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AGH, A NOVEL HEMOGLOBIN-DERIVED PEPTIDE, TARGETS SEROTONERGIC RECEPTORS TO INDUCE ANTINOCICEPTION IN MICE, WITHOUT INDUCING ANXIETY-DEPRESSIVE-LIKE BEHAVIOR

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Abstract
AGH (AGHLDDLPGALSAL), a novel bioactive peptide derived from the alpha-chain of hemoglobin, induces peripheral antinociceptive effect in rats. In the present study the systemic antinociceptive profileof AGH was examined in mice evaluated by the hot plate test or in submitted to the CFA-induced mechanical hyperalgesia and evaluated by electronic von Frey. The mechanisms involved on AGH-induced antinociception as well as anxiety-depressive-like behaviorwere also evaluated. Intraperitoneal administration of AGH (25 mg/ Kg) reversed CFA-induced non-opioid mechanical allodynia at the 3rd and 6th h after its injection. On the other hand, an opioid-mediated antinociception was observed when mice were evaluated at the hot plate test. Intraperitoneal pretreatment with methysergide (5-HT serotonergic receptor antagonist) attenuated antinociceptive effect induced byAGH on CFA-induced hyperalgesia. However, yohimbine (α2-adrenergic receptor antagonist) did not affect antinociception. To investigate whether AGH could induce changes in spontaneous motor activity or anxiety-depressive-like behavior, mice were evaluated by the open field test, tail suspension test and forced swimming test, respectively. No behavioral changes were observed for AGH-treated mice. These results demonstrate that AGH induces systemic antinociception, by targeting serotonergic receptors and without inducing behavioral changes in mice.

HIGHLIGHTS

- AGH reverses CFA-induced mechanical allodynia in mice
- Serotonergic receptors mediate AGH-induced antinociception
- AGH does not induce anxiety-depressive-like behavior

ABBREVIATIONS

SCA: substrate capture assay
CFA: Complete Freund's adjuvant

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NLX: Naloxone
Met: Methysergide
Yo: Yoimbine

INTRODUCTION

Hemorphins are endogenous peptides belonging to the family of ‘‘nonclassical’’ or ‘‘atypical’’ opioid peptides. They are generated by enzymatic hydrolysis of the beta-, kappa-, gamma-, or epson-chain of the blood protein hemoglobin(Nyberg et al., 1989). These peptides have been identified as naturally occurring peptides in brain(Barkhudaryan et al., 1993; Brent et al., 1995; Chang et al., 1980; Erchegyi et al., 1992; Glamsta et al., 1991; Karelin et al., 1994), plasma(Glamsta et al., 1993),cerebrospinal fluid(Glamsta et al., 1992)and spinal cord(NishimuraandHazato, 1993)and presentseveral biological activities including effects on spatial learning(Lee et al., 2012), transient hypotension (Barkhudaryan et al., 1992), inflammation(Sandersonet al., 1998)and analgesia(Cheng et al., 2012). They are generated by proteolytic cleavage of large precursor proteins followed by storage in secretory vesicles from where they are released upon cell stimulation(Gomes et al., 2010). For example, the β-globin fragment of hemoglobin in mammalian tissues serves as the endogenous precursor for the generation of LVV- and VV-hemorphins, while in erythrocytes the predominant peptides are V-hemorphins and hemorphins(Ivanovet al., 1997; Yatskin et al., 1998).The opioid activity of these peptides was demonstrated by their naloxone-reversible ability to inhibit the contractions of electrically-stimulated guinea-pig ileum(Brantl et al., 1986; Kosterlitz et al., 1969; Moisan et al., 1998).

A novel class of endogenous hemoglobin-derived peptides called hemopressins was first generated using the mutated catalytically inactive thimetoligopeptidase (EC 3.4.24.15, EP24.15) in a substrate capture assay (SCA) to identify novel bioactive peptides(Rioli et al., 2003).These peptides are derived from either alpha hemoglobin (hemopressin, RVD-hemopressin-alpha and VD-hemopressin-alpha) or beta hemoglobin (VD-hemopressin-beta)(Rioli et al., 2003; Heimann et al., 2007).Hemopressintargets CB1 receptor, modulating its signalingand exhibits antinociceptive effects in inflammatory pain models(Heimann et al., 2007; HamaandSagen, 2011; Toniolo et al., 2014).

