Central Opioidergic System Interplay with Histamine on Food Intake in Neonatal Chicks: Role of μ-Opioid and H1/H3 Receptors

ABSTRACT

The present study was designed to examine the role of Opioidergic and Histaminergic systems on feeding behavior in 3-hour food deprived neonatal meat-type chicks. In experiment 1, chicks received intracerebroventricular (ICV) injection of (A) control solution, (B) α-FMH (alpha fluoromethyl histidine; 250 nmol), (C) DAMGO (μ-opioid receptor agonist, 125 pmol) and (D) α-FMH + DAMGO. Experiments 2-4 were similar to experiment 1, except chicken ICV injected with Chlorpheniramine (histamine H1 receptors antagonist; 300 nmol), famotidine (histamine H2 receptors antagonist; 82 nmol) and Thioperamide (histamine H3 receptors antagonist; 300 nmol) instead of the α-FMH. In experiments 5-8, birds ICV injected with the same procedure as experiments 1-4, except they were injected with DPDPE (δ-opioid receptor agonist, 40 nmol) instead of DAMGO. Experiments 9-12 were similar to the experiments 1-4, except neonatal broilers ICV were injected with U-50488H (κ-opioid receptor agonist, 30 nmol) instead of DAMGO. Then the cumulative food intake was measured until 120 min post injection. According to the results, ICV injection of DAMGO, significantly decreased food intake (p<0.05) while DPDPE and U-50488H increased feeding behavior compared to the control group (p<0.05). Co-administration of the α-FMH and DAMGO significantly inhibited hypophagic effect of the DAMGO in neonatal broilers (p<0.05). Also, Chlorpheniramine significantly inhibited DAMGO-induced feeding behavior in neonatal chicks (p<0.05). In addition, co-administration of the Thioperamide + DAMGO significantly amplified the hypophagic effect of the DAMGO in neonatal chicks (p<0.05). However, famotidine had no effect on food intake induced by DAMGO (p>0.05). Also, the hyperphagic effect of DPDPE and U-50488H had no affect by α-FMH, Chlorpheniramine, famotidine and Thioperamide (p>0.05). These results suggested that an interconnection between central opioidergic and histaminergic systems on feeding behavior is mediated via μ-opioid and H1/H3 receptors in neonatal broilers.

INTRODUCTION

Feeding behavior is a very complex neurochemical pathway which is regulated hierarchically from the central nervous system (CNS) and the peripheral nervous system (PNS) (Jonaidi et al., 2012). Complex physiological interaction exists on food intake and expenditure by afferent signals and efferent effectors between the CNS and gastrointestinal tract (Hassanpour et al., 2015). In the CNS, this process is regulated by complex neurochemical mechanisms in the hypothalamic nuclei, striatum, amygdala and arcuate nucleus (ARC) (D’Addario et al., 2014). Several neurotransmitters in the CNS have been identified where food intake is regulated (Ladepeche et al., 2013).
Histamine is one of the main neurotransmitters which express in the paraventricular nucleus (PVN) and ventromedial hypothalamus (VMH) of the brain (Giannoni et al., 2009; Blandina et al., 2012). To date, 4 subtypes of histamine receptors have been identified including H₁, H₂, H₃ and H₄ in the several parts of CNS (Schneider et al., 2014). The central histaminergic (HAergic) system has the key role in feeding behavior (Rozov et al., 2014) where the ICV injection of histamine decreases food intake whereas ICV injection of alpha-fluoromethylhistidine (α-FMH, selective inhibitor of the histidine decarboxylase as histamine synthesizing enzyme) or chlorpheniramine (histamine H₁ receptor antagonist) increases food intake in rats (Morimoto et al., 2001) and chicken (Kawakami et al., 2000). It is well documented that appetite is regulated by the interaction of various neurotransmitters and complex network (Branch et al., 2013).

