morphine exposure in early life (5 μg sc/day/7 days from P8) promotes a hyperalgesic response to noxious events in adult life of rats, which was reversed by a NMDA receptor antagonist [47]. Another study described

nociceptive changes at P17 and at P55 after fentanyl infusion (50 µg/Kg/hour for 72 hours at P14–P16) [48]. Both models described before illustrate that the repetitive administration of an opioid agonist causes long-term alterations in the nociceptive response. On the other hand, in the present study, we show that a single administration of fentanyl combined with S(+)-ketamine at P14 was able to produce altered nociceptive response (analgesia) in the medium term.

Another important neurotransmission system in the nociceptive response is the purinergic system [6]. In addition, in a previous study, our group showed that repeated opioid agonist administration in early life alters E-NTPDase activity and gene expression in the rat spinal cord [12]. In the present study, we show that a single administration of S(+)-ketamine/fentanyl at P14 was able to change adenine nucleotide hydrolysis in synaptosomes of the spinal cord at short (P14) and intermediate (P30) timepoints, associated with increased AMPase activity at both. Highlighting that, the increased AMPase activity at P14 and the increased ATPase activity at P30 were reversed by the surgical procedure. We can suggest that the increased level of extracellular ATP due to invasive procedures [4,5,49] reverses the increased AMPase activity (FK-SUR group) since both ATP and ADP levels inhibit its activity [50]. It is known that the NTPDase 2 or ecto-ATPase has preference for nucleotide triphosphates over the diphosphates (30:1), thus, we suggest that NTPDase 2 is the enzyme involved in the effect observed at P30. In addition, it is important to consider that the ADP generated by enzymatic degradation of ATP is a potent agonist at P2Y [51,52], thereby decreasing the excitatory neurotransmission in sensory neurons and minimizing the ATP algogenic side-effects [53]. Thus, considering that the endogenous adenosine is involved in the physiological control of pain and opioid antinociception [54], we suggest that the increase in AMP hydrolysis observed at both ages generates higher concentrations of extracellular adenosine and may partially explain the analgesia observed in the animals (at least in P30).

On the other hand, the animals that received isoflurane presented a reduction in the ATPase, ADPase, and AMPase activities in spinal cord of rats in the short term (at P14). These effects can be explained by the alteration in the fluidity of cell membranes arising from the action of inhalational anesthetic, since the ectonucleotidases are anchored in the cell membranes. We highlighted that the surgical procedure was able to reverse these effects that may result from increased level of extracellular ATP. This ATP increase is due to a variety of mechanisms, including mechanical stimulation, vesicular release with other neurotransmitters (e.g., acetylcholine, noradrenaline, glutamate, GABA, or neuropeptide Y) or cellular damage (e.g., surgery or hypoxia) [4,5,49]. Therefore, we suggest that the last effect observed from surgery on ATP/ADP hydrolysis may be related to a compensatory effect of the enzyme activity to control probable increased extracellular ATP levels caused by cell lysis.

In addition, we hypothesize that the enzyme probably involved in our result is the NTPDase 1 or ecto-apyrase that hydrolyzes nucleotide triphosphates and diphosphates at a 1:1 proportion. The activation of NTPDase 1 promotes the conversion of extracellular ATP into AMP, and consequently AMP is converted into adenosine by ecto-5'nucleotidase without ADP production. Based on our results, even with the inhibition of NTPDase 1, ATP could be converted to ADP by other ecto-ATPases present on the cell surface.

Moreover, we compared the ectonucleotidase activities between the two experimental designs and found that, at P14, the ISO group presented a decreased ATPase activity in relation to other groups, and decreased ADPase activity in relation to FK and FK-SUR

groups. The ISO, ISO-SUR, and FK groups presented decreased AMPase activity in relation to the FK group. The surgery procedure reversed the alteration in the ATPase activity after isoflurane exposure, showing that this enzyme is sensitive to noxious intervention; however, the surgery did not reverse after FK exposure where the drug effect is longer. In relation to AMPase activity, we showed an opposite effect, since this change was only observed in the FK group. We suggest that these enzyme activities can vary according to the type of intervention. The exact mechanism of action of inhalator anesthetics is not currently well established, but it is believed that it involves membrane fluidity, and this is an important point since the ectonucleotidase enzymes are anchored in the cytoplasmic membranes. On the other hand, at P30, the FK-SUR presented increased AMPase activity in relation to ISO and ISO-SUR. In this case, we can suggest that the early surgical procedure (with fentanyl and ketamine) induced long-term spinal cord sensitization as shown by increased of 5'ectonucleotidase activity.