Recent data identified a new endogenous bioactive peptide derived from hemoglobin alpha-chainnamed AGH (AGHLDDLPGALSAL)(Ribeiro et al., 2013). AGH was generated using a previously characterized substrate capture assay that uses a catalytically inactive form of the thimetoligopeptidase(Ribeiro et al., 2003)combined with isotopic labeling and mass spectrometry in order to identify new bioactive peptides.Pharmacological assays demonstrate that AGH inhibits peripheral inflammatory hyperalgesiain rats(Ribeiro et al., 2013). Previous peptidomics studies have identified the AGH as well as many other natural peptides derived from hemoglobin alpha-chain containing this sequence, further suggesting that AGH is a natural endogenous peptide with possible physiological relevance(Berti et al., 2009; Gelman et al., 2010;Gomes et al., 2010; Ribeiro et al., 2013). Considering its lack of opioid-like effects on the central nervous system, AGH could have potential to treat inflammatory pain. Given that the original sequence of AGH is present in many other natural peptides (Gelman et al., 2010) it appears that AGH is the prototype of a new class of bioactive peptides derived from hemoglobin.

The aim of the present study was to evaluate the possible mechanisms involved on AGH-induced antinociception, as well as to identify possible behavioral changes induced by this peptide in mice.

MATERIAL & METHODS

2.1. Animals

Male C57BL6 mice weighing 20-25 g, age-matched, were used throughout this study. Animals were maintained under controlled light cycle (12/12 h) and temperature (21 ± 2°C) with free access to food and water. Throughout the experiments, animals were managed using the principles and guidelines for the care of laboratory animals in

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studies involving pain and were approved by the Ethics Committee on the Use of Animals at University of São Paulo (CEUA, protocol number 85/2013).

2.2. Mechanical allodynia

Testing for mechanical allodynia (Von Frey filaments - Touch-Test® Sensory Evaluators - North Coast Medical) was performed according to the method of Chaplanet al. (1994). Mice were placed individually in plastic cages with a wire bottom, which allowed access to their paws. To reduce stress, mice were habituated to the experimental environment one day before the first measurement. At the day of the test, the animals were placed in the cages 30 min before the beginning of each measurement. The area tested was the mid-plantar left paw.

2.3. Hot plate test

Thermal threshold was evaluated by the Hot plate test (JacobandRamabadran1978).Mice were placed on a metal surface kept at 52.5±0.5ºC (Hot Plate, Ugo Basile®). The time interval between placement and behavioral responses (licking or flinching of the paws, or jumping) was recorded as response latency. Animals were tested before AGH administration in order to obtain a baseline and a 25 sec cutoff was used to minimize tissue damage. Results were analyzed by comparing the difference between pre- and post-treatment.

2.4.Anxiety-depressive-like behavior

2.4.1. Open field test

Possible changes in motor activity provoked by the AGH were investigated in an open-field arenaas described (Broadhurst, 1960). Each animal was individually placed in the center of the open field and behavioral parameters recorded for 3 min. Hand-operated counters were used to score ambulation (locomotion) frequency (number of floor units entered), and rearing frequency (number of times the animal stood on hind legs).

2.4.2. Forced swimming test

Mice were placed in cylindrical plexiglass tank (50 cm high and 30 cm diameter) filled with water (22ºC) up to a level of 25 cm from the bottom.The animal´s behavior during the first 5 mim of the swim test was scored by a trained observer and the following measures were taken: (1) time spent swimming, defined as moving all four limbs, swimming around the tank or diving and (2) time spent floating, defined as remaining immobile with only occasional slight movements to keep the body balanced and the nose above the water. Animals were exposed to a pretest for 15 min, 24 h prior to the 5-min swim test. The pretest facilitates the development of immobility during the test session and increases the sensitivity for detecting antidepressant behavioral effects (Porsolt et al., 1977; Borsini et al., 1989).

2.4.3. Tail suspension test

Animals were suspended 50 cm above the floor by means of an adhesive tape, placed approximately 1 cm from the tip of the tail. The time during which mice remained immobile was quantified during a test period of 6 min. Mice were considered immobile only when they hung passively and completely motionless. A camera was mounted facing the tail suspension test arena and all the test events were recorded. Two experienced observers independently scored the behavior blindly and an average of the two scores was used as a final score(Steru et al., 1985).

