UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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Influence of Environmental Enrichment on Morphine-Exposed Neonate Rats:
Effect on Neurodevelopment and Long-term Memory

Natalia de Paula Silveira

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Influence of Environmental Enrichment on Morphine-Exposed Neonate Rats:

Effect on Neurodevelopment and Long-term Memory

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Highlights

- Morphine early in life may affect neural development;
- Environmental enrichment alters long-term memory in neonate rats;
- Environmental enrichment is a promising tool to treat neonatal noxious stimuli.

Abstract

The stressful stimuli exposure and the use of drugs in early life may affect neurodevelopment, altering behaviors, memory and nociceptive response. These factors have been related to the delay in recovery of patients after medical procedures in hospitals. In this study, we investigated the influence of environmental enrichment (EE) on morphine exposure effect on neurodevelopment and memory of neonate rats. 28 pups were divided into four experimental groups: saline + standard housing (SS), saline + environmental enrichment (SE), morphine + standard housing (MS) and morphine + environmental enrichment (ME). The newborns received daily (P8-P14) subcutaneous injections of saline (5 µl) or morphine (5 µg/5 µl), and they were submitted to manipulation or EE (P8-P21). The righting reflex and the negative geotaxis, the object recognition and the hot plate tests were used to evaluate neuromotor reflexes, long-term memory and thermal hyperalgesia, respectively. Animals that received morphine showed longer neuromotor reflexes response time compared to the saline group, an effect that was age-dependent. Animals that received morphine showed less ability to recognize a new object in the environment, an effect that was partially reversed by EE. Nociceptive response was not altered for morphine nor EE. These findings demonstrate that neonatal morphine exposure alters the neurodevelopment and long-term memory in pup rats, without affecting nociceptive response. Thus, EE can be a promising non-pharmacological treatment to the consequences of neonatal noxious stimuli.

Keywords: morphine; nociception; pup rats; long-term memory; neurodevelopment.

1. INTRODUCTION

The use of opioids, such as morphine, has increased in neonatal intensive care units (NICUs) (Pacifici, 2016; Maciel et al., 2019) due to changes and advances in the identification, understanding, and treatment of pain in children. However, it has been documented that exposure to opioids can have deleterious effects on the development of embryo and newborns, causing distress and discomfort (Pereira et al., 2007; Pacifici, 2016). Environmental Enrichment (EE) is a non-pharmacological strategy used to improve neurodevelopment, combining stimuli that improve sensory, cognitive and motor skills. Furthermore, EE can benefit animals in their ability to learn and to memorize, stimulating curiosity in exploring the environment (Piazza et al., 2014).

The first reflexes in newborns are representative of the development of motor skills. The observation of such reflexes enables to identify persistence or delay in neurodevelopment, which may be related to behavioral changes in adult life (Heyser, 2004; Berk, 2006). Thus, in early childhood, exposure to drugs has been related to alterations in neurodevelopment, for example, long-term memory impairment (Lee et al., 2014).

Neurotransmission systems that drive painful stimuli to the cerebral cortex are already present in preterm and term newborn babies (Guinsburg et al., 2000). Newborn babies are susceptible to changes in sensitivity to painful stimuli, indexed by hyperalgesia and allodynia (Grunau et al., 2001), and to stressors (Grunau et al., 2004). Clinical evidence demonstrates that the exposure to painful events in childhood may be related to psychiatric disorders in adulthood such as anxiety, depression, fear, and schizophrenia (Reichert et al., 2000; McGrath et al., 2008; and Pillai Riddell, 2013, Victoria and Murphy, 2016), suggesting that the exposure to aversive events in childhood can have lifelong consequences.

Studies in animal models have shown that at the 1st postnatal day (P1), opioid receptors are already widely distributed in the central nervous system (CNS), with μ (mu) and k (kappa)

receptors predominating in this period. The peak density of μ receptors occurs on the seventh postnatal day (P7) and gradually declines until the third week of life, when it reaches the levels that are found in adults (Beland and Fitzgerald, 2001; Kar and Quirion, 1995). It has also been shown that changes caused by morphine during the prenatal period may have an impact on synaptic plasticity processes throughout development (Schrottet et al., 2008). Previous studies from our group have shown that animals repeatedly exposed to morphine in early life exhibit hypernociceptive behavior in adult life (Rozisky et al., 2011). In addition, exposure to morphine early in life may lead to changes in the developing CNS, with long-term neurochemical and behavioral changes in rodents (Rozisky et al. 2008, Oliveira et al. 2017).