Opioids are known as inhibitory neurotransmitters and 3 receptor subtypes are identified, mu (µ), delta (δ) and kappa (κ), belonging to the G protein-coupled receptors (GPCRs) (Filizola & Devi, 2013). Opioids are responsible in numerous physiologic functions such as pain modulation, respiratory, neuroendocrine and reward and food intake regulation (Kaneko et al., 2012). The intracerebroventricular (ICV) injection of [D-Ala², NMe-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO) and β-casomorphin (µ-opioid receptor agonists) induce hypophagia while [D-Pen², ⁵]-enkephalin (DPDPE) (δ-opioid receptor agonist) exerts orexigenic effects in mammals (Kaneko et al., 2012). The ICV injection of µ-opioid receptor agonists induces hypophagia while δ-opioid receptor agonist and U-50488H (κ-opioid receptor agonist) has a hyperphagic effect in neonatal layer and broiler chicks (Bungo et al., 2004; Shiraishi et al., 2008, Shojaei et al., 2015; Zendehdel et al., 2016a).

Based on the literature, an interconnection exists between central HAergic and opioidergic systems in some areas in the brain. Endomorphins have the highest affinity in the amygdala, PVN and dorsomedial hypothalamus with histaminergic neurons and might regulate arousal and sedative behaviors (Koneru et al., 2009). It is reported that Naloxone-induced water intake decreased by blockade of H₁ and H₂ receptors in male rats (Oryan et al. 2004). The interaction of the histaminergic and opioidergic systems in the hippocampus mediates pain from originating (Mojtahedin et al., 2008). Co-injection of the histamine H₁ receptor antagonists and morphine, increased analgesic activity in the acute trigeminal model of pain in rats (Khalilzadeh et al., 2017). Despite the researches being done on interaction of the central HAergic and opioidergic systems, there is no report on interaction of these two systems on feeding behavior in mammals and poultry. It is known that central food intake regulation is dissimilar between mammals and birds (Zendehdel & Hassanpour, 2014). It is logical to assume the regulatory mechanisms governing these processes in birds (Hassanpour et al., 2015). Therefore, the current study was designed for the first time to determine the possible interconnection of the central opioidergic and HAergic systems on feeding behavior in neonatal meat-type chicks.

**MATERIALS AND METHODS**

**Animals**

A total 528 male meat-type one-day-old chickens (Ross 308) were purchased from a local hatchery (Mahan Co. Iran). Birds were kept as flocks for 2 days then randomly transferred into individual cages at a temperature of 30 ± 1°C with 50 ± 2 percent humidity (Olanrewaju et al., 2006). A commercial diet provided during the study containing 21% crude protein and 2850 kcal/kg of metabolizable energy (Chineh Co. Iran) (table). All birds had free access to food and fresh water during the study. Just 3 hours prior the
ICV injections, chicken were food deprived (FD3) but had free access to water. The injections were applied to all birds at 5 days of age. Animal handling and experimental procedures were performed according to the Guide for the Care and Use of Laboratory animals by the National Institutes of Health (USA) and the current laws of the Iranian government for animal care.

**Experimental Drugs**

DAMGO (µ-opioid receptor agonist), DPDPE (δ-opioid receptor agonist), U-50488H (κ-opioid receptor agonist), α-FMH (alpha fluoromethyl histidine; histidine decarboxylase inhibitor), Chlorpheniramine (histamine H1 receptors antagonist), famotidine (histamine H2 receptors antagonist), Thioperamide (histamine H3 receptors antagonist) and Evans blue were purchased from Sigma Co. (Sigma, USA) and Tocris Co. (UK). The drugs were first dissolved in absolute dimethyl sulfoxide (DMSO) then diluted with 0.85% saline containing Evans blue at a ratio of 1/250. DMSO with this ratio does not have cytotoxic effect (Blevins et al., 2002; Qi et al., 2008).