2.5. Pharmacological treatments

AGH(Proteimax Biotechnology) was diluted in sterile saline and injected intraperitoneal (200 µl) or intraplantar (30 µl) at the dose of 25 mg/Kg.Complete Freund's adjuvant (CFA, 25 µL, SIGMA) was administered intraplantar.Involvement of opioidson AGH-inducedantinociceptionwas evaluated usingnaloxone (10 mg/kg, s.c., Rhodia) injected 20 min before the nociceptive test. Morphine was administered i.p at the dose of 20 mg/Kg. Fluoxetine, a selective serotonin reuptake inhibitor, (40mg/kg, UniãoQuímica) was administered orally.Methysergide (serotonergic receptor antagonist, 5 mg/kg, St Louis, MO, USA, a) or yohimbine (α-adrenergic

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receptor antagonist, 5 mg/kg, St Louis, MO, USA) werediluted in sterile saline and wereadministered by intraperitoneal route 30 minutes before intraperitoneal AGH injection(Abrahão et al., 2013).

2.6. Statistical analysis

Results were expressed as the mean±SEM. Statistical analyses of data were generated by using GraphPad Prism, version 4.02 (GraphPad).A value of $p<0.05$ indicated a significant difference.Statistical comparison of more than two groups was performed using analysis of variance (ANOVA), followed by Bonferroni's test. Statistical comparison for treatment over time was performed using two way ANOVA followed by Bonferroni's test.

RESULT

3.1. AGH inhibits CFA-induced hyperalgesia in mice

Mice received intraplantar injection of CFA (25 µL) and immediately afterreceivedintraperitoneal administration of different concentrations of AGH (15,25 or50 mg/Kg). Nociceptive test was performed before any treatment (basal, time 0) and after different times of CFA injection. CFA(n=9) induced mechanical allodyniain mice, in all evaluated times, characterized by a significant decrease on pain thresholdwhen compared to control group injected with saline (n=8) [$F(24,204)=2.97$, $p<0.0001$](Fig. 1).AGH (n=7) reversed mechanical allodynia only at the intermediate dose (25mg/Kg) [$F(4,204)=22.15$], atthe 3rd($p<0.001$) and 6th($p<0.01$) hof evaluation (Fig. 1).AGH treatment by itself did not change the nociceptive threshold of animals in all evaluated times (data not shown).

3.2. Opioids are not involved in AGH-induced antinociception in mice

To evaluate whether opioid receptors could be involved on AGH-induced antinociception, mice were injected with CFA (25 µL) and immediately after received 25 mg/Kg of AGH i.p. (n=4). Naloxone (10 mg/ Kg; n=5) was injected subcutaneously 20 min before the nociceptive test and interestingly naloxone treatmentdid not interfere with AGH-induced antinociception[$F(2,39)=9.46$, $p=0.0029$](Fig. 2).In order to confirm whether opioid involvement on AGH-induced antinociception could be related to a peripheral effect, mice received intraplantar injection of CFA (25 µL) concomitantly with AGH (0.5, 1 or 2.5 µg). Nociceptive test was performed before any treatment (basal, time 0) and after different times of CFA injection. Once again CFA (n=4) induced mechanical allodynia in mice, in all evaluated times (Fig. 3). AGH reversed mechanical allodynia in all evaluated concentrations (Fig. 3 A-C). At the concentration of 0.5 µg(n=4) the antinociception was detected at 30 min and from 3h up to 24 h [$F(3,72)=14.97$, $p=0.0002$] (Figure 3A). At the concentration of 1 µg(n=4) the antinociception was detected atall evaluated times [$F(12,54)=6.69$, $p<0.0001$] (Figure 3B). Finally, at 2.5 µg (n=4) the antinociception was detected at all evaluated times after one single administration of the peptide [$F(3,72)=145.67$, $p<0.0001$] (Figure 3C). More interesting was the fact that naloxone completely reversed AGH-induced antinocipetion of mice injected intraplantar with AGH 2.5 µg (n=4)[$F(3,12)=39.12$, $p<0.0001$](Fig. 4). AGH treatment by itself did not change the nociceptive threshold d of animals in all evaluated times (data not shown).

In order to evaluate a possible supraspinal response involved on AGH induced antinociception, mice were evaluated at the Hot Plate test. Since this model is sensitive to opioid drugs, animals injected with morphine (20 mg/ Kg i.p.) were evaluated as positive controls of inhibition of nociceptive response. Nociceptive behavior was evaluated before (basal, time 0) and 3 h after intraperitoneal injection of AGH (25 mg/ Kg). Results demonstrate that AGH (n=18) induced a robust antinociceptive effect on mice, very similar to that observed for the group of animals injected with morphine (n=11)[$F(3,43)=20.46$, $p<0.0001$](Fig. 5). More interesting was the fact that naloxone (10 mg/ Kg s.c., n=11) treatment completely blocked AGH-induced antinociception[$F(3,43)=13.17$, $p<0.0001$] (Fig. 5).

