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Research report

Involvement of cannabinoid receptors in the amygdala and prefrontal cortex of rats in fear learning, consolidation, retrieval and extinction

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HIGHLIGHTS

- Drug study on fear memory and cannabinoids in prefrontal cortex and amygdala.
- Cannabinoids reduce fear learning in amygdala and prefrontal cortex.
- Cannabinoid antagonists impair fear extinction in the prefrontal cortex.

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ABSTRACT

Cannabinoid receptors 1 (CB1R) have been shown to be a crucial part of the neuromodulatory endocannabinoid system which is involved in emotional learning and memory.

We here investigated in rats the role of CB1R in the basolateral amygdala (BLA) and medial prefrontal cortex (mPFC) in different phases of fear learning, memory and extinction. We used the fear potentiated startle paradigm to measure the effects of local microinfusion of the CB1R agonist WIN 55,212-2 (WIN) or the CB1R antagonist AM251 on acquisition, consolidation, retrieval and extinction of fear.

No effects on fear acquisition of WIN or AM251 were found in the BLA or mPFC. WIN impaired fear retrieval in the BLA and in mPFC. Also, WIN reduced fear consolidation in the BLA but not in the mPFC. AM251 decreased fear consolidation after mPFC infusion. Likewise, fear extinction was impaired by AM251 infused into the mPFC.

Our data indicate that fear memory consolidation and retrieval, as well as extinction are regulated differentially by amygdaloid and cortical CB1R.

1. Introduction

Endocannabinoid signalling regulates transmitter release via cannabinoid receptors 1 (CB1R) that are located presynaptically on several transmitter systems in the brain [1–3]. CB1R inhibit transmitter release as heteroreceptors mainly on glutamatergic and GABAergic nerve terminals in the cerebellum and in the forebrain. Hence, CB1R and endocannabinoid signalling are in strategic positions to regulate a variety of cognitive functions, such as learning and memory. For example, enhancement of endocannabinoid signalling by the inhibition of its metabolizing enzyme modulates acquisition and extinction of spatial memory [4], the selective CB1R-antagonist AM251 has an amnestic effect in the inhibitory avoidance paradigm [5], and the synthetic CB1R agonist WIN 55,212-2 (WIN) impairs spatial memory retrieval [6]. In addition the genetic deletion of CB1R in mice impaired the extinction of conditioned fear [7]. Many recent studies support the important role of endocannabinoid signalling in mnemonic processes [8–14]. However, there are still some open questions concerning the involvement of CB1R with respect to different brain systems and different phases of learning and memory.

We here focus on fear conditioning and fear memory. Classical fear conditioning comprises different phases of learning and memory processes such as acquisition, consolidation, retrieval and extinction [15,16]. Recently, different paradigms of fear conditioning were used to study the role of endocannabinoid signalling in fear conditioning. But the differences of for example olfactory fear conditioning [12], auditory fear conditioning [11], contextual fear conditioning [10], visual fear conditioning [17] and trace fear conditioning [13] make direct comparisons between these studies difficult. Furthermore, different species (mice or rats), knockout and wildtype animals and various drugs in different doses were used in these studies. Therefore, we here investigated in a systematic approach in rats the role of CB1R in the basolateral complex of the amygdala (BLA) and the medial prefrontal cortex (mPFC) in different phases of fear learning. We used the fear-potentiated startle (FPS) paradigm and tested the effects of the CB1R agonist WIN and

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the antagonist AM251 on acquisition, consolidation, retrieval and extinction of fear after local microinfusion of the drugs.

2. Materials and methods

2.1. Subjects

A total of 265 adult male Wistar rats (Hannover strain, Harlan-Winkelmann, Borchen, Germany) weighing between 250 and 300 g were used. Animals were housed in groups of five in Macrolon cages (type IV) under standard conditions on a 12 h light-dark cycle with lights on at 7 o'clock. All experimental procedures were performed during the rats' light cycle. They received free access to tap water and were maintained on their experimental body weight by controlled feeding of 12 g rodent chow per rat per day.