The small number of animals in the ectonucleotidase activity assays is an important limitation in our study. However, we found a statistically significant effect with this number of animals, thus there was no need for a higher number. In addition, previous studies of our group used the same number of animals [55,56], and it is in agreement with international ethical recommendations for animal number reduction (Guide for the Care and Use Of Laboratory Animals, 8th ed, 2011).

In conclusion, our results demonstrate the importance of extending studies related to general anesthetics administered during the CNS maturation period when different biological systems are being developed or maturing. We observed alterations in the nociceptive response and neurochemical alterations of ectonucleotidases at short and/or intermediate timepoints after a single exposure to general anesthetics at P14, and these effects were mainly related to fentanyl and S(+)-ketamine. Further studies are necessary to elucidate the mechanisms involved in the observed alterations. In addition, these findings indicate the importance of evaluating the clinical consequences of anesthetics administration in early life and highlight the need for further studies involving these agents.

CONSENT

It is not applicable for the present manuscript.

ETHICAL APPROVAL

The Institutional Committee for Animal Care and Use approved the experimental protocol (GPPG-HCPA protocol No: 08149). All authors hereby declare that "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) were followed. All experiments were performed in accordance with the NIH Publications No. 85-23, Brazilian Community's Council Directive of October 8, 2008 (Law No. 11.794) and conformed to the Guide for the Care and Use of Laboratory Animals, 8th ed, 2011. Animal handling and all experiments were performed in accordance with Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council 2003) for animal welfare and measures were taken to minimize animal pain and discomfort. The experiment used the number of animals necessary to produce reliable scientific data. To control for the possible effect of outliers, we excluded those rats that did not present any response in the behavioral tests. All the experimenters were blinded to the treatment conditions during the post-treatment behavioral test.

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COMPETING INTERESTS

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

REFERENCES

1. Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J Neurosci. 2003;23:876–82.
2. Mellon RD, Simone AF, Rappaport BA. Use of anesthetic agents in neonates and young children. Anesth Analg. 2007;104:509–20.
3. Young C, Jevtovic-Todorovic V, Qin YQ, Tenkova T, Wang H, Labruyere J, John W, Olney JW. Potential of ketamine and midazolam, individually or in combination, to induce apoptotic neurodegeneration in the infant mouse brain. Br J Pharmacol. 2005;146(2):189–197.
4. Burnstock G, Williams M. P2 purinergic receptors: modulation of cell function and therapeutic potential. J Pharmacol Exp. 2000;295:862–69.
5. Burnstock G. Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev. 2007;87:659–797.
6. Donnelly-Roberts DL, Jarvis MF. Discovery of P2X7 receptor-selective antagonists offers new insights into P2X7 receptor function and indicates a role in chronic pain states. Br J Pharmacol. 2007;151(5):571–9.
7. Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H. Purinergic signalling in the nervous system: an overview. Trends Neurosci. 2009;32(1):19–29.
8. Chen W, Guidotti G. Soluble apyrases release adp during ATP hydrolysis. Biochem Biophys Res Commun. 2001;282(1):90–5.
9. Agteresch HJ, Dagnelie PC, van den Berg JW, Wilson JL. Adenosine triphosphate: established and potential clinical applications. Drugs. 1999;58:211–32.
10. Torres IL, Battastini AM, Buffon A, Fürstenau CR, Siqueira I, Sarkis JJ, Dalmaz C, Ferreira MB. Ecto-nucleotidase activities in spinal cord of rats changes as function of age. Int J Dev Neurosci. 2003;21(8):425–9.
11. de Paula Cognato G, Bruno AN, Vuaden FC, Sarkis JJ, Bonan CD. Ontogenetic profile of ectonucleotidase activities from brain synaptosomes of pilocarpine-treated rats. Int J Dev Neurosci. 2005;23(8):703–9.
12. Rozisky JR, da Silva RS, Adachi LS, Capiotti KM, Ramos DB, Bogo MR, Bonan CD, Sarkis JJ, Torres IL. Neonatal morphine exposure alters E-NTPDase activity and gene expression pattern in spinal cord and cerebral cortex of rats. Eur J Pharmacol. 2010;642(1–3):72–6.

