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Research report

The endocannabinoid system and associative learning and memory in zebrafish

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HIGHLIGHTS

- Zebrafish medial pallium is identified to be crucially involved in emotional associative learning and memory.
- Zebrafish medial pallium is known to contain high density of endocannabinoid receptor CB1.
- Acute activation/inactivation of CB1 has no effect on memory retrieval from associative memory in zebrafish.
- Chronic activation/inactivation of CB1 enhances or disrupts associative acquisition learning depending on the learning motivation.

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ABSTRACT

In zebrafish the medial pallium of the dorsal telencephalon represents an amygdala homolog structure, which is crucially involved in emotional associative learning and memory. Similar to the mammalian amygdala, the medial pallium contains a high density of endocannabinoid receptor CB1. To elucidate the role of the zebrafish endocannabinoid system in associative learning, we tested the influence of acute and chronic administration of receptor agonists (THC, WIN55,212-2) and antagonists (Rimonabant, AM-281) on two different learning paradigms. In an appetitively motivated two-alternative choice paradigm, animals learned to associate a certain color with a food reward. In a second set-up, a fish shuttle-box, animals associated the onset of a light stimulus with the occurrence of a subsequent electric shock (avoidance conditioning). Once fish successfully had learned to solve these behavioral tasks, acute receptor activation or inactivation had no effect on memory retrieval, suggesting that established associative memories were stable and not alterable by the endocannabinoid system. In both learning tasks, chronic treatment with receptor antagonists improved acquisition learning, and additionally facilitated reversal learning during color discrimination. In contrast, chronic CB1 activation prevented aversively motivated acquisition learning, while different effects were found on appetitively motivated acquisition learning. While THC significantly improved behavioral performance, WIN55,212-2 significantly impaired color association. Our findings suggest that the zebrafish endocannabinoid system can modulate associative learning and memory. Stimulation of the CB1 receptor might play a more specific role in acquisition and storage of aversive learning and memory, while CB1 blocking induces general enhancement of cognitive functions.

1. Introduction

Associative learning is defined as the ability to acquire and consolidate the relationship between the occurrence of a particular sensory stimulus and a subsequent consequence, which can have an either positive or negative relevance [1,2]. Typically food rewards are positive consequences for associative learning, while electric shocks or noxious substances are applied for punishment or visceral discomforts [3,4]. Both types of associative learning have been investigated in the zebrafish (*Danio rerio*) [5–7] involving different sensory modalities. Often animals were trained in visual association tasks [8–11], but also olfactory [12] and gustatory [13] based associative learning was investigated.

Developmental and hodological findings suggest that the medial pallium (Pm) represents an amygdala homologous structure in the teleost dorsal telencephalon [14–17]. This assumption is supported by lesion studies in goldfish (*Carassius auratus*). Destruction of Pm resulted in an impaired retention of a previously acquired strategy during active avoidance conditioning [18–20]. Furthermore, mapping of neuronal activity by measuring expression of c-fos mRNA

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revealed that Pm serves as a brain center for emotionally guided behavior in zebrafish [21]. Hence, the Pm is an essential structure for associative learning.

The cannabinoid receptor 1 (CB1) represents the major target for endo- and exogenous cannabinoids in the central nervous system of vertebrates [22,23]. Lam et al. [24] investigated the distribution of CB1 mRNA expression in the brain of adult zebrafish. On the level of the dorsal telencephalon, the Pm showed most intense staining compared to other regions. This led to the assumption that the endocannabinoid system is crucially involved in Pm related processes like the fish's emotional state or associative memory. Actually, acute treatment of zebrafish trained in a color discrimination paradigm with the CB1 agonist THC (Δ^9-tetrahydrocannabinol) did not affect memory retrieval, while a dose dependent anxiolytic effect was suggested [25].

The present work aimed to determine the influence of acute and chronic treatment with CB1 agonists and antagonists on behavioral performance, associative learning and memory retrieval in zebrafish trained either in an appetitively motivated color discrimination task or in an aversively motivated active avoidance paradigm in a fish shuttle-box.

2. Materials and methods

2.1. Animals

Zebrafish ($n = 132$; *D. rerio*, AB wild type strain, 30–40 mm body length, one year of age, both sexes) were bred in-house and accomodated communally (maximum $n = 100$) in 200-l aquaria with aerated and filtered water at 25–27 °C, a substrate of sand and different hiding places (plants, stones and potsherds). Animals were subjected to a 12:12 h light/dark cycle and maintained on a daily diet of pellet fish food (Sera, Heinsberg, Germany). Before beginning of learning Experiment 1 (see Section 2.3), participating individuals ($n = 85$) were not fed for four days. During the experiments, animals did not get extra food besides rewards from the tests. Zebrafish ($n = 47$) taken for learning experiment 2 (see Section 2.4), were not food deprived.