The experiments were performed in accordance with the National Institutes of Health ethical guidelines for the care and use of laboratory animals for experiments and were approved by the local animal care committee and authorities.

2.2. Experimental procedures

Upon arrival, the rats were allowed to acclimatize to the vivarium for three days with access to food and water ad libitum. Afterwards, feeding was reduced to 12 g per animal per day and daily handling was conducted for one week. Then, stereotaxic surgeries were performed and guide cannulae were either implanted into the prelimbic part (PrL) of mPFC or into the BLA. Three weeks after arriving in the laboratory animals underwent an ASR matching procedure, which was done in order to individually identify the acoustic startle stimulus intensity for each animal that differed least from the mean value of the group. This matching procedure was done in order to reduce the variability of different baseline ASR reactivity. Three days later the fear conditioning training was commenced. At 24 h following the fear conditioning session, animals were returned to the same chambers and presented with the first FPS test. During the next three days the same FPS test was executed in a 24 h rhythm and again at 14 days following these tests. Three to four days later some of the animals were also tested in the elevated plus-maze. After completion of the experiments all laboratory animals were killed by an overdose of chloral hydrate (in accordance with the Animal Welfare Act).

2.3. Matching, fear conditioning and fear potentiated startle

For matching, fear conditioning and FPS measuring a Startle Response System (TSE Systems, Bad Homburg) was used. Startle-boxes consisted of two different sets of cages (Set I and II) with Plexiglas doors and floors resting on a piezo-sensitive platform mounted inside of a sound attenuated and ventilated chamber. Vibrations of the cages caused by the whole body acoustic startle response (ASR) were transduced into analogy signals and then digitized and stored by a computer using the TSE software. For fear conditioning an electrical stimuli were delivered through a floor grid (only Set I). All acoustic startle stimuli (AS) were presented as broadband noise at the respective sound levels. Pure sine wave tones of 4 kHz and a sound pressure level of 72 decibel (dB) were used as conditioned stimuli (CS). At the beginning of each session, animals were placed into the startle chambers for a 5-min acclimatization period without stimuli. All boxes were equipped with a fan that produced a broadband noise level of 60 dB (sound pressure level).

2.4. Matching

During the matching procedure animals were exposed to AS of four different intensities (90, 95, 100, 105 dB). Each stimulus was presented eight times for 30 ms. Altogether, a total of 32 stimuli were presented in pseudo randomized order. Interstimulus intervals (ITI) differed from 20 to 30 s and were also presented in pseudo randomized order.

2.5. Fear conditioning

Conditioning was performed by pairing a CS with a foot shock. During training a tone (4 kHz, 72 dB, 4000 ms) was presented eight times with a pseudo randomized delay between the presentations (90–180 s). After 3500 ms duration the CS was paired with the delivery of a 0.5 mA foot shock (500 ms) as an unconditioned stimulus (US).

2.6. FPS test

In order to evaluate FPS as an operational measure of fear the AS intensity from the matching was used was each rat individually. Following an acclimatization period of 5 min this individually defined AS was presented ten times (Block I). Then, 30 trials followed in pseudo randomized order consisting of three different types of trials. (1) Ten trials involved the presentation of the AS (Block II), (2) ten trials involved the presentation of the CS, for 4000 ms, and, at 3500 ms after its onset, the simultaneous presentation of the AS. (3) Ten trials involved the presentation of a neutral signal tone (10 kHz, 72 dB), for 4000 ms, and, at 3500 ms after its onset, the simultaneous presentation of the AS. Finally, ten AS alone presentations were presented (Block III). Interstimulus intervals differed from 20 to 30 s. The FPS was calculated as the percent difference between the ASR in the presence or in the absence of the CS (100% × [AS with CS − AS alone]/AS alone) according to previous studies [18–20].

