

# L-histidine enhances learning in stressed zebrafish

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The aim of the present study was to determine the effect of the histaminergic precursor L-histidine and the H<sub>3</sub> receptor antagonist thioperamide on the learning process of zebrafish submitted or not to confinement stress. On each of the 5 consecutive days of experiment (D1, D2, D3, D4, D5), animals had to associate an interruption of the aquarium air supply with food offering. Non-stressed zebrafish received an intraperitoneal injection of 100 mg/kg L-histidine, 10 mg/kg thioperamide or saline after training. Stressed animals received drug treatment and then were submitted to confinement stress for 1 h before the learning procedure. Time to approach the feeder was measured (in seconds) and was considered to be indicative of learning. A decrease in time to approach the feeder was observed in the saline-treated group (D1 = 141.92 ± 13.57; D3 = 55 ± 13.54), indicating learning. A delay in learning of stressed animals treated with saline was observed (D1 = 217.5 ± 25.66). L-histidine facilitated learning in stressed (D1 = 118.68 ± 13.9; D2 = 45.88 ± 8.2) and non-stressed (D1 = 151.11 ± 19.20; D5 = 62 ± 14.68) animals. Thioperamide inhibited learning in non-stressed (D1 = 110.38 ± 9.49; D4 = 58.79 ± 16.83) and stressed animals (D1 = 167.3 ± 26.39; D5 = 172.15 ± 27.35). L-histidine prevented the increase in blood glucose after one session of confinement (L-histidine = 65.88 ± 4.50; control = 53 ± 3.50 mg/dL). These results suggest that the histaminergic system enhances learning and modulates stress responses in zebrafish.

Key words: *Danio rerio*; Conditioning; Feed trial; Thioperamide; H<sub>3</sub> receptor; Blood glucose

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## Introduction

The histaminergic system is involved in different physiological and behavior processes such as wakefulness, sleep-wake cycle, appetite control, learning, memory, and emotion (for reviews, see Refs. 1 and 2).

Histaminergic neurons are present in the caudal hypothalamus, sending out axons that innervate most parts of the central nervous system. All vertebrates studied contain a well-conserved system of histaminergic neurons (3-6). In zebrafish, all the histaminergic neurons are concentrated in the vicinity of the posterior recess. The morphology, intracellular storage, distribution, and projection patterns

in the brain seem to have been highly conserved throughout evolution, or at least among teleosts and mammals (7).

The zebrafish has received attention because it is useful in genetic studies, for its rapid life cycle, easy handling and genetic manipulation (8). Recently, these animals have been used in behavioral studies, but few investigations have used this species for the study of the role of the histaminergic system in learning (2).

Several studies have shown that the manipulation of the histaminergic central system results in modification of animal behavior in different learning paradigms, although with contradictory results. Both facilitatory and inhibitory effects of histaminergic drugs have been reported in mam-

mals and fish (2,9).

The histaminergic system is also involved in stress-related processes. The highest histaminergic fiber density is found in the hypothalamus, where histamine is involved in the regulation of autonomic and neuroendocrine functions (10). The stress system coordinates the adaptive responses of the organism to stressors of any kind. Activation of the stress system leads to behavioral and peripheral changes that improve the ability of the organism to adjust homeostasis and increase its chances of survival (11). One of the first physiological alterations is the secretion of glucocorticoids and catecholamines, hormones that are responsible for energy mobilization to be used for the reestablishment of homeostasis (12).

The stress response of teleost fish is similar to that of land vertebrates, including function, with elevated consumption and transfer of oxygen, mobilization of energy substrates, decreased energy use for growth and reproduction, and suppressive effects on immune functions (13). Fish respond to stressors with the release of glucocorticoids and catecholamines, which raise glucose production (14). Although the increase in glucose is only a secondary response to stress that follows the release of glucocorticoids and catecholamines, it has been used as an indicator of stress (14,15).

There is evidence that a relationship between learning impairment and stress exists. Both learned helplessness and chronic mild stress significantly decreased the cognitive performance of stressed mice in the water maze task (16). The same investigators observed that chronic mild stress induces the activation of the neuroendocrine and neuroimmune systems, which potentially promote cell damage and decreased neurogenesis and impair cognitive function in mice (17). A deleterious effect of stress on fish cognition was also observed, since isolation stress inhibited active avoidance learning in goldfish (18). The capacity of fish to evoke a stress response to a neutral stimulus was also demonstrated (19,20). Moreover, it was observed that chronically elevated blood cortisol levels in a line of rainbow trout with high responsiveness to a stressor (21)

and chronic administration of exogenous cortisol to fish (22) interfered with cognitive performance.

