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Research article

Cannabinoid modulation of zebrafish fear learning and its functional analysis investigated by c-Fos expression

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ABSTRACT

It has been shown that zebrafish fear learning proceeds in the same way as reported for rodents. However, in zebrafish fear learning it is possible to substitute the use of electric shocks as unconditioned stimulus and utilize the inborn fear responses to the alarm substance Schreckstoff, instead. The skin extract Schreckstoff elicits typical fear reactions such as preferred bottom dwelling, swimming in a tighter shoal, erratic movements and freezing. This natural fear behavior can be transferred from Schreckstoff to any other sensory stimulus by associative conditioning (fear learning). We presented Schreckstoff simultaneously with a red light stimulus and tested the effectiveness of fear learning during memory retrieval. The two brain regions known to be relevant for learning in zebrafish are the medial and the lateral pallium of the dorsal telencephalon, both containing rich expressions of the endocannabinoid receptor CB1. To test the influence of the zebrafish endocannabinoid system on fear acquisition learning, an experimental group of ten fish was pretreated with the CB1 receptor agonist THC (Δ^9-tetrahydrocannabinol; 100 nM for 1 h). We found that CB1 activation significantly inhibited acquisition of fear learning, possibly by impairing stimulus encoding processes in pallial areas. This was supported by analyzes of c-Fos expression in the brains of experimental animals. Schreckstoff exposure during fear acquisition learning and memory retrieval during red light presentation increased the number of labelled cells in pallial structures, but in no other brain region investigated (e.g. striatum, thalamus, and habenula). THC administration before fear conditioning significantly decreased c-Fos expression in these structures to a level similar to the control group without Schreckstoff experience, suggesting that Schreckstoff induced fear learning requires brain circuits restricted mainly to pallial regions of the dorsal telencephalon.

1. Introduction

Normal fear, defined as a normal reaction to a real or imagined threat, is an essential behavior shown in response to physical and emotional danger (Gullone, 2000). If an organism cannot feel it, it is unable to protect itself from legitimate threats. Fear processing in a mouse brain corresponds well with fear processing in a human brain. When a menacing stimulus is encountered, the thalamus activates the amygdala even before it informs the brain parts responsible for higher cognitive processes (Penzo et al., 2015). The amygdala then activates a so-called fight-or-flight response, triggering typical symptoms such as heart rate increase, startle response, and sweating. The similarities of fear responses in the brains of mice and humans have allowed to investigate the neuronal circuitry and molecular processes of fear and fear behaviors more extensively than other basic emotions (Tovote et al., 2015). For this purpose, a fear-conditioning paradigm is routinely used to study fear learning and memory in rodents (Shin, 2012): in a typical experiment, the animal is placed in a fear conditioning chamber. After the initial familiarization, an aversive stimulus (e.g. an electric foot shock) is paired several times with an auditory conditioned stimulus (CS, tone) within the novel environment. When tested at various time points after training, rats and mice exhibit marked fear, measured by freezing behavior in response to re-presentation of either the context (contextual fear conditioning) or the auditory CS delivered in a different context (cued fear conditioning). Cued fear conditioning allows the investigation of amygdala-dependent memory processes, whereas contextual fear conditioning relies on both the amygdala and the hippocampus (Phillips and LeDoux, 1992). These two structures are both characterized by high expressions of endocannabinoid receptor CB1, suggesting a fundamental role of the endocannabinoid system in regulating fear learning (Mackie, 2005). It has been reported that local i.p. injection of the selective CB1 ligand WIN 55,212-2 (WIN) impairs contextual fear conditioning but does not modify the cued freezing behavior elicited by tone presentation (Pamplona and Takahashi, 2006). Because of these findings, it was suggested that cannabinoid agonists affect selectively the hippocampus-dependent aversive memories in rats.

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However, micro-infusion of WIN in the rat basolateral amygdala has no effect on fear acquisition learning, but on fear consolidation and retrieval (Kuhnert et al., 2013). In contrast, other authors have reported that injection of WIN into the basolateral amygdala impairs fear acquisition and consolidation, but not retrieval, while injecting the drug into the ventral subiculum of the hippocampus impairs fear retrieval (Segev and Akirav, 2011). These findings suggest that cannabinoid signaling differentially affects memory depending on the task, the brain region, and the memory stage.

In the zebrafish brain two structures exist, which are supposed to be functional homologues to the mammalian amygdala and hippocampus; these are the medial and the lateral pallium, respectively (Mueller et al., 2011, von Trotha et al., 2014). Lesion experiments as well as investigations of immediate early gene expressions highlighted both regions to play a critical role at initializing and controlling anxiety and learned fear behavior (Lau et al., 2011, Martin et al., 2011, Portavella et al., 2004). And as found for the mammalian counterparts, both structures contain high densities of CB1 (Aoki et al., 2013, Lam et al., 2006).

Since several years, the zebrafish is a respected model organism in behavioral neuroscience, and besides several appetitively motivated learning tasks, it is also used for investigating aversive and fear learning (Maximino et al., 2010). Principally, fear conditioning in zebrafish proceeds in the same way as in rodents. However, it is possible to relinquish painful electroshocks by using an inborn behavioral response to the presentation of Schreckstoff, instead (von Frisch, 1941). Schreckstoff can be detected through olfaction at very low concentrations, and it induces alarm- or fear behaviors, which are expressed in preferred bottom dwelling, swimming in a tighter shoal and increased freezing behavior, or erratic movements (fast zic-zac swimming) (Speedie and Gerlai, 2008). During zebrafish fear conditioning, a CS (e.g. red light) is presented together with Schreckstoff. A single stimulus pairing is sufficient for later release of the conditioned fear behavior on presentation of the CS alone (Hall and Suboski, 1995).