The exposure to stressful stimuli is an environmental factor related to alteration in the neural development process (Lai et al., 2006; Aisa et al., 2009). Thus, the environment is a determining factor from birth to early adulthood, when the CNS is more susceptible to environmental alterations (Fernández-Teruel et al., 2002; Nunes et al., 2003). However, there is little information on the ideal period for EE exposure, like a non-pharmacological neuroprotection feature in neonates. EE studies have been conducted to study the impact of environmental conditions on behavioral, morphological and functional adaptations in different experimental models (Segovia et al., 2009; Fox et al., 2006).

EE has been related to the alteration of neuroplastic processes such as neurogenesis (Van Praag et al., 2000, Kotloski et al., 2015), neurotrophin levels (Rossi et al., 2006), neuronal survival, synaptogenesis and cell proliferation (Mohammed et al., 2002). Previous studies have shown that animals that grew up in an enriched environment have increased cortical mass and neurotransmitter levels, such as noradrenaline and dopamine (Naka et al., 2002, O'Shea et al., 1983), as well as, a better performance in learning tasks (Kobayashi et al., 2002). However, there are a limited number of studies about the effects of EE on the nociceptive response.

Finally, the EE preclinical studies have a high relevance, since positive- effects can be observed (Durán-Carabali et al., 2018; Khalaji et al., 2018).

Taken together, preclinical studies using EE are necessary to a better understanding of its effects in improving cognition and neuroprotection in experimental animal models. Thus, the aim of this study was to evaluate the short term effects of early EE in neurodevelopment, long-term memory and nociceptive response after neonatal morphine exposure in male rats.

2. MATERIAL AND METHODS

2.1 Animals

A total of 28 male Wistar rat pups were used in the present study. Pups were kept together with their mothers in polypropylene home cages (70 x 70 x 50 cm) with the floor covered using sawdust bedding. Animals were maintained on a standard 12 h dark/light cycle (lights on at 7:00 a.m.) in a temperature-controlled environment (22 ± 2 ºC), and had *ad libitum* access to water and standard rodent chow. Female pups were not used because of the influence of hormonal oscilations on the nociceptive process and drug response (Ribeiro et al., 2005). At birth, litters were standardized according to Silveira et al. (Silveira et al., 2010, 2011), with minor modifications, in 7 male pups per dam. Standardization of litters in the first day after birth promotes a uniform nutritional condition for all pups (Tanaka et al., 2004). All the procedures described in this experiment were approved by the Institutional Committee for Animal Care and Use (GPPG/HCPA protocol #18-0187), conducted in compliance with the Brazilian law #11.794 (2008) and its normative regulations (MCTI, 2013a; MCTI, 2013b) and the Laboratory Guide for the Care and Use of Animals (The National Academies Press, Eighth Edition, 2011). Vigorous attempts were made to minimize animal suffering, and decrease external sources of pain and discomfort, as well as to use the minimum number of animals required to produce reliable scientific data.

2.2 Environmental Enrichment

The early EE protocol was adapted from Pereira et al. (2007; 2008), and consists of free exploration of objects, by the mother and the puppies, inside the acrylic box-dwelling. The following enrichment artifacts were used: tow nest, aluminum ramp, racing wheel, plastic tunnel, cotton nests, rope, craft paper nest, plastic dog bone, and wooden slabs covered with edible paint (Khalaji et al., 2018; Neal et al., 2018). EE was carried out between P8 and P21 for the enriched groups, during which weekly housecleaning was performed and, concurrently, the replacement of objects to maintain the novelty factor. All pups were maintained with their dams until weaning (P21).

2.3 Experimental design

After birth and standardization of litters, animals were randomly assigned to one of the following groups: saline + standard housing (SS); saline + enriched housing (SE); morphine + standard housing (MS) or morphine + enriched housing (ME). Animals were treated (saline or morphine) from P8 to P14 and exposed to EE or manipulation between P8 and P21. Behavioral tests were blindly performed, always at the same time of the day (2:00 p.m.). Initial reflex tests were carried out from P9 to P20. Pups were tested for nociception and memory on P20.