13. Medeiros LF, Rozisky JR, de Souza A, Hidalgo MP, Netto CA, Caumo W, Battastini AM, Torres IL. Lifetime behavioural changes after exposure to anaesthetics in infant rats. Behav Brain Res. 2011;218(1):51–6.
14. Medeiros LF, de Souza AC, de Souza A, Cioato SG, Scarabelot VL, Caumo W, Fernandes LC, Torres IL. Fentanyl administration in infant rats produces long-term behavioral responses. Int J Dev Neurosci. 2012;30(1):25–30.
15. Fitzgerald M, Anand KJ. Developmental neuroanatomy and neurophysiology of pain. In: Schechter NL, Berde CB, Yaster M (ed). Pain in infant, children, and adolescents. Williams & Wilkins, Baltimore. 1993;11–31.
16. Fitzgerald M. Nat Neurosci Rev. 2005;6:507–520.
17. Smith JC, Corbin TJ, McCabe JG, Bolon B. Isoflurane with morphine is a suitable anaesthetic regimen for embryo transfer in the production of transgenic rats. Lab Anim. 2004;38:38–43.
18. Reich DL, Silvay G. Ketamine: an update on the first twenty-five years of clinical experience. Can J Anaesth. 1989;36:186–97.
19. Freo U, Ori C. Effects of anesthesia and recovery from ketamine racemate and enantiomers on regional cerebral glucose metabolism in rats. Anesthesiology. 2004;100:1172–8.
20. Danneman PJ, Mandrell TD. Evaluation of five agents/methods for anesthesia of neonatal rats. Lab Anim Sci. 1997;47:386–95.
21. Rice JE, Vannucci RC, Brierley JB. The influence of immaturity on hypoxic–ischemic brain damage in the rat. Ann Neurol. 1981;9:131–41.
22. Tai YH, Wang YH, Wang JJ, Tao PL, Tung CS, Wong CS. Amitriptyline suppresses neuroinflammation and up-regulates glutamate transporters in morphine-tolerant rats. Pain. 2006;124(1–2):77–86.
23. Tjølsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. Pain. 1992;51(1):5–17.
24. Netto CA, Siegfried B, Izquierdo I. Analgesia induced by exposure to a novel environment in rats: effect of concurrent and post-training stressful stimulation. Behav Neural Biol. 1987;48(2):304–9.
25. Martindale J, Bland-Ward PA, Chessell IP. Inhibition of C-fibre mediated sensory transmission in the rat following intraplantar formalin. Neurosci Lett. 2001;316:33–36.
26. Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain. 1977;4:161–74.
27. Shibata M, Ohkubo T, Takahashi H, Inoki R. Modified formalin test: characteristic biphasic pain response. Pain. 1998;38:347–52.
28. Castilho VM, Macedo CE, Brandão ML. Role of benzodiazepine and serotonergic mechanisms in conditioned freezing and antinociception using electrical stimulation of the dorsal periaqueductal gray as unconditioned stimulus in rats. Psychopharmacology (Berl). 2002;165(1):77–85.
29. Tseng LF, Tang R. Different mechanisms mediate beta-endorphin- and morphine-induced inhibition of the tail-flick response in rats. J Pharmacol Exp Ther. 1990;252:546–51.
30. Cepeda MS, Bonney I, Moyano J, Carr DB. Corticotropin-releasing factor hormone (CRH) produces analgesia in a thermal injury model independent of its effect on a systemic beta-endorphin and corticosterone. Regul Pept. 2004;118:39–43.
31. Nagy AK, Houser C, Delgado-Escueta AV. Rapid preparation of synaptosomes from mammalian brain using nontoxic isosmotic gradient (Percoll). J Neurochem. 1984;43:1114–23.