Since the histaminergic system is involved in stress and in the learning process, the aim of the present study was to investigate the effect of the histaminergic precursor L-histidine and the H<sub>3</sub> receptor antagonist thioperamide on the learning process of zebrafish submitted or not to confinement stress.

The experiments carried out in this study complied with the norms of the Brazilian Neuroscience and Behavior Society (SBNeC), based on the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

## Material and Methods

### Animals

A total of 133 naive zebrafish of unknown sex were used. An acclimatization interval of at least 1 week was allowed from the time of purchase of the fish to the beginning of the experiment. The animals were placed in 30-L aquaria (30 animals per aquarium) at 18-22°C with constant filtering and aeration, under a natural light cycle and fed flake food (Wardly Corporation, USA) five times a week.

### Experimental design

The animals were weighed and placed individually in the aquaria two days before the beginning of the experiments and did not receive food during this period. The experiment was carried out on five consecutive days (D1, D2, D3, D4, and D5), one fish at a time. The procedure was the same on all 5 training days. The air supply was turned off and after 30 s food was offered inside the feeder. The time (in seconds) the fish took to enter the feeding area from the moment the air supply was turned off was recorded and considered to be indicative of learning. Animals that did not eat on the first training day were excluded.

The experiment involved stressing and non-stressing conditions. In the first one, animals received drug treatment 5 min after each training procedure and in the second, 5 min after receiving the drug injections (on the 5 training days), the animals were confined for 1 h by a plastic transparent barrier in an area corresponding to 8% of the total aquarium area. One hour after confinement, the animals were submitted to the training procedure as described above. The animals were divided into the experimental groups described in Table 1. Mean ( $\pm$  SEM) fish weights were not different for both treatments (ANOVA).

### Experimental aquarium

The aquaria used in this experiment were 25 cm long,

**Table 1.** Description of the experimental groups.

Experimental condition	N	Drug	Dose	Fish body weight (g)
Non-stressed	26	Saline	-	0.45 $\pm$ 0.03
Non-stressed	25	L-histidine	100 mg/kg	0.49 $\pm$ 0.03
Non-stressed	24	Thioperamide	10 mg/kg	0.44 $\pm$ 0.03
Stressed	18	Saline	-	0.54 $\pm$ 0.03
Stressed	18	L-histidine	100 mg/kg	0.48 $\pm$ 0.05
Stressed	22	Thioperamide	10 mg/kg	0.43 $\pm$ 0.03

11.5 cm wide and 15 cm high and the water was constantly aired. A transparent plastic barrier was fixed 6 cm from one side of the aquarium, limiting a feeding area. A plastic transparent cylinder was placed inside this area, where food was offered during the experiments.

### Drugs

The drugs used in both experiments were the histamine precursor L-histidine dissolved in saline to a concentration of 50 mg/mL and the H<sub>3</sub> receptor antagonist, thioperamide maleate salt (Sigma, USA) dissolved in saline at a concentration of 5 mg/mL. The drugs were administered intraperitoneally (*ip*) in a volume of 2 mL/kg body weight using a 10- $\mu$ L syringe (Hamilton, model 7105KH, USA). These doses were chosen on the basis of the literature (23,24).

Saline was used as control. Both drugs and vehicle were stored in coded Eppendorf tubes under refrigeration. The code was unknown to the experimenter at the time of the tests.

### Blood glucose analysis

Blood glucose analysis was carried out in animals selected from the second experiment after training on the first and the fifth day of confinement. This analysis was also carried out in one group of non-stressed and non-injected animals used as control.

The animals were decapitated and blood glucose was determined using Accu-Check Advantage II blood glucose strips and the Accu-Check Advantage monitor (Roche Diagnostics, Germany).