2.4 Pharmacological Treatment

Rats received daily (P8 – P14) mid-scapular subcutaneous injection of either saline (5 µl, SS and SE groups) or morphine (5 µg / 5 µl, MS and ME groups). This dose was chosen based on previous studies of our research group (Rozisky et al., 2008, 2010, 2011, 2012a, 2012b). The neurological development in rats at P8 is similar to that of a human newborn, which justifies starting treatment at this stage (Fitzgerald e Anand, 1993). Moreover, it is accepted that they are physiologically immature (Pattinson e Fitzgerald, 2004), since this period is

characterized by large cerebral and neuroplastic variations of the developing pain system (Rabinowicz et al., 1996; Kim et al., 1996; Shapiro, 2001). All treatments were given at the same time each day (11:00 a.m). One milliliter of morphine sulfate was diluted in 9 mL of sterile 0.9% NaCl. Morphine Sulfate (Dimorf® 10 mg/mL, Cristália Ltda., São Paulo, Brasil) was provided by Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil.

2.5 Development of Neuromotor Reflexes

2.5.1 Negative geotaxis

The negative geotaxis test consists of a postural reaction, making the animal face upwards, when placed on a sloped surface facing downwards. The test was carried out at P10, P12, P14, P16, P18 and P20. Pups were placed on a 35 cm-long inclined platform (45º slope), facing the downslope. The animals were expected to turn around 180º to face upwards, and climb up the board with their forepaws reaching the upper edge of the board. The latency to turn 180º was registered using a digital chronometer. The results of the test were considered negative if the pups did not succeed in this task within the observed time period of 60 s (Motz and Alberts, 2005; de Oliveira et al., 2017).

2.5.2 Righting Reflex

The righting reflex test was carried out on P9 and P10. Pups were placed in a supine position, and the latency (s) to turn over their longitudinal axis to restore a normal prone position was measured. This was considered fully achieved when the pups turned 180º around their longitudinal axis, and their four paws were in contact with the plane surface within the observed 60 s time frame (Karalis et al. 2011; de Oliveira et al. 2017).

2.6 Long-term Memory Test

2.6.1 Object Recognition

The object recognition test was performed in an open field (60 x 40 x 50 cm) (protocol adapted from Myskiw et al., 2008 and Benetti et al., 2009). The test consists of three phases: habituation, training and testing. During the habituation phase (P18) the animal explored the open field for 5 minutes. On the day of the training session (P19) the animals were placed in the arena in the presence of two distinct objects (A and B) and stayed in place to explore them for 5 minutes. The test session was performed 24 hours after the training session (P20) to assess long-term memory. In the test phase, a copy identical to the old object (A or B) was placed in the open field along with a new T-shaped object (C) and the animal was again placed in the arena and tested for 5 minutes. Exploration of an object was defined as directing the nose to the object at a distance of equal to or less than 2 cm or touching it with the nose. The time spent exploring each object was recorded by the researcher and expressed as the percentage of the total exploration time in seconds (Rossato et al., 2007). The objects were build using plastic LEGO blocks (Leffa et al., 2016). The test was videorecorded and afterwards blindly analyzed.

2.7 Nociceptive Test

2.7.1 Hot Plate Test (HP)

The HP test is used as an indicator of the supraspinal pain process and evaluates thermal nociceptive threshold (Ossipov et al., 1995). HP was performed at P20. Briefly, 24 hours prior to the test, all animals were habituated to the apparatus for 5 min to avoid novelty-induced analgesia (Netto et al., 1987). Licking the paws or jumping responses were considered to be the result of supraspinal sensory integration (Caggiula et al., 1995; Rubinsteinet et al., 1996). Animals were placed in glass funnels on the heated surface (55 ± 0.1 °C), and the time between the placement of the rat and the first response (i.e., foot licking, jumping, or rapid removing of

paws) was identified as the latency for the nociceptive response. Additionally, a cutoff time of 20 s was employed to avoid tissue damage (Woolfe and Macdonald, 1944).

2.8 Statistical analysis

The results of the neurobehavioral tests and object recognition were analyzed using a generalized estimation equation (GEE). The nociceptive behavioral test was evaluated using non-parametric Kruskall Walis test. To control for possible effect of outliers, those animals that did not present behavioral responses were excluded from the analysis. Data were expressed as mean ± standard error of the mean (SEM). All results were considered statistically significant if $p < 0.05$. The SPSS 20.0 software was used for statistics.