32. Battastini AMO, da Rocha JBT, Barcellos CK, Dias RD, Sarkis JJF. Characterization of an ATP diphosphohydrolase (EC 3.6.1.5) in synaptosomes from cerebral cortex of adult rats. Neurochem Res. 1991;16:1303–10.
33. Chan K, Delfert D, Junger KD. A direct colorimetric assay for Ca + 2- ATPase activity. Anal Biochem. 1986;157:375–80.
34. Heymann D, Reddington M, Kreutzberg GW. Subcellular localization of 5′-nucleotidase in rat brain. J Neurochem. 1984;43:971–78.
35. Bradford MMA. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem. 1976;72:218–51.
36. Irwin S, Houde RW, Bennett DR, Hendershot LC, Steevers MH. The effects of morphine, methadone and meperidine on some reflex responses of spinal animals to nociceptive stimulation. J Pharmacol Exp Ther. 1951;101:132–43.
37. Sinclair JG, Main CD, Lo GF. Spinal vs supraspinal actions of morphine on the rat tail-flick reflex. Pain. 1988;33:357–62.
38. Mitchell D, Hellon RF () Neuronal and behavioral responses in rats during noxious stimulation of the tail. Proc R Soc Lond B Biol Sci. 1977;197:169–94.
39. Le Bars D, Gozariu M, Cadden SW () Animal Models of Nociception. Pharmacol Rev. 2001;53:597–652.
40. Fitzgerald M. Developmental biology of inflammatory pain. Brit J Anaes. 1995;75:177–85.
41. Soriano SG, Anand KJS. Anesthetics and brain toxicity. Curr Opin Anaesthesiol. 2005;18(3):293–7.
42. Krasowski MD, Harrison NL. General anaesthetic actions on ligand-gated ion channels. Cell Mol Life Sci. 1999;55:1278–303.
43. Dilger JP. The effects of general anaesthetics on ligand-gated ion channels. Br J Anaesth. 2002;89:41–51.
44. Garthwaite J. NDMA receptors, neuronal development and neurodegeneration. In: Watkins JC, Collinridge GL (Eds). The NMDA receptor. Oxford: IRL Press. 1989;187–205.
45. Kalb RG, Hockfield S. The distribution of spinal cord N-methyl-D-aspartate receptors is developmentally regulated. Society for Neuroscience Abstracts. 1991;17:1534.
46. Pattinson D, Fitzgerald M () The neurobiology of infant pain: Development of excitatory and inhibitory neurotransmission in the spinal dorsal horn. Reg Anesth Pain Med. 2004;29:36–44.
47. Rozisky JR, Medeiros LF, Adachi LS, Espinosa J, de Souza A, Neto AS, Bonan CD, Caumo W, Torres IL. Morphine exposure in early life increases nociceptive behavior in a rat formalin tonic pain model in adult life. Brain Res. 2011;1367:122–9.
48. Thornton SR, Smith FL. Long-term alterations in opiate antinociception resulting from infant fentanyl tolerance and dependence. Eur J Pharmacol. 1998;363(2–3):113–9.
49. North RA. Molecular physiology of P2X receptors. Physiol Rev. 2002;82:1013–1067.
50. Cunha RA, Sebastião AM. Extracellular metabolism of adenine nucleotides and adenosine in the innervated skeletal muscle of the frog. Eur J Pharmacol. 1991;197:83–92.
51. Burnstock G. Purinergic signalling. Br J Pharmacol. 2006;147(Suppl 1):S172–81.
52. Zimmermann H. Nucleotide signaling in nervous system development. Pflugers Arch. 2006;452(5):573–88.
53. Gerevich Z, Borvendeg SJ, Schröder W, Franke H, Wirkner K, Nörenberg W, Fürts S, Gillen C, Illes P. Inhibition of N-type voltage-activated calcium channels in rat dorsal root ganglion neurons by P2Y receptors is a possible mechanism of ADP-induced analgesia. J Neurosci. 2004;24(4):797–807.

54. Bennett GJ. Update on the neurophysiology of pain transmission and modulation: focus on the NMDA-receptor. J Pain Symptom Manage. 2000;19:S2–S6.
55. Rozisky JR, Vendite D, Fontella FU, Nonose Y, Laste G, Dalmaz C, Caumo W, Torres IL. Morphine treatment in early life alters glutamate uptake in the spinal synaptosomes of adult rats. Neurosci Lett. 2012;31;529(1):51–4.
56. Torres IL, Buffon A, Dantas G, Fürstenau CR, Böhmer AE, Battastini AM, Sarkis JJ, Dalmaz C, Ferreira MB. Chronic stress effects on adenine nucleotide hydrolysis in the blood serum and brain structures of rats. Pharmacol Biochem Behav. 2002;74(1):181–6.

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