**ICV injection procedures**

The birds were randomly allocated into 12 experimental groups (each experiment includes 4 groups, n=11 in each group). Prior to each experiment, the chicks were weighed and based on their body weight, divided into experimental groups so the average weight between treatment groups was as uniform as possible. The ICV injection was applied using a microsyringe (Hamilton, Switzerland) without anesthesia according to the technique previously described by Davis et al., (1979) and Furuse et al., (1997) where the head of the birds was held with an acrylic device while the bill holder was 45º and calvarium parallel to the surface of table (Van Tienhoven & Juhasz, 1962). A hole was drilled in a plate where the skull overlaid immediately over the right lateral ventricle. A microsyringe was inserted into the right ventricle via the hole and the tip of the needle penetrated 4 mm beneath the skin of the skull. It is revealed that, there is no injection-induced physiological stress using this method in neonatal chicks (Saito et al., 2005). Each chick received an ICV injection (with vehicle or drug solution) in a volume of 10 μL (Furuse et al., 1999). The control group received a control solution (DMSO/saline mixture containing Evan’s blue, 10 μL) (Furuse et al., 1999). Right away after the injection, FD3 the birds returned to their individual cages and supplied fresh water and food (pre-weighed). Cumulative food intake (gr) was measured at 30, 60 and 120 minutes post the injection. Food consumption was calculated as a percentage of body weight to minimize the impact of the body weight on the amount of food intake. Each bird was used just once in each experimental group. At the end of the experiments, the accuracy of the placement of the injection in the ventricle was verified by presence of Evans blue followed by slicing the frozen brain tissue. All experimental procedures were done from 8:00 A.M. until 3:30 P.M.

**Feeding experiments**

To investigate the interconnection of opioidergic and histaminergic systems on cumulative food intake in neonatal meat-type birds, 12 experiments designed (each experiment contains 4 groups (A-D) within 11 replicates in each group) were used. In experiment 1, FD3 the chicks received a dose of the ICV injection of (A) control solution, (B) α-FMH (alpha fluoromethyl histidine; 250 nmol), (C) DAMGO (µ-opioid receptor agonist, 125 pmol) and (D) α-FMH + DAMGO. Experiments 2-4 were similar to experiment 1, except FD3 chicks were ICV injected with chlorpheniramine (histamine H1 receptors antagonist; 300 nmol), famotidine (histamine H2 receptors antagonist; 82 nmol) and thioperamide (histamine H3 receptors antagonist; 300 nmol) instead of α-FMH. In experiment 5, FD3 chicken received a dose of the ICV injection of (A) control solution, (B) α-FMH (250 nmol), (C) DAMGO (µ-opioid receptor agonist, 125 pmol) and (D) α-FMH + DPDPE. Experiments 6-8 were similar to experiment 1, except FD3 birds were ICV injected with chlorpheniramine (histamine H1 receptors antagonist; 300 nmol), famotidine (histamine H2 receptors antagonist; 82 nmol) and thioperamide (histamine H3 receptors antagonist; 300 nmol) instead of α-FMH. In experiment 9, FD3 chicken received a dose of the ICV injection of (A) control solution, (B) α-FMH (250 nmol), (C) U-50488H (κ-opioid receptor agonist; 30 nmol) and (D) α-FMH + U-50488H. Experiments 10-12 were similar to experiment 1, except FD3 chicks received ICV injection of the chlorpheniramine (histamine H1 receptors antagonist; 300 nmol), famotidine (histamine H2 receptors antagonist; 82 nmol) and thioperamide (histamine H3 receptors antagonist; 300 nmol) instead of α-FMH. Each bird was injected once only. These doses of drugs were calculated based on the previous studies (Bungo et al., 2004, 2005; Taati et al., 2009; Shojaei et al., 2015; Zendehdel et al., 2015, 2016a, b) and our pilot studies (un-published data). Right away after the injection, chickens were returned to their individual cages and provided ad libitum food (pre-weighed) and water. Cumulative food intake was recorded at 30, 60 and 120 minutes post injection.
Statistical analysis

Data is presented as mean ± SEM (standard error of the mean). Cumulative food intake (as percent of body weight) was analyzed by repeated measure two-way analysis of variance (ANOVA) using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). For treatment showing a main effect by ANOVA, means were compared by Tukey-Kramer test. p<0.05 was considered as significant differences between treatments.

RESULTS

Effects and interactions of central HAergic and opioidergic systems on cumulative food intake in FD3 neonatal meat-type chicks are shown in figures 1-12. In this study to examine the possible interaction between these two systems, effective and sub-effective doses of pharmacological agents were administered to confront nullifying effects of the agents. In experiment 1, ICV injection of the DAMGO (μ opioid receptors agonist, 125 pmol) significantly decreased food intake until 120 min post injection compared to the control group (p<0.05). The ICV injection of the sub effective dose of the α-FMH (alpha fluoromethyl histidine; 250 nmol) had no effect on cumulative food intake compared to the control group (p>0.05). Co-administration of the α-FMH and DAMGO significantly inhibited the hypophagic effect of the DAMGO in neonatal broilers [treatment effect: F (3, 80) = 162.1, p<0.0001; time effect: F (2, 80) = 541.3, p<0.0001; treatment and time interaction: F (6, 80) = 28.53, p<0.0001; Fig. 1].