3.3. AGH does not interfere with the spontaneous Motor Activity of mice or induce anxiety-depressive-like behavior in mice

AGH showed a strong and significant opioid-dependent antinociceptive effect at the 3rd h after its intraperitoneal administration and, considering that following systemic administration, AGH could cross the blood brain barrier to

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activate central circuits such as nociceptive regions in the periaqueductal grey matter and dorsal raphe nucleus and considering that both von Frey and Hot Plate tests use stimulus that elicit motor response and that Hot Plate test elicits a supraspinal-mediated response, it is plausible to hypothesize that AGH could induce central behavioral effects such as anxiety-depressive-like behaviors. To test this hypothesis we evaluated whether AGH could induce behavioral changes on animals.

Mice received different doses of AGH (1, 25, 50, 75 and 100mg/ Kg; i.p.) and after 3 h the general activity was evaluated by the open-field test. Results show that AGH did not change both locomotion [$F(5)=1.824$, $p<0.0001$]and rearing [$F(5)=0.645$, $p>0.05$]frequencies of animals in all evaluated doses when compared with the control group of mice injected with saline (Fig. 6).

Finally we evaluated the effect of AGH on depressive-like behavior of mice using forced swimming test or tail suspension test. Mice were injected with AGH (25 mg/Kg; i.p.) and after 3 h were evaluated on either forced swim [$F(5)=69.71$, $p<0.0001$] AGH did not induce changes on any evaluated parameter (Fig. 7).

3.4. Serotonergic but not adrenergic system is involved in AGH-induced antinociception in mice

We examined the possible involvement of serotonergic and adrenergic systems in AGH-induced antinociception. The pretreatment with yohimbine (YO; α2 adrenergic receptors antagonist) did not affect AGH-induced antinociception[$F(3, 15)=78.32$, $p<0.0001$] (Fig. 8A). However, the blockade of serotoninergic receptor with systemic pre-administration of methysergide (MET; serotonergic receptors antagonist), completely abolished both the AGH-induced analgesia [$F(3, 16)=17.17$, $p<0.0001$](Fig. 8B). The treatment with methysergide or yohimbine itself did not affect the hyperalgesic effect induced by CFA (Fig. 8).

DISCUSSION

AGH, a novel hemoglobin-derived peptide, inhibits peripheral inflammatory hyperalgesic responses in rats through an indirect activation of *mu* opioid receptors (Ribeiro et al., 2013). In this study,AGH-induced antinociception was investigatedin mice. We found that intraplantar injection of 25 µL of CFA induced a decrease on pain threshold of mice from 15 min up to 24 h of evaluation. This decrease was in agreement with data from the literature demonstrating that intraplantar injection of CFA in the hind paw of both mice and rats is a classical experimental model for the study of inflammatory pain (Ohsawa et al., 2000; Aoki et al., 2014). Intraperitoneal injection of AGH on CFA-injected mice was able to completely block signs of pain from the 3rd h of administration, supporting the idea that AGH induces true antinociception in an inflammatory pain model. Also, it was observed that AGH has a long lasting effect in blocking hind paw hypersensitivity; once the anti-hyperalgesic effect was observed up to 24 h after peptide administration. One interesting point is the fact that AGH was effective only at the dose of 25 mg/Kg with no antinociceptive effect observed when either loweror higher doses of the peptide were administered. Also, intraperitoneal administration of AGH was able to block mechanical allodynia of mice, demonstrating that AGH has a systemic action.

The first identified peptide using the SCA assay was the nonapeptidehemopressin (Hp). Hp is demonstrated to induce antinocicetpive effect on inflammatory pain models(Hama and Sagen, 2011;) with the same intensity regardless the evaluated dose of the peptide(Heimann et al., 2007; Dale et al., 2005), suggesting that the tendency of Hp to self-assemble to form nanostructure aggregates(Bomar et al., 2012) might protect it from rapid degradation in an *in vivo* environment(Gomes et al., 2010)supporting the duration of its effect and the lack of dose/response effect. Considering that AGH is also a peptide, identified by the same SCA assay using the same endopeptidase (EP24.15) as a substrate, it is possible to speculate that both peptides could share some similarities. That hypothesis could explain the pattern of antinociceptive effect observed.