2.7. Surgery

Rats were anesthetized with an intraperitoneal injection of chloral hydrate (360 mg/kg, Sigma–Aldrich, Steinheim, Germany) and placed in a stereotaxic device. Subsequent supplements of chloral hydrate were administered intraperitoneally if necessary. Incisions were made in the scalp to expose the skull, burr holes were drilled, and the dura overlying the BLA or mPFC was perforated. All rats were bilaterally implanted with stainless steel guide cannulae (21 gauge) aiming 1 mm above the intended injection site in either mPFC (PrL) or BLA. These cannulae were permanently secured to the skull with dental cement as well as bone screws and closed by removable mandrins before and between behavioural experiments. A post-surgery recovery period of 6–10 days was allowed.

Stereotactic coordinates based on Bregma according to the atlas of Paxinos and Watson [21] used for the final injection sites were for prelimbic mPFC: anteroposterior (AP) +2.7 mm, latero-medial (LM) ± 0.8 mm, dorso-ventral (DV) −3.7 mm; BLA: AP −3.1 mm, LM ±5.0 mm, DV −6.0 mm.

2.8. Microinjection

Bilateral injections of WIN, AM-251 or vehicle were carried out on hand-restrained rats with the injection cannulae (26 gauge) extending 1 mm beyond the tip of the guide cannula using micro-litre syringes (1 μl, SGE Analytical Science, Ringwood, Australia) attached to flexible polyethylene tubes. The rate of injection was 0.15 μl/min, and the injection cannulae were left in place for an additional 1 min to allow adequate absorption of WIN, AM-251 or vehicle by the surrounding tissue. According to the different learning and memory processes investigated the time interval between microinjection and testing differed.

2.9. Drugs

For intra-cerebral microinjection, a dose of 5 μg/0.3 μl WIN 55,212-2 (Sigma–Aldrich, Steinheim, Germany) or 1 μg/0.3 μl AM-251 was used and bilaterally infused into the region of interest. WIN and AM-251 were each dissolved in dimethyl sulfoxide (DMSO; Sigma–Aldrich, Steinheim, Germany) and Tween-80 and diluted in Ringer's solution for injection Braun (Braun AG, Melsungen, Germany) at a ratio of 10% DMSO, 3% Tween80 to 85% Ringer's solution for injection. A composite of the solvents of the same ratio served as control. Drugs were freshly prepared before being used.

2.10. Histology

After completion of the experiments the rats were deeply anaesthetized with 720 mg/kg chloral hydrate and transcardially perfused with 0.1 M phosphate-buffered formaldehyde (pH 7.4). Brains were removed from the skull, post-fixed in a sucrose-formalin solution (30 g Sucrose, Merck, Darmstadt, Germany; 20 ml of 37% formalin, Merck, Darmstadt) for two days. Afterwards, the sucrose–formalin solution was replaced by a 30% sucrose solution (in 0.2 M phosphate buffer) and the brains were again stored for two days. Subsequently, the brains were cut into 40-μm coronal sections on a cryostat microtome. To verify the appropriate location of tips of the infusion cannulae, the sections were Nissl-stained with thionin and analyzed using a light microscope (Axioscop, Zeiss, Göttingen). The atlas of Paxinos and Watson (1998) was used for the re-construction of the injections sites.

2.11. Statistical analysis

The descriptive statistics is based on means, and variance is indicated by the standard error of the mean (±SEM). All analyses were performed with the statistical software SigmaStat (version 2.03 for Windows). Results were analyzed using one-way or two-way analyses of variance (ANOVA). After significant ANOVAs, differences between groups were evaluated by post hoc Tukey's *t* test. A *P*-value <0.05 was considered to represent a significant effect.

3. Results

3.1. Histology

A total of 265 animals underwent stereotactic surgery. The target areas were missed in nine animals. Data from these rats were not included in the analysis. Three animals died during surgery. Fig. 1A–D shows the localization of the injection cannulae in the BLA. In 127 rats evaluation of thionine-stained brain sections (Fig. 2A and B) using light microscopy indicated that the target area