In experiment 2, hypophagia was observed after the ICV injection of DAMGO (125 pmol) in FD3 neonatal chicken, compared to the control group (p<0.05). The ICV injection of the chlorpheniramine (histamine H1 receptors antagonist; 300 nmol) had no effect on food intake in comparison to the control group (p>0.05). Co-injection of the chlorpheniramine + DAMGO significantly inhibited the hypophagic effect of the DAMGO in neonatal meat-type chicken [treatment effect: F (3, 80) = 416.2, p<0.0001; time effect: F (2, 80) = 985.13, p<0.0001; treatment and time interaction: F (6, 80) = 5.37, p<0.0001; Fig. 2].

In experiment 3, significant decrease in food intake was observed after the ICV injection of DAMGO (125 pmol) in birds compared to the control group (p<0.05). The ICV injection of the famotidine (histamine H2 receptors antagonist; 82 nmol) had no effect on food intake in comparison to the control group (p>0.05). Co-injection of the famotidine + DAMGO was not able to change DAMGO-induced hypophagia [treatment effect: F (3, 80) = 89.35, p<0.0001; time effect: F (2, 80) = 549.7, p<0.0001; treatment and time interaction: F (6, 80) = 9.17, p<0.0001; Fig. 3].
In experiment 4, the ICV injection of the DAMGO (125 pmol) significantly decreased food intake in comparison to the control group ($p<0.05$). No significant effect was observed on food intake by the ICV injection of thioperamide (histamine H3 receptors antagonist; 300 nmol). Co-administration of the Thioperamide + DAMGO amplified hypophagic effect of the DAMGO in neonatal chicks [treatment effect: $F (3, 80) = 119.61, p<0.0001$; time effect: $F (2, 80) = 859.14, p<0.0001$; treatment and time interaction: $F (6, 80) = 43.12, p<0.0001$; Fig. 4].

In experiment 5, the ICV injection of the α-FMH (250 nmol) had no significant effect on food intake ($p>0.05$). Hyperphagia was observed after the ICV injection of DPDPE (β-opioid receptor agonist; 40 pmol) in FD3 neonatal birds ($p<0.05$). Co-administration of the α-FMH + DPDPE had no significant effect on β-opioid receptors agonist-induced hyperphagia in neonatal chicks [treatment effect: $F (3, 80) = 74.46, p<0.0001$; time effect: $F (2, 80) = 750.71, p<0.0001$; treatment and time interaction: $F (6, 80) = 7.52, p<0.0001$; Fig. 5].

In experiment 6, no effect was observed after the ICV injection of the chlorpheniramine (300 nmol) in chicks. ICV injection of the DPDPE (40 pmol) significantly increased food intake in FD3 neonatal birds compared to the control group ($p<0.05$). Co-injection of the Chlorpheniramine + DPDPE was not able to change hyperphagic effect of the DPDPE in neonatal birds [treatment effect: $F (3, 80) = 31.83, p<0.0001$; time effect: $F (2, 80) = 518.96, p<0.0001$; treatment and time interaction: $F (6, 80) = 13.28, p<0.0001$; Fig. 6].

In experiment 7, hyperphagia was observed after the ICV injection of the DPDPE (40 pmol) in neonatal broilers compared to the control ($p<0.05$). Administration of the famotidine (82 nmol) had no effect on the food consumption in FD3 neonatal birds ($p>0.05$). Co-injection of the DPDPE + famotidine had no effect on hyperphagic effect of the DPDPE in FD3 neonatal birds [treatment effect: $F (3, 80) = 83.19, p<0.0001$; time effect: $F (2, 80) = 472.21, p<0.0001$; treatment and time interaction: $F (6, 80) = 4.16, p<0.0001$; Fig. 7].
In experiment 8, no significant effect was observed on food intake after the ICV injection of the thioperamide (300 nmol) (p>0.05). ICV injection of the DPDPE (40 pmol) significantly increased food intake in F3 neonatal birds compared to the control group (p<0.05). While, the ICV injection of the DPDPE + famotidine was not able to affect hyperphagic effect of the DPDPE in F3 neonatal birds [treatment effect: F (3, 80) = 39.14, p<0.0001; time effect: F (2, 80) = 548.15, p<0.0001; treatment and time interaction: F (6, 80) = 5.17; p<0.001; Fig. 8].