The mechanism of AGH-mediated inhibition of CFA-induced mechanical allodinya in mice was first examined using naloxone, an unspecific opioid-receptor antagonist. Results presented herein demonstrate that subcutaneous injection of naloxone was not able to reverse AGH-induced antinociception, thus suggesting that opioids are not involved on the observed antinociceptive effect. This data are in contrast withdata from the literatureshowing that intraplantar injection of AGH was able to inhibit signs of inflammatory pain in rats, mediated by opioids(Ribeiro et

al., 2013).The discrepancy between these results and the results presented here could be attributed, in part, to the differences of pain models used (carrageenan vs. CFA), to methods of evaluation (Paw pressure test vs. up-down method with von Frey filaments), to differences in routes of administration (intraplantar vs. intraperitoneal) of AGH or differences in species evaluated (mouse vs. rat).To test this hypothesis, mice were injected intraplantar with AGH concomitant with CFA. Interestingly, our results demonstrate that AGH induced an opioid-dependent antinociception when a local injection was performed. Moreover, in this case AGH was able to reverse nociception in all evaluated concentrations and at the higher dose the antinociception was observed for at least 24 h after one single administration.

To test whether AGH could induce a central mediated antinociceptive effect, mice were evaluated at the hot plate testand results demonstrate that AGH induced a potent antinociception, 3 h after its administration very similar to that observed when animals were injected with morphine. Moreover, subcutaneous injection of naloxone completely reversed AGH-induced antinociception at the hot plate test, thus supporting the idea that opioids are involved on AGH-induced antinociception. Hot plate test is widely used to investigate centrally acting analgesic drugs (Hiruma-Lima et al., 2000; Srinivasan et al., 2003; Moniruzzaman et al., 2015) andis consideredto be selective for opioid-like substances, used particularly for the evaluation of central pain at the supraspinaland spinal levels, since it includes both neurogenic and central mechanisms of nociception (Janssen et al., 1963; Taher et al., 2015).

As mentioned above, AGH was first identified through SCA using EP24.15 enzyme as a substrate(Ribeiro et al., 2013). Previously identified peptides using this approach were further demonstrated to act directly in GPCRs, as both agonists and antagonists(Heimann et al., 2007; Gomes et al., 2009). AGH, instead, had been proved to be unable to bind opioid or cannabinoid receptors, the first deduced targets for its effects(Ribeiro et al., 2013). Thus, differently from the other peptides, AGH could act as a modulator of 24.15 activities. In fact, it was already demonstrated, on a fluorescent substrate assay, that AGH reduces cleavage efficiency of 24.15(Ribeiro et al., 2013). Furthermore, it was recently demonstrated that naturally occurring peptides, including some identified with 24.15-SCA, modulate interactions of cytosolic proteins with 24.15(Russo et al., 2012). In this sense, it is demonstrated that 24.15 cleaves enkephalins and dynorphins precursors, thus generating these endogenous opioids(Orlowski et al., 1989; Dahms andMentlein, 1992).As our previous results showed opioid-dependent effect without AGH/opioid receptor interaction, it is possible to speculate that AGH could enhance endogenous opioids through the modulation of 24.15/substrate interaction(Ribeiro et al., 2013)and this in turn would lead to a localized production of opioids.This hypothesis could also explain the absence of side effects, like sedation or motor effects, which could be observed at the open field, forced swimming and tail suspension tests. In this sense, results presented herein demonstrate that administration of different doses of AGH did not influence the locomotor activity of the animals, which could alter their performance in the nociceptive tests presently used. Moreover, no signs of depression were observed after AGH treatment in mice submitted to the forced swimming test or to the tail suspension test. The forced swimming test (FST) is a behavioral model that has a good predictive value for antidepressant potency in humans(Willner, 1984)and is sensitive to antidepressant drugs after acute administration (Borsini et al., 1989).The tail suspension test (TST), a derivative of the forced swimming test (FST) , shares with the FST the ability to induce a state of immobility in animals, which is claimed to reproduce a condition akin to human depression (Renshaw et al., 2009; Simpson et al., 2012). Antidepressant drugs are reported to reduce the immobility time of mice in both tests (Renshaw et al., 2009; Lee et al., 2012) and several reports have shown that their effectiveness correlates significantly with clinical potency(Simpson et al., 2012). As mentioned above, AGH treatment did not induce any significant alteration on mice reinforcing the hypothesis that AGH induces true systemic antinociception in mice.