In this project, we investigated zebrafish fear learning and memory and highlighted the role of the endocannabinoid system during acquisition learning in order to compare our results with those found in rodents. To do so, we trained zebrafish to associate a red light stimulus with the occurrence of Schreckstoff in the tank water. We tested the influence of treatment with the CB1 agonist THC (Δ^9-tetrahydrocannabinol) on memory acquisition. After finishing behavioral experiments, we looked for expression of the immediate early gene product c-Fos to highlight the brain network behind zebrafish fear learning and identify the possible stages of cannabinoid influences.

2. Material and methods

2.1. Animals and housing

WT-wild type zebrafish ($n = 60$; *Danio rerio*, 30–40 mm body length, one year of age, both sexes) were bred in-house and held communally (maximum $n = 100$) in 200-l aquaria with aerated and filtered water at 28 ± 1 °C, a substrate of sand and plants, stones and potsherds for hiding places. Animals were kept under a 12:12 h light/dark cycle and maintained on a daily diet of red bloodworms and pellet fish food (Sera, Heinsberg, Germany).

Before beginning of the experiments, zebrafish were transferred to the behavioral laboratory and were housed with 5 animals each in three separate compartments of the test aquarium (Fig. 1A), each equipped with a heater and a water filter. A ceiling-mounted white light tube, approximately 50 cm above the tank kept the 12:12 h light/dark cycle. All fish were naïve to the drug treatment as well as the experimental paradigm. For habituation to the experimental procedure, a daily water exchange was performed always at the same time for one week, with the animals staying in their tanks. During the water exchange, 90% of the tank water in each compartment was replaced by stagnant tap water of the same temperature.

All fish were housed according to randomly assigned groups, e.g. 1 - control, 2 - acquisition, 3 - retrieval, 4 - vehicle or 5 - THC. Group 1 ($n = 10$) received the control treatment and got distilled water during acquisition instead of the Schreckstoff solution. Group 2 ($n = 15$) did the acquisition procedure with application of Schreckstoff during presentation of the red light only. In contrast, the fish of group 3 ($n = 15$) performed also the retrieval session on the day following acquisition. The groups 4 and 5 (each $n = 10$) were treated in the same way as group 3, but these two groups were pharmacologically treated before the acquisition procedure, in order to test the influence of the endocannabinoid system on fear acquisition learning. During acclimatization and behavioral testing, fish were fed once a day in the morning (~ 11:00) with flake food. Testing occurred between 1 pm and 3 pm. Fish behavior was recorded during the experiments with video-cameras (Panasonic, WV CD20) located in front of the test-tank and stored on a personal computer with the freeware VirtualDub (Avery Lee, USA). The experiments followed the guidelines of the animal welfare laws

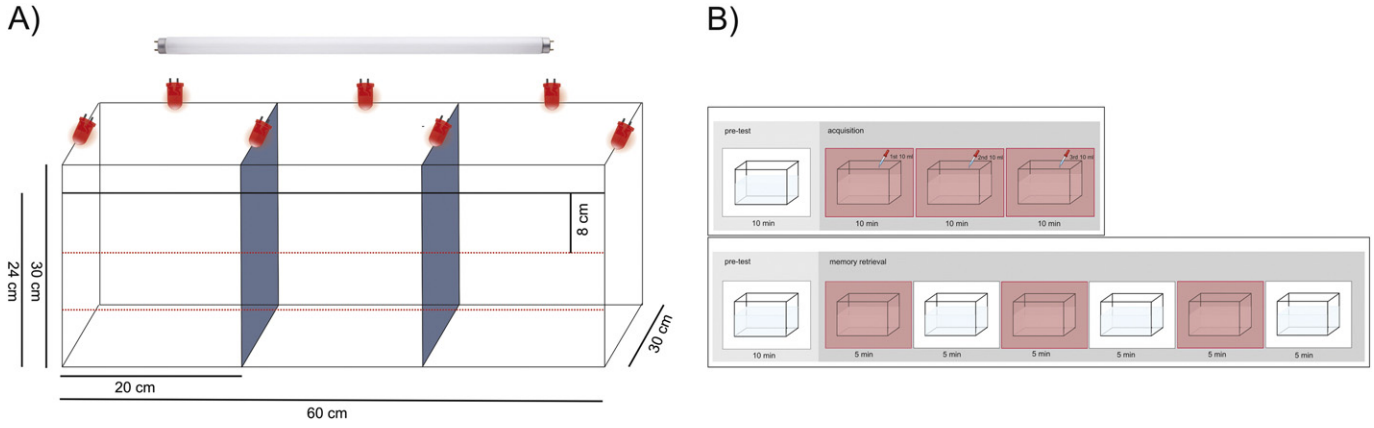

Fig. 1. A) The experimental tank was divided in three equal compartments. In each compartment, a group of five fish was placed. The experimental tank was illuminated either by a white light mounted above or by seven red LEDs. A video camera in front of each compartment recorded the behavior of the fish during the acquisition and the retrieval sessions. To evaluate bottom dwelling behavior, the tank was divided into three horizontal layers. B) Before acquisition learning and memory retrieval sessions, the behavior of each group was pre-tested under white light. During acquisition, the white light was switched off and the red light LEDs were switched on while three doses of 10 ml Schreckstoff solution were applied into each compartment every 10 min. During memory retrieval, the light stimulus changed between red and white light every 5 min.