3. RESULTS

3.1 Neuromotor development

3.1.1 Negative Geotaxis

On P10, P12, P14, P16, P18 and P20 all pups were submitted to the negative geotaxis test. GEE statistic results indicate an effect of age and group interaction * time (GEE: Wald χ_2= 100,465; 15, $p < 0.05$), demonstrating a developmental effect. On P12, the SS group presented a faster response than the group SE (GEE: Wald χ_2= 8,562; 3, $p < 0.05$). In P16 the groups receiving morphine showed a delay in response compared to saline groups (GEE: Wald χ_2 =30,463; 3, $p < 0.05$). At P20, the MS group presented slower performance compared to the SS group (GEE: Wald χ_2= 11,364; 3, $p < 0.05$) (Figure 1).

---------- Insert Fig. 1----------

3.1.2 Righting Reflex

On P9 and P10, the pups were submitted to the righting reflex test. GEE indicated statistically significant differences between the groups on the two assessment days, with group interaction * time (Wald $\chi2$ = 33,107; 3, p <0,05). The analysis showed that in P9 the animals of the MS group took longer time to return to the position of ventral decubitus compared to the animals of group SE and ME (Wald $\chi2$ = 27,223; 3, $p < 0,05$). In P10 the animals of both MS and ME groups presented longer reflex response time when compared to the animals of both SS and SE groups (Wald $\chi2$ = 115,470; 3, $p < 0,05$) (Figure 2).

---------- Insert Fig. 2 ----------

3.1.3 Object Recognition

In P20 the animals were submitted to the object recognition test. GEE indicated statistically significant differences between the groups, there is interaction group * object (Wald

$\chi 2 = 43{,}351$; 3, $p < 0{,}05$). The animals of group SE and MS interacted more with the old object in relation to the SS group. The animals in the ME group presented significant difference in relation to the SE and MS groups, the MS group interacted for longer time with the old object, and the EE partially reversed the morphine effect in the ME group (Wald $\chi 2 = 14{,}0806$; 3, $p < 0{,}05$). Regarding the new object, the MS group was statistically different from the other groups, suggesting a long-term memory imparment of these animals (Wald $\chi 2 = 129{,}037$; 3, $p < 0{,}05$) (Figure 3).

---------- Insert Fig. 3----------

3.1.3 Hot Plate Test

In P20 the animals were submitted to the hot plate test. The nonparametric Kruskal Wallis test showed no significant difference between groups in the thermal nociception test (Wald $\chi 2 = 1{,}004$; 3, $p = 0{,}791$) (Table 1).

---------- Insert Table 1----------

4. DISCUSSION

In the present study, we showed that animals that received morphine from P8 to P14 presented an impairment in neuromotor reflexes and negative effects on long-term memory. It is important to report that part of these effects was age-dependent, and that EE from P8 to P21 has partially reversed these negative outcomes. Moreover, neither morphine nor EE altered the pup rats thermal nociceptive response.

The evaluations of the righting reflex and the negative geotaxis showed that early-life morphine exposure was able to affect development neuromotor in an age-dependent manner. On P9, rats that received morphine without enriched environmental showed longer neuromotor reflexes response time than rats receiving saline injections or morphine-injected associated to EE. Thus, EE on P9 was able to revert the neurodevelopmental impairment. On P10, both groups receiving morphine presented a slower neuromotor response than saline injected animals. It was also demonstrated by the negative geotaxis that rats receiving morphine have a worse performance on these test, but only on P16 and P20. EE was able to improve the performance on saline-injected pups on P12, demonstrating age- and treatment-dependent effects. These findings demonstrate that morphine has a negative effect on the development of motor skills in neonate rats. Motor reflexes are fundamental for the adaptation of the neonate to the environment (Golan and Huleihel, 2006) and changes in its development may affect its perception and the way it reacts to the environment (Schuch et al., 2016). Preclinical and clinical studies demonstrate that sensory and motor skills are fundamental for the development of newborns (Zafeiriou et al., 1999; de Oliveira et al., 2017). Therefore, changes in biological and behavioral processes may be markers of changes in adult life (Heyser, 2004; Schuch et al., 2016).

Corroborating our findings, a recent study from our research group demonstrated that newborn rats exposed to morphine and maternal deprivation early in life show a delay in the

development of neonatal reflexes, evidenced by the inferior performance in the latency of the righting reflex (P8-P10), an effect that is related to treatment with morphine. In addition, the negative geotaxis test (P8, P10 and P12) also showed a lower performance score in the groups that received morphine and submitted to maternal deprivation. These data demonstrate that morphine and/or stress exposures in early life adversely affect brain development and increase the risk of occurrences of behavioral changes (de Oliveira et al., 2017).