The roles of opioid, serotonergic, and adrenergic receptors in the regulation of modulation of nociceptive processing have been demonstrated in many previous studies (SchmaussandYaksh, 1984; Yaksh, 1984). In an attempt to characterize the mechanism by which AGH induces antinociception, we finally evaluated the effects of the blockade of serotonin (5HT), and α2-adrenergic receptors in AH-induced antinociception, and we observed that serotonergic receptors appear to be involved in AGH-induced antinociception, once pretreatment of mice withmethysergide, a nonspecific 5-HT1/2 receptor antagonist, blocked the AGHs' antinociceptiveactivity.

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In conclusion, our data demonstrates that AGH exhibits strong, long lasting antinociceptive properties under local and systemic administration by targeting serotonin receptors. Such effect isabsent of neither motor nor anxiety-depressive-like side effects. Data presented herein reinforce the potential prominent role that AGH plays in the control of pain. These properties make AGH an attractive candidate scaffold for the development of novel therapeutics for the treatment of pain.

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FIGURE LEGENDS

Figure 1. Effect of systemic administration of AGH on mechanical allodynia induced by CFA in mice. Animals received intraplantar injection of CFA (25 µL) and were evaluated by the von Frey test before (basal, time 0) and after 15 min, 1, 3, 6, 12 and 24hof treatment. AGH was administered intraperitoneal (i.p.) immediately after CFA at

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the doses of 1, 15, 25 or 50 mg/kg. The control group of animals received saline (Sal) and underwent the same protocol. Data represent mean values ± S.E.M..Statistically significant differences vs. CFA+SAL(*p<0.05, **p<0.001)are indicated. Two-way ANOVA followed by Bonferroni's multiple comparison post-test

Figure 2. Effect of naloxone on AGH-induced antinociception.Animals received intraplantar injection of CFA (25 µL) and immediately after received AGH i.p. at the dose of 25 mg/kg. The von Frey test was applied before (basal, time 0) and after 3, 10 and 24 h of treatments. Naloxone was administered subcutaneously (10 mg/ kg) 20 min before the 3 h measurement. Data represent mean values±S.E.M.. Statistically significant differences vs. CFA+SAL+SAL (*p<0.05, **p<0.001) are indicated. Two-way ANOVA followed by Bonferroni's multiple comparison post-test.

Figure 3. Effect of local administration of AGH on mechanical allodynia induced by CFA in mice. Animals received intraplantar injection of CFA (25 µL) concomitantly with AGH at the concentrations of 0.5 µg (A), 1 µg (B) or 2.5 µg (C) and were evaluated by the von Frey test before (basal, time 0) and after 15 min, 1, 3, 5, 8 and 24 h of treatment. AGH was administered intraplantarconcomitantly with CFA at the doses of 1, 15, 25 or 50 mg/kg. The control group of animals received saline (SAL) and underwent the same protocol. Data represent mean values±S.E.M. Data represent mean values±S.E.M.. Statistically significant differences vs. CFA+SAL (*p<0.05, **p<0.001) are indicated. Two-way ANOVA followed by Bonferroni's multiple comparison post-test.

Figure 4. Effect of naloxone on local antinociception induced by AGH.Animals received intraplantar injection of CFA (25 µL) concomitantly with AGH at the concentration of 2.5 µg. The von Frey test was applied before (basal, time 0) and 3 haftertreatments. Naloxone was administered subcutaneously (10 mg/ kg) 20 min before the 3 h measurement. Data represent mean values ± S.E.M. Data represent mean values±S.E.M.. Statistically significant differences vs. CFA+SAL+SAL (***p<0.001) and vs SAL (#p<0.001) are indicated. Two-way ANOVA followed by Bonferroni's multiple comparison post-test.

Figure 5. Effect of systemic administration of AGH on thermal hyperalgesia of mice evaluated by the hot plate test. Mice received intraperitoneal injection of AGH (25 mg/kg) or morphine (10 mg/ Kg) and were evaluated at the Hot plate test before (basal, time 0) and after 3 h after treatments. Naloxone was administered subcutaneously (10 mg/ kg) 20 min before the 3 h measurement. Data represent mean values±S.E.M. Statistically significant differences vs. Saline (***p<0.001) are indicated. Two-way ANOVA followed by Bonferroni's multiple comparison post-test.