In experiment 9, ICV administration of the U-50488H (κ-opioid receptor agonist; 30 nmol), DPDPE (κ-opioid receptor agonist; 40 pmol) and combination of thioperamide plus DPDPE on cumulative food intake (gr/100 gr BW) in neonatal chicks. Data are expressed as mean ± SEM. Different letters (a and b) indicate significant differences between treatments at each time (p<0.05).

In experiment 10, the ICV injection of the chlorpheniramine (300 nmol) had no effect on cumulative food intake compared to the control group (p>0.05). ICV administration of the U-50488H (30 nmol) had hyperphagic effect compared to control group (p<0.05). Co-administration of the Chlorpheniramine + U-50488H had no effect on κ-opioid receptor agonist-induced hyperphagia in neonatal broilers [treatment effect: F (3, 80) = 117.39, p<0.0001; time effect: F (2, 80) = 450.8, p<0.0001; treatment and time interaction: F (6, 80) = 8.25; p<0.0001; Fig. 10].

In experiment 11, ICV administration of the 82 nmol of the famotidine had no effect on feeding behavior compared to the control group (p>0.05). ICV administration of the 30 nmol of the U-50488H increased cumulative food intake compared to the control group (p<0.05). Co-administration of the famotidine + U-50488H had no effect on κ-opioid receptor agonist-induced hyperphagia in neonatal broilers [treatment effect: F (3, 80) = 82.06, p<0.0001; time effect: F (2, 80) = 246.37, p<0.0001; treatment and time interaction: F (6, 80) = 5.09; p<0.0001; Fig. 11].
In experiment 12, the ICV injection of the 30 nmol U-50488H increased cumulative food intake compared to the control (p<0.05); while thioperamide (300 nmol) had no effect on feeding behavior compared to the control group (p>0.05). Injection of the Thioperamide + U-50488H had no effect on κ-opioid receptor agonist-induced hyperphagia in neonatal broilers [treatment effect: F (3, 80) = 58.94, p<0.0001; time effect: F (2, 80) = 639.25, p<0.0001; treatment and time interaction: F (6, 80) = 5.36; p<0.0001; Fig. 12].

**DISCUSSION**

The present study was designed for the first time to investigate the possible interconnection of the opioidergic system with histamine on food intake in neonatal broiler chicks. To the best of our knowledge, this is the first report on the interaction of the central HAergic and opioidergic systems on appetite regulation in FD₃ neonatal broiler chicks. In this study we used sub effective doses of the HAergic antagonists and effective doses of the opioid receptors agonists to determine possible interconnection between HAergic and opioidergic systems on food intake in FD₃ broiler chicks. The results obtained imply that the ICV injection of DAMGO decreased food intake while DPDPE increased cumulative food intake (gr/100g BW) in neonatal chicks. Data are expressed as mean ± SEM. Different letters (a and b) indicate significant differences between treatments at each time (p<0.05).

**Figure 12** – Effects of intracerebroventricular injection of control solution, thioperamide (histamine H3 receptors antagonist; 300 nmol), U-50488H (κ-opioid receptor agonist; 30 nmol) and combination of thioperamide plus U-50488H on cumulative food intake (gr/100g BW) in neonatal chicks. Data are expressed as mean ± SEM. Different letters (a and b) indicate significant differences between treatments at each time (p<0.05).

Hᵢ receptors are known as hypophagic receptors in rats (Morimoto et al., 2001) and broiler chickens (Taati et al., 2010). Anorexic effects reported for Hᵢ receptors in broilers (Meade and Denbow, 2001) and thioperamide decreases cumulative food intake in broilers (Taati et al., 2010). In poultry, histamine mediates its effect via H₁ receptors (Zendehehdel et al., 2015) but controversial reports exist for H₃ receptors. Taati et al., (2009) reported that ICV injection of the thioperamide(300 and 600 nmol) decreased food intake in food-deprived broilers (Taati et al., 2009). Scarce information exists about expression of the H₄ receptors in poultry brain (Zendehehdel et al., 2015). The ICV injection of thioperamide had no effect on feeding behavior in fasted or non-deprived rats in the lighting period (Passani et al., 2011) while decreased appetite in the dark period when central histamine is at low levels. Perhaps, it affects when histaminergic system activity is low (Passani et al., 2011). Blockade of the H₄ receptors decreases food intake in rats (Chiba et al., 2009) and injection of the H₁ receptor antagonists attenuated effects of the H₄ antagonists in rats (Hancock & Brune, 2005).