The long-term memory results achieved by the object recognition test show that morphine-injected exposure rats to non-enriched environment have impaired ability to recognize a new object in the environment, while remaining most of the time interacting with the old object. EE was able to reverse this effect in the enriched group. EE induces neuronal changes and it is able to revert stress effects, especially in cognitive tasks (Sale et al., 2009). Thus, EA has been used to evaluate memory performance and it alters the neurogenesis process (Sampedro-Piquero, P. and Begega, A., 2016). However, there are rare studies that evaluate the EE effects in the morphine-exposed neonates, especially at long-term memory (Lee et al., 2014; Shen et al., 2013). Thus we can suggest that EE is a potential therapeutic strategy. A study conducted by Shih and colleagues (2012) showed long-term cognitive impairment induced by exposure to anesthetics in childhood. In addition, EE is also a useful method for the prevention of brain changes induced by maternal separation, a potent stressor for neonates (Khalaji et al., 2018; Sale et al., 2017).

On the other hand, in the present study, no difference between groups in the nociceptive response evaluated on P20 was demonstrated. Previous findings from our group have shown that exposure to stressors in the neonatal period, such as maternal deprivation, is also related to changes in nociceptive response assessed in P21 (Ströher et al., 2019). In addition, neonatal exposure to morphine induces an improvement in morphine-induced analgesia from P8 to P16, probably related to neonate neurodevelopment (Rozisky et al., 2008).

We may therefore emphasize the present findings as potential harming effects of morphine taken in early-life and protective effects of EE. It may be a promising noninvasive strategy to ameliorate deficits in the maturation of the nervous system and promote the recovery of sensory functions in pathological conditions that affect the brain in developing ages (Sale et al., 2009).

5. CONCLUSION

In summary, our study demonstrates that premature exposure to morphine negatively affects behavioral development and long-term memory in neonate rats, and that EE may be a non-pharmacological alternative to avoid the deleterious effects induced by repeated administration of morphine in a period of high neuroplasticity. In this way, the preclinical studies involving this type of intervention are of high relevance.

6. STUDY LIMITATIONS

This main limitation of the present study is the small number of the sample, which may have influenced our results.

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Declaration of Interest

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

Contributors

This work counted on the collaboration of all the authors:

Coordination of the study: Iraci L.S. Torres

Elaboration of the study project and the experimental design: Carla de Oliveira, Natalia P. Silveira and Iraci L.S. Torres.

Elaboration of the manuscript: Natalia P. Silveira, Dirson Stein, Carla Oliveira and Iraci L.S. Torres.

Statistical analysis: Iraci L.S. Torres, Vanessa L. Scarabelot; Bettega Costa Lopes and Dirson Stein.

Treatments and behavioral tests: Roberta Ströher; Lisiane S Silva; Natalia P. Silveira.

All authors read and approved the final version of this manuscript.

LEGENDS

Figure 1: Negative Geotaxis. Data are presented as the mean ± standard error of the mean (N=28). Groups: saline + standard housing (SS); saline + enriched housing (SE); morphine + standard housing (MS); morphine + enriched housing (ME). GEE showed an effect of group GEE: Wald χ_2= 100,465; 15, $p < 0.05$. The differences between groups were assessed at each time point (P9-P20) separately, and number of different symbols. * $p < 0.05$; ** $p < 0.01$.

Figure 2: Righting Reflex. Data are presented as the mean ± standard error of the mean (N = 28). Groups: saline + standard housing (SS); saline + enriched housing (SE); morphine + standard housing (MS); morphine + enriched housing (ME). GEE showed interaction group × time (Wald $\chi2$ = 33,107; 3, $p < 0.05$). The differences between groups were assessed at each time point (P9-P10) separately, and number of different symbols. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Figure 3: Object Recognition. Data are presented as the mean ± standard error of the mean (N=28). Groups: saline + standard housing (SS); saline + enriched housing (SE); morphine + standard housing (MS); morphine + enriched housing (ME). **Old Object.** GEE showed interaction group × interaction object (Wald $\chi2$ = 33,107; 3, $p < 0,05$). **New Object.** GEE showed interaction group × interaction object (Wald $\chi2$ = 129,037; 3, $p < 0,05$). Differences between groups were evaluated for the objects separately, and the number of different symbols. *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$.