Figure 6. Effect of AGH on general activity of mice. Mice were injected intraperitoneal with AGH at the doses of 1, 25, 50, 75 and 100 mg/kg and were evaluated by the open-field test. Locomotion (**A**) and rearing (**B**) frequencies were determined 3 h after AGH administration. Mice injected with saline underwent the same experimental protocol and were evaluated as control groups. Data represent mean values ± S.E.M. One-way ANOVA followed by by Bonferroni's multiple comparison post-test.

Figure 7. Effect of AGH on depressive-like behavior. Animals received AGH (25 mg/Kg; i.p.; ■) and after 3 h were evaluated by the forced swimming test (**A**) or tail suspension test (**B**). Animals injected with saline (□) underwent the same protocols and were evaluated as control group. Fluoxetine (40 mg/kg; ■) was used as positive control of tail suspension test and administered byoral route 1 h before the tail suspension test. Data represent mean values± S.E.M. Statistically significant differences vs. Saline or vs AGH (***p<0.001) are indicated. One-way ANOVA followed by by Bonferroni's multiple comparison post-test.

Figure 8. Effect of methysergide and yohimbine on AGH-induced antinociception. Animals received intraplantar injection of CFA (25 µL) and immediately after received AGH i.p. at the dose of 25 mg/kg. The von Frey test was applied before (basal, time 0) and after 3, 10 and 24 h of treatments. Yohimbine (A) or methysergide (B) (5 mg/ kg) were administered by intraperitoneal route 30 minutes before intraperitoneal AGH injection. Data represent mean values±S.E.M. Statistically significant differences vs. CFA+SAL+SAL (***p<0.001) are indicated. Two-way ANOVA followed by Bonferroni's multiple comparison post-test.

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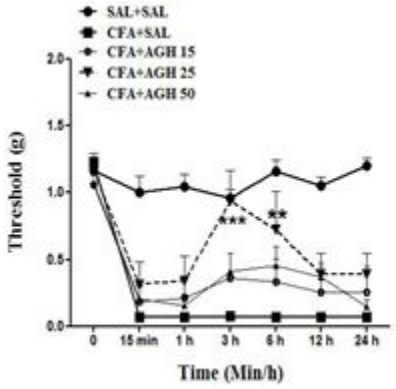
Figure 1



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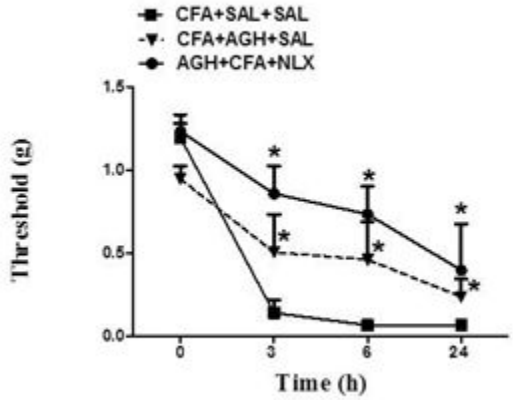

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Figure 3

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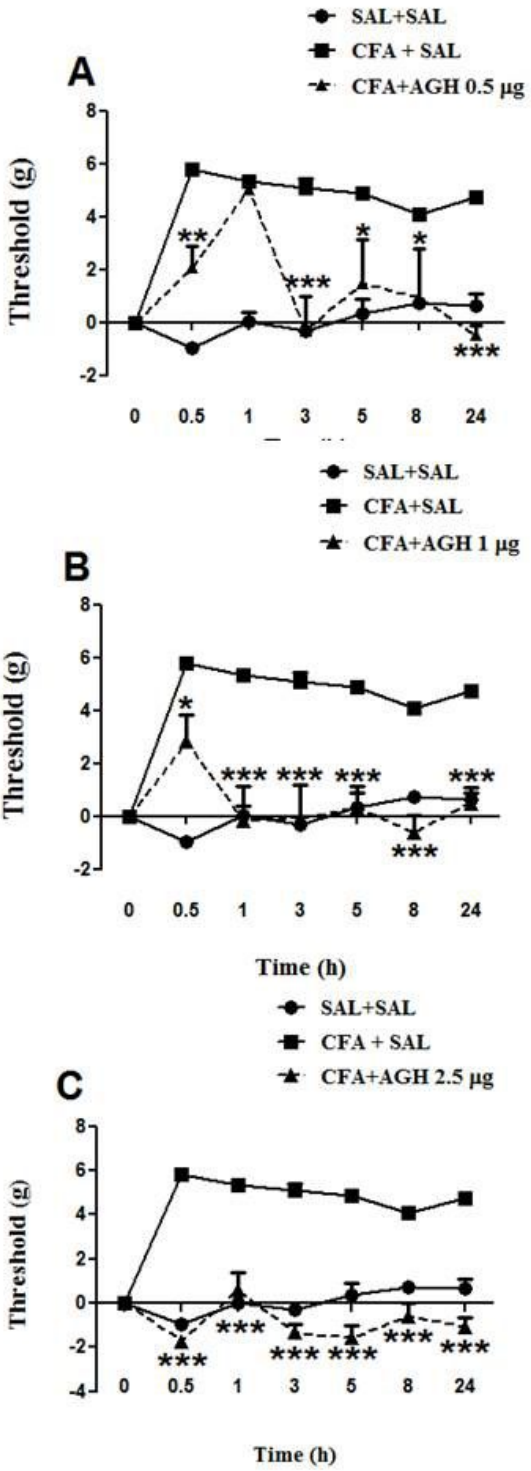