As observed, α-FMH and chlorpheniramine inhibited hypophagic effect of the DAMGO in neonatal broilers. Additionally, co-administration of the histamine H₃ receptors antagonist (Thioperamide) with DAMGO significantly amplified hypophagic effect of the DAMGO in neonatal chicks. It is reported that Thioperamide induced anti-nociception mediates via the endogenous opioid system (Khalilzadeh et al., 2010). ICV injection of thioperamide increased the nociceptive threshold at supraspinal level in a rat (Mobarakeh et al., 2009). Also, Hough et al., (1997) reported that the ICV injection of the thioperamide had no analgesic activities in nociception tests in rats while analgesic and hyperalgesic effects reported by ICV administration of the thioperamide and R-α-methylhistamine in rats (Malmberg-Aiello et al., 1994). The H₃ receptors, exerted inhibitory effects on the morphine-induced anti-nociception at the spinal level (Mobarakeh et al., 2009). The anti-nociceptive effect of the histamine was reversed by the ICV injection of the naloxone into periaqueductal gray (Khalilzadeh et al., 2010). A close relationship reported between H₁ receptor and μ-opioid receptor in scratching behavior in mice, where co-injection of the histamine and morphine caused scratching and simultaneous
administration of morphine and histamine had an additive effect. Naloxone and chlorpheniramine reserved histamine-induced scratching behavior (Nakasone et al., 2016). Anticonvulsant action observed by activation of the H receptors whereas inhibition of H receptors induced proconvulsant effects (Amini-Khoei et al., 2015). Co-injection of morphine with H1 and H3 agonists/antagonists reversed their effects on PTZ-induced seizure (Amini-Khoei et al., 2015). Pretreatment with H3 antagonist decreased the amisulpride-induced seizures in mice (Rehri et al., 2011). Co-injection of chlorpheniramine with morphine potentiates the anti-nociceptive activity of morphine in the acetic acid-induced visceral pain - in rats (Zanboori et al., 2008).

Based on the literature, the histaminergic system mediates some of the central effects of morphine. However, there is no report on their interaction on feeding behavior. Histamine impresses its effect via agoutirelated protein (AgRP), neuropeptide Y (NPY), cocaine and amphetamine regulated transcript neurons and histamine regulated transcript neurons (Zendehdel et al., 2015). Also, the interconnection exists between opioidergic system, NPY and AgRP neurons in the ARC (Zendehdel et al., 2015). However, the neural pathway between opioidergic system and NPY and AgRP is not identified in poultry's hypothalamus (Dodo et al., 2005). ICV injection of DAMGO increased µ-opioid receptor mRNA expression in ARC of rats (Zheng et al., 2007). Perhaps the interaction of these systems on food intake regulation happens in these nuclei of the hypothalamus. However, neuroanatomic and pharmacological researches needed to determine their possible neural interconnection.

In conclusion, the new findings of the current study suggested ICV injection of the α-FMH + DAMGO or chlorpheniramine + DAMGO decreased DAMGO-induced hypophagia in neonatal chicks. It seems that the interaction exists among central opioidergic and HAergic systems on feeding behavior mediates via µ-opioid and H1/H3 receptors in neonatal broilers. There was no previous study on the role of central opioidergic and HAergic systems on food intake in poultry. Most research on central food intake regulation was done with rat models. So, authors were not able to compare their results with it. This information can be used as base data on central feeding behavior in poultry. It is suggested that further investigation needs to be done to determine direct cellular and molecular signaling pathways of the HAergic and opioidergic systems with other receptors in physiology of food intake regulation in domestic fowls.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

INFORMED CONSENT

This manuscript does not contain any studies with human subjects performed by any of the authors.

HUMAN AND ANIMAL RIGHTS

All experiments were executed according to the Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal ethics committee.

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