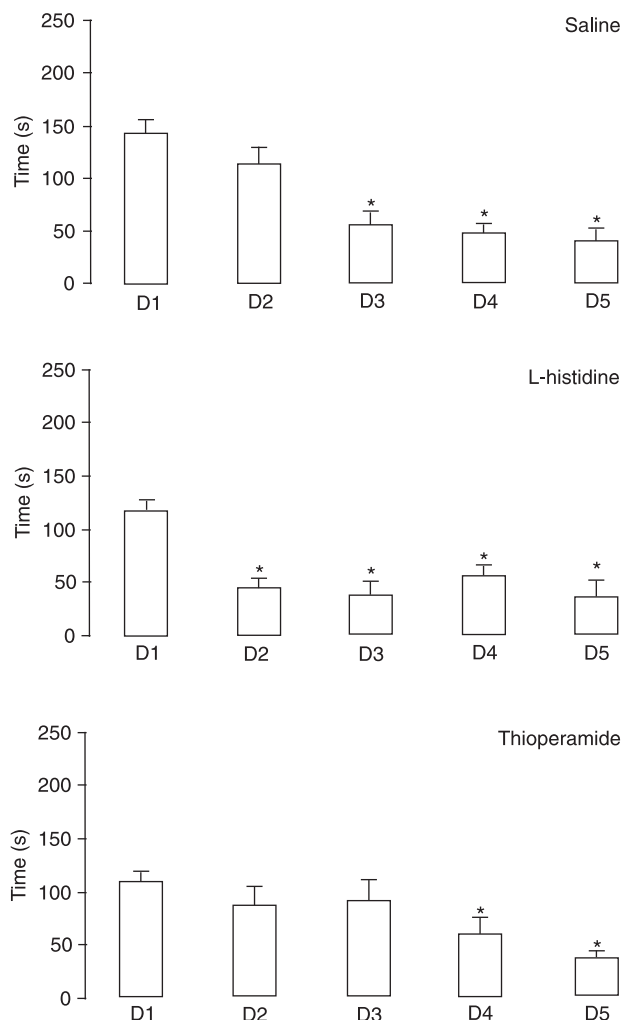
### Statistical analysis

Statistical analysis was carried out using the GB-STAT School Pack software, version 1997. Data are reported as means  $\pm$  SEM. Since the data obtained in the two experiments were not homogeneously distributed, they were analyzed by the non-parametric Friedman test followed by the Dunn multiple comparison test when appropriate. To determine if confinement affected the learning of the animals, the latencies to enter the feeding area by the animals treated with saline in experiments 1 and 2 were compared by the Mann-Whitney U-test, with the level of significance set at  $P < 0.05$ . Since the data for the glucose experiment were homogeneous, they were analyzed by parametric two-way-ANOVA followed by the Student-Newman-Keuls test.

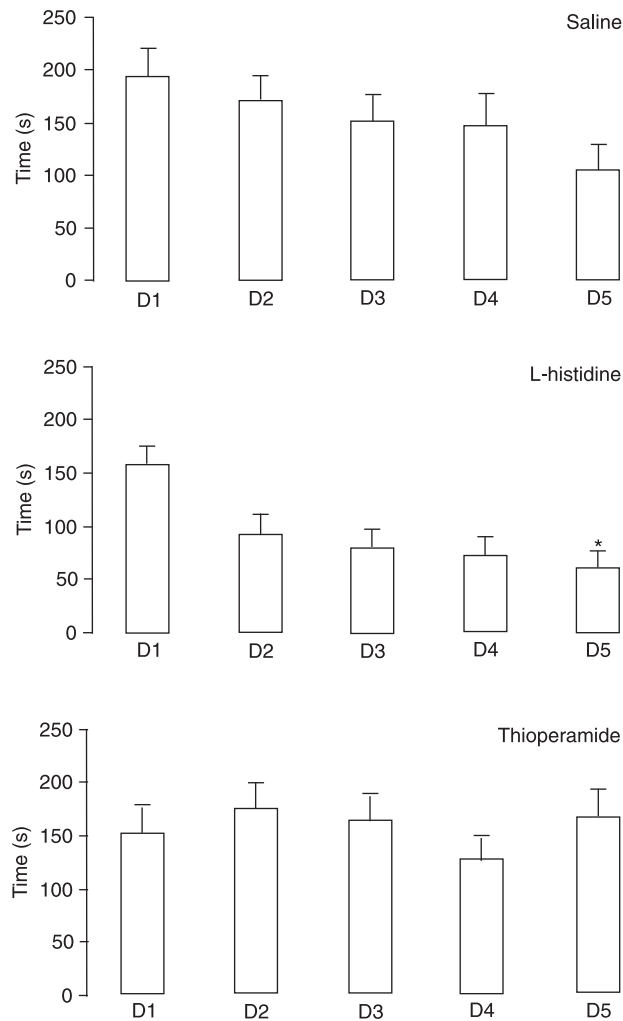
### Results

The data for the non-stressed animals are shown in

Figure 1. The animals treated with saline after training presented a significant decrease of the latency to enter the feeding area after day 3 ( $P < 0.0001$ , d.f. = 4,  $\chi^2 = 35,752$ , Friedman test;  $P < 0.05$ , Dunn test). Animals treated with thioperamide presented a significant decrease of the latency to enter the feeding area after day 4 ( $P < 0.0001$ , d.f. = 4,  $\chi^2 = 30,025$ , Friedman test;  $P < 0.05$ , Dunn test), while the group treated with L-histidine presented a significant decrease of the latency to enter the feeding area after day 2 ( $P \leq 0.0001$ , d.f. = 4,  $\chi^2 = 30.744$ , Friedman test;  $P < 0.05$ , Dunn test).