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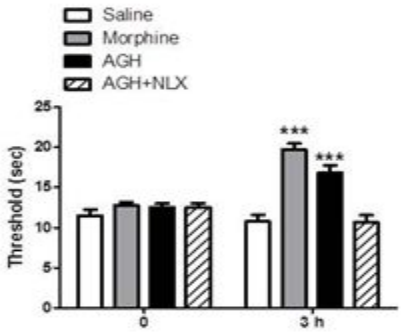
Figure 4

Figure 5



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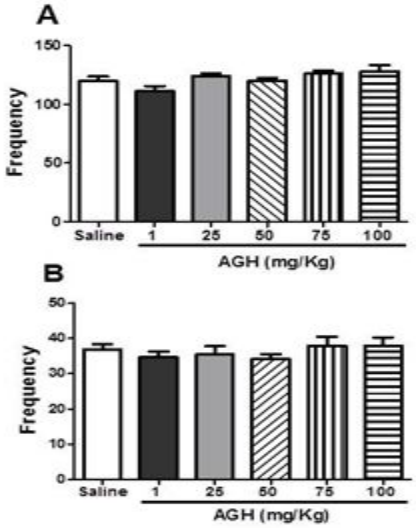

Figure 7

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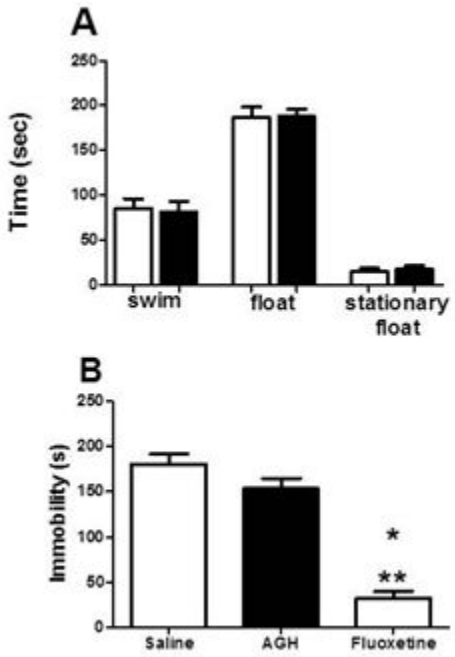
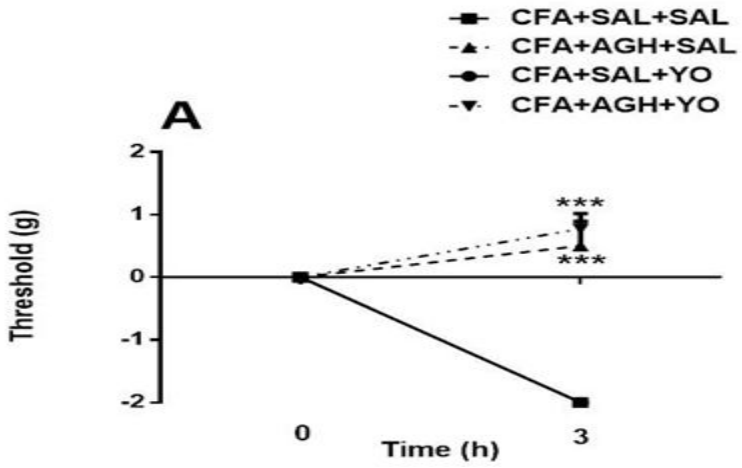


Figure 8



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