Table 1. Hot Plate Test. The nonparametric Kruskal Wallis test indicated that there was no statistically significant difference between groups on the latency to respond on the thermal nociception test (Wald $\chi2$ = 1,004; 3, $p > 0.05$).

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Figure 1

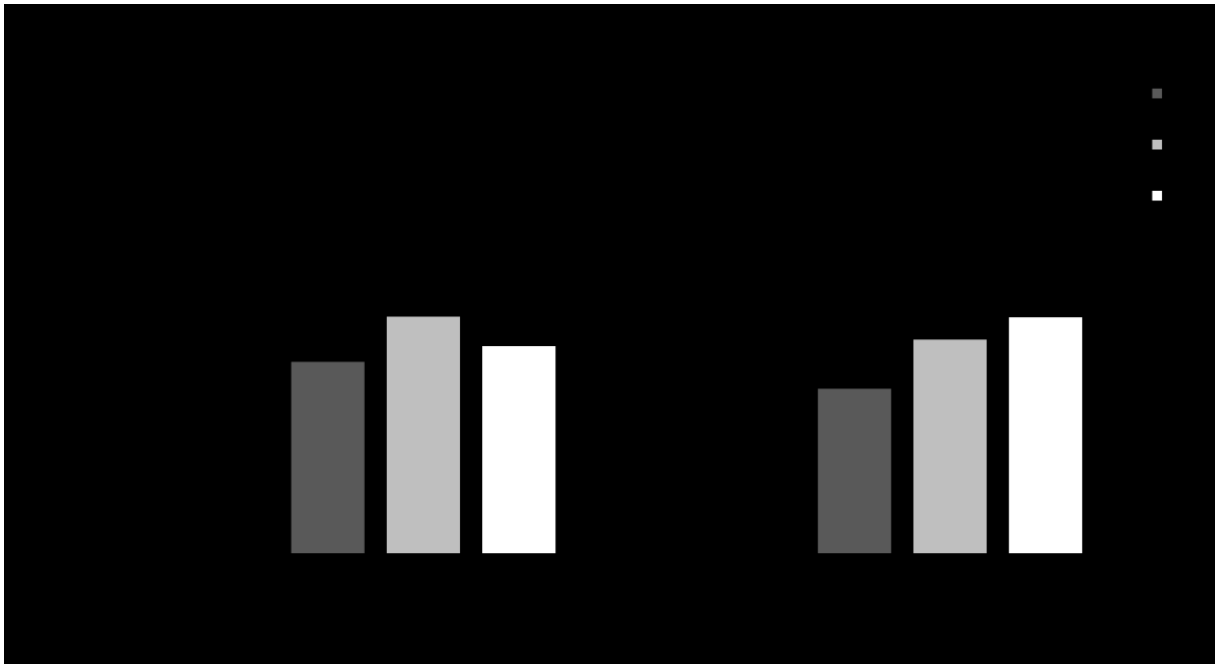

Figure 2

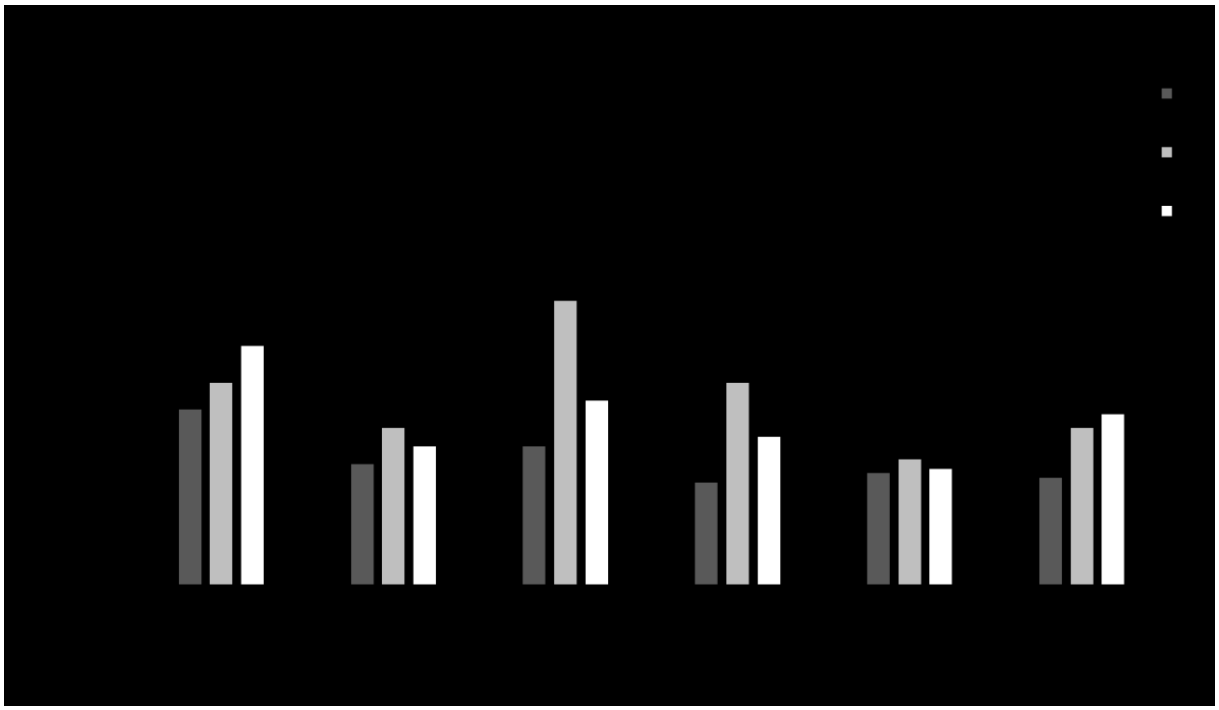

Figure 3

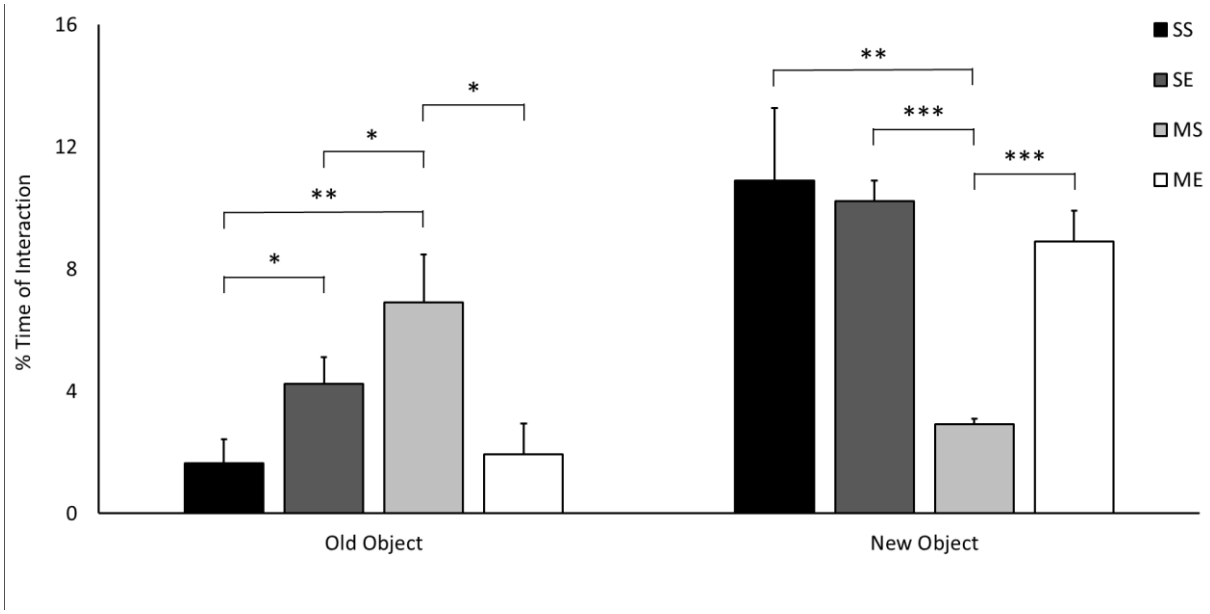
16
12
8
4
0
% Time of Interaction
SS
SE
MS
ME
*
**
*
*
**
***
***
Old Object
New Object

Table 1 – Hot Plate test.

Group	**Latency (s)**			*N*
	Median	*Minimum*	*Maximum*	
SS	7.00	5.00	8.00	7
SE	7.60	6.00	9.00	7
MS	7.33	3.00	10.00	7
ME	7.00	6.00	11.00	7

SS = saline + standard housing; SE = saline + environmental enrichment; MS = morphine + satandard housing; ME = morphine + environmental enrichment. No significant differences between groups.

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Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

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State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

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