Before beginning of the experiments, zebrafish were transferred to the test laboratory and were housed in groups of five animals in 12-l aquaria equipped with a heater and a water filter. Half of the aquariumwater was exchanged once a week. The experiments started after an acclimatization period of three days. All fish were drug naive prior to the experiments.

The experiments followed the guidelines of the animal welfare laws and were approved by the Animal Care and Use Committee of the state of North Rhine-Westphalia, Germany.

2.2. Drug application

To check how activation or inactivation of cannabinoid receptors changes behavioral performances in the two learning tasks, zebrafish were administered the CB1 agonists THC (THC-Pharm GmbH, Germany) or WIN55,212-2 (Sigma–Aldrich, Germany), or the CB1 antagonists Rimonabant (THC-Pharm GmbH, Germany) or AM-281 (Tocris, UK) as described before [25]. For the drug treatment animals were swimming in a beaker containing 100 nM drugs in 2.5 liter of tap-water for one hour. To reach the desired concentration, 250 μl of a 1 mM stock solution (3.14 mg THC or 5.23 mg WIN55,212-2, 268.6 μl ethanol, 700 μl cremophor, 9 ml $aqua_{dest}$; 4.6 mg Rimonabant or 5.57 mg AM-281, 300 μl ethanol, 700 μl cremophor, 9 ml $aqua_{dest}$) was added to the aquarium water. Control animals were kept in tap-water containing the same amount of vehicle. Zebrafish were either tested for an acute effect and treated only once, or they were used for the investigation of chronic pharmacological influences and received a daily treatment before each training.

2.3. Experiment 1 – color discrimination learning

The color discimination paradigm followed the same protocol as described earlier [25] and is here described only briefly: The set-up for color discrimination learning was a 50-l aquarium that was divided into three compartments. A central starting chamber was separated from two choice-compartments by gray PVC-walls, each containing a central lockable door. At the outer sides of the choice-compartments, removable colored foils were fixed to the aquarium walls. Each foil was illuminated by a light bulb from behind, coloring the respective choice compartment and thus providing a color stimulus for learning. The light intensities of colors in the two choice-compartments were set equally to 500 lx.

In a two-alternative choice task, zebrafish learned to associate a green color stimulus (S^+) with a food reward. First, the animals were habituated to the unknown environment for two days. All individuals of one group were placed together in the starting chamber for 30 min, with both doors open and with two identical green color-stimuli. The group could freely explore the aquarium. Each time a door was passed, the animals were food rewarded with small pieces of blood worms. On the following two days, animals were set individually into the starting chamber, while the procedure was otherwise the same as on the previous days. After the habituation period, zebrafish were trained individually by releasing single fish into the starting chamber. After two minutes of acclimatization, the first trial started by opening both gates simultaneously. Fish learned to discriminate between two colors (green and red) by swimming to the color defined as positive (green: S^+) to get a food reward. If a fish swam to the negative color (red: S^-), it was not rewarded and returned to the starting chamber. Before a new trial started, the two colored foils for S^+ and S^- were re-arranged according to a pseudorandom schedule [26]. The same color combination of S^+ and S^- was used during each training day. Daily sessions consisted of 4–8 trials for each individual. Correct or wrong decisions and the time from opening the gates until the fish swam through one of the doors were noted for each trial. Once the group had reached the learning criterion of more than 70% correct choices on three consecutive days, tests with acute pharmacological treatment were performed ($n = 14$ THC test, $n = 13$ Rimonabant test).

To investigate the effect of chronic pharmacological treatment on learning and memory, other groups of fish ($n = 11$ control, $n = 11$ vehicle, $n = 8$ THC, $n = 9$ WIN55,212-2, $n = 10$ Rimonabant, $n = 9$ AM-281) were treated with the drugs before each test for 13 days. Fish were trained as decribed above for seven days followed by six days of reversal training, during which choice of the previously incorrect color (red) was now rewarded and the previously correct color (green) was now incorrect. The procedure of daily training and measurements were the same as that used in the previous discrimination learning.

2.4. Experiment 2 – active avoidance learning

For active avoidance learning, single zebrafish were trained in a fish-shuttle box modified after Pradel et al. [5]. A 12-l tank was divided by an opaque barrier into two equal compartments. The barrier was 10 cm high and the water level was set at 12 cm to allow the animals to freely pass over the barrier from one compartment into the other. There was a red foil at the end of each tank wall illuminated by a white LED behind it. In each compartment at the long sides of the tank, there were two steel electrode plates (13 cm × 16 cm). The steel electrodes were connected to a shocker unit consisting of an accupulser (Model A310, World Precision Instruments, Sarasota, USA) and a stimulus generator (Model