**Figure 1.** Latencies to enter the feeding area of non-stressed animals treated with saline (N = 26), 100 mg/kg L-histidine (N = 25) and 10 mg/kg thioperamide (N = 24) on the 5 training days (D1, D2, D3, D4, and D5). Data are reported as means  $\pm$  SEM. \* $P < 0.05$  compared to D1 (Friedman test followed by the Dunn test when appropriate).



**Figure 2.** Latencies to enter the feeding area of animals submitted to confinement stress and treated with saline (N = 18), 100 mg/kg L-histidine (N = 18) and 10 mg/kg thioperamide (N = 22) on the 5 training days (D1, D2, D3, D4, and D5). Data are reported as means  $\pm$  SEM. \*P < 0.05 compared to D1 (Friedman test followed by the Dunn test when appropriate).

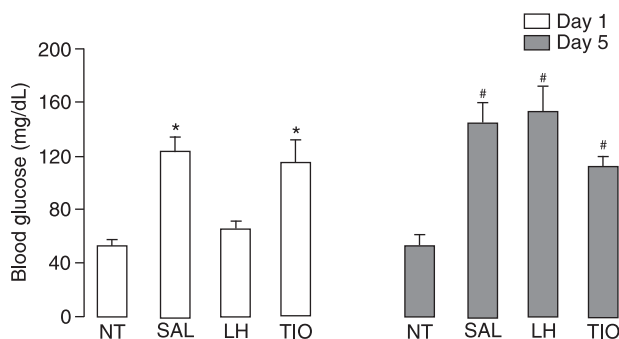
The data for stressed animals are shown in Figure 2. The animals submitted to the stress procedure and treated with saline presented a decreased latency to enter the feeding area ( $P = 0.0482$ , Friedman test, d.f. = 4,  $\chi^2 = 9.5778$ ); however, the Dunn multiple comparisons test did not reveal differences between training days. Animals treated with L-histidine presented a significant decrease in the latency to enter the feeding area, with a statistically significant difference between days 1 and 5 ( $P = 0.0012$ , Friedman test, d.f. = 4,  $\chi^2 = 17.9889$ ;  $P < 0.05$ , Dunn test). The animals treated with thioperamide did not present a decrease in latency to enter the feeding area as observed in the animals treated with saline or L-histidine ( $P = 0.6172$ , Friedman test, d.f. = 4,  $\chi^2 = 2.6545$ ).

#### Effect of stress on the latency to enter the feeding area on day 1 of training

The Mann-Whitney U-test indicated that the animals of experiment 1 presented a significantly lower latency to enter the feeding area on the first day of training (D1:  $141.92 \pm 13.57$ ) than the animals submitted to stress on the same day of training (D1 =  $217.5 \pm 25.66$ ;  $P = 0.02$ , Mann-Whitney U-test).

#### Effect of stress on blood glucose

Two-way ANOVA indicated a difference between the different groups ( $P = 0.0083$ , d.f. = 41,  $F = 4.52$ ) and days of treatment ( $P < 0.001$ , d.f. = 1,  $F = 25.72$ ; Figure 3). The Student-Newman-Keuls test indicated that the animals submitted to the stress procedure and treated with saline presented higher blood glucose levels compared to control animals that were not submitted to the stress and did not receive drug treatment. The blood glucose level of stressed animals that received thioperamide was higher than that of the control group, while stressed animals that received L-histidine presented a level similar to that of the control group.



**Figure 3.** Blood glucose levels of animals pre-treated with saline (SAL, N = 10), 100 mg/kg L-histidine (LH, N = 8), or 10 mg/kg thioperamide (TIO, N = 9) and submitted to one (day 1, D1) or five sessions of confinement stress (day 5 (D5), SAL: N = 13, LH: N = 10, TIO: N = 10). Animals not submitted to confinement stress (NT) that did not receive drug treatment were used as blood glucose control after 1 (N = 6) or 5 days (N = 7) in the experimental aquarium. Data are reported as means  $\pm$  SEM. \*P < 0.05 compared to NT-D1; #P < 0.05 compared to NT-D5 (two-way ANOVA followed by the Student-Newman-Keuls test).

After 5 days of stress exposure, animals treated with saline, L-histidine and thioperamide presented higher blood glucose levels compared to control.

## Discussion

In the present study, animals had to associate the interruption of aeration with food offering. Animals submitted to the learning procedure that received saline presented a decrease in the latency to enter the feeding area, indicating that the animals were able to learn the task.

The animals treated with L-histidine also presented a decrease of the latency to enter the feeding area. Indeed, the drug treatment facilitated learning. Facilitatory effects of histamine and L-histidine have been observed in previous studies. Both intracerebroventricular injection of histamine and *ip* injection of histidine presented proactive effects against memory deficits induced by nucleus basalis lesion in rats (25). In goldfish, L-histidine had a facilitatory effect on appetitive learning (26).

In zebrafish, the injection of the histidine decarboxylase inhibitor, alpha-fluoromethylhistidine, significantly increased the time to find the goal tank in a T-maze, suggesting that the lack of histamine may impair long-term memory (2). L-histidine is a histaminergic precursor, which increases brain histamine levels. Thus, the memory facilitation observed in the L-histidine-treated group may be the result of an increase in brain histamine levels following L-histidine administration.

Animals treated with thioperamide presented a delay in learning, indicating the inhibitory effect of this drug. Since thioperamide is an H<sub>3</sub> receptor antagonist that increases brain histamine (27,28), a facilitatory effect of this drug was expected. Many studies have indicated that H<sub>3</sub> receptor blockade by selective antagonists improves cognition in different animal models of learning involving mice and rats, such as one-trial inhibitory avoidance task (29), two-trial place recognition test (30) and spatial learning (23).

Besides being histaminergic auto-receptors, H<sub>3</sub> receptors also function as presynaptic heteroreceptors controlling the release of serotonin (31), acetylcholine (32) and several other neurotransmitters (33,34). It has been shown that histaminergic H<sub>3</sub> antagonists (ciproxifan, clobenpropit and thioperamide), directly administered to the basolateral amygdala, decreased spontaneous acetylcholine release. Also, rats receiving intra-basolateral amygdala injections of the H<sub>3</sub> antagonists at doses similar to those inhibiting spontaneous acetylcholine release showed memory consolidation impairment of contextual fear conditioning (35). A possible inhibition of acetylcholine release resulting from the treatment with thioperamide could be responsible for

the learning deficit observed in the group that received this drug.

Since evidence suggests that there is a relationship between learning impairment and stress (36), and that brain histamine is related to both processes (1,10), we investigated learning in animals submitted to confinement stress and treated with histaminergic drugs (L-histidine and thioperamide). Blood glucose analysis was carried out in order to observe the effectiveness of confinement as a stressor and also the possible effects of the drugs on stress. Some studies have indicated that blood glucose analysis carried out using blood glucose monitors may not always be reliable. For example, humidity or temperature may interfere with the monitor reading (37). However, in the present study, the blood glucose levels of non-stressed animals were similar to the plasma glucose levels of non-stressed Nile tilapia determined by a colorimetric method (15,38). Despite the different types of stressors used in these studies, such as electroshock, a social stressor (38) and air emersion (15), the glucose increase following confinement stress was similar to the increase observed in the present study. Thus, although the use of a blood glucose monitor is not the standard method used in stress studies, it proved to be a reliable method to establish if stress is present.

Blood glucose analysis indicated that a single confinement session enhanced the blood glucose level of the animals, indicating stress. Previous work had already shown that confinement is an effective stressor, which causes enhanced cortisol release (39).

Despite the confinement, stressed animals treated with saline were able to learn the task. However, this procedure delayed the learning of the task, since animals submitted to the stress presented higher latencies to enter the feeding area than non-stressed animals. Other studies have shown that different kinds of stress are capable of delaying or impairing learning (18,40) and it seems that cognitive impairment caused by stress is related to specific alterations in brain homeostasis, involving the neuroimmune and neuroendocrine systems as well as neurogenesis (17).

The stressed animals treated with L-histidine presented a facilitation of learning since the results for this group were similar to those observed in the non-stressed group. It seems that this amino acid attenuated the memory disruption caused by stress. This drug also attenuated the memory disruption induced by immobilization stress in rodents (40). In addition, blood analysis indicated that this drug blocked the blood glucose increase caused by confinement. Thus, the facilitatory effect observed in this group might be secondary to the effect of L-histidine on stress.

Further studies are needed to investigate this question.

While thioperamide delayed learning in non-stressed animals, it prevented this occurrence in stressed fish. It is possible that a decrease of acetylcholine release induced by this drug added to the impairment of learning caused by the stress procedure could result in the blockage of learning observed in this group.

Taken together, the results of this experiment show

that the histaminergic system enhances learning and modulates stress responses in zebrafish.

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