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Metabotropic Glutamate Receptors 7 within the Nucleus Accumbens are Involved in Relief Learning in Rats

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Abstract: Relief learning is an appetitive association of a formally neutral cue with relief induced by the offset of an aversive stimulus. Since the nucleus accumbens mediates relief learning and accumbal metabotropic glutamate receptors 7 (mGluR7) modulate appetitive-like processes, we hypothesized that accumbal mGluR7 may be involved in the modulation of relief learning. Therefore, we injected the allosteric mGluR7 agonist AMN082 into the nucleus accumbens and tested the effects of these injections on acquisition and expression of relief memory, as well as on the reactivity to electric stimuli. AMN082 injections blocked acquisition but not expression of relief memory. In addition, accumbal AMN082 injections strongly reduced the locomotor reactivity to electric stimuli indicating antinociceptive effects. These antinociceptive effects might be causal for the blockade of relief learning after AMN082 injections. Taken together, the present study indicates that functional activation of accumbal mGluR7 has antinociceptive effects that interfere with relief learning.

Keywords: AMN082, mGluR7, nucleus accumbens, pain, rat, relief.

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1. INTRODUCTION

An aversive event can induce very different learning experiences. It is well known that cues preceding an aversive event are acquired as conditioned fear stimuli (fear CS). However, cues associated with the relief from an aversive event lead to a very different memory. Instead of later triggering conditioned fear responses, like it is the case for a fear CS [1-4], such cues induce appetitive-like behavioral changes such as approach behavior and startle attenuation [summarized in 5]. This learning phenomenon is called 'relief learning' and has been demonstrated in flies [6, 7], rats [8, 9] and humans [8, 10].

Typically, a relief learning experiment consists of two phases. In the first phase, the learning or acquisition session, the to-be-learned cue (*e.g*. a light) is repeatedly presented shorty after an aversive event (unconditioned stimulus (US), *e.g*. an electric stimulus). One day later in a second phase, the retention or expression session, the behavioral effects of the now learned cue are measured. One of the behavioral changes that can be observed during exposure to a relief CS is the attenuation of the startle response [8-11]. The startle paradigm is a robust and well established measure of emotional valence. Both innate and learned 'emotional' stimuli are able to modulate the startle response [12-15].

We recently demonstrated that the nucleus accumbens is required for the acquisition and/or expression of conditioned relief [8, 9]. The goal of the present study was to further investigate the neuropharmacology of relief learning. Based on the idea that relief is rewarding [5] and on published data demonstrating that mGluR7 within the nucleus accumbens is involved in reward-related learning [16-18], we hypothesized that mGluR7 may also play a role in relief learning. Therefore, we injected the allosteric mGluR7 agonist AMN082 [19] into the nucleus accumbens and tested the effects of these injections on the acquisition and expression of conditioned relief, as well as on the locomotor reactivity to aversive electric stimuli.

2. MATERIAL AND METHODS

2.1. Animals

Fifty adult male Sprague Dawley rats aged between 2- 3 months (250-350 g) at the time of the surgery were used in this experiment. They were kept in groups of 4 to 6 animals per cage under a light:dark cycle of 12h:12h (lights on 6:00 am) and had free access to water and food. All experiments and surgeries were done during the light phase. The experiments were performed in accordance with international guidelines for the use of animals in experiments (2010/63/ EU) and were approved by the local ethical committee (Landesverwaltungsamt Sachsen-Anhalt, Az. 42502-2-1172 UniMD).

2.2. Implantation of Intra-accumbal Cannulas

The animals were anesthetized with an isoflurane/oxygen mixture (5% isoflurane for induction, then 2.0-2.5%) and fixed into a stereotaxic apparatus. The skull was exposed and stainless steel guide cannulas (custom-made; diameter: 0.7 mm, length: 8.0 mm) were bilaterally implanted aiming at NAC: 1.2 mm rostral, ± 1.5 mm lateral, and 7.4 mm ventral

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to the bregma [20]. Cannulas were fixed with dental cement and anchoring screws. After the surgery, there was a recovery period of 5-7 days.

2.3. Apparatus

We used a startle system with six chambers (SR-LAB, San Diego Instruments, USA). Each chamber consisted of a stable platform holding a horizontal, cylindrical, transparent animal enclosure (9 cm x 16 cm). A piezoelectric motion sensor measured the animals' movements. The digitalized output signal of this sensor (sampling rate: 1 kHz) was sent to a computer for further analysis. For the measurement of the startle magnitudes, the average readout of the sensor in the time window 10-30 ms after startle stimulus onset was used. To measure reactivity to the electric stimuli, the average readout of the sensor during the presentation of the electric stimulus was used [cf. 21, 22].

For relief conditioning, aversive electric stimuli (unconditioned stimulus, US) and light stimuli (conditioned stimulus, CS) were used [cf. 8, 9, 21]. The light stimulus was produced by a 10 W bulb, had an intensity of ~1000 lux and duration of 5 s. The electric stimuli were administered *via* a floor grid (6 bars with 5 mm diameter, distance: 10 mm), had an intensity of 0.4 mA and a duration of 0.5 s. For the application of acoustic stimuli, a loudspeaker mounted onthe ceiling of the box was used. During all tests, a background noise with an intensity of 50 dB SPL was presented to mask environmental noises. The acoustic startle stimulus was a noise burst with an intensity of 96 dB SPL and duration of 40 ms. For testing the reactivity to electric stimuli, stimulus intensities of 0.0, 0.1, 0.2, 0.3 and 0.4 mA were used.

2.4. Behavioral Protocol

2.4.1. Experiment 1: Effects of AMN082 Injections on Acquisition of Relief Memory

Day 1 (baseline session): Animals (n = 28) were put in the chambers and after 5 minutes of acclimation, 10 startle stimuli were delivered with an inter-trial interval of 30 s (see also Fig. (**1**)). Then, the animals were put back into their home cages. Based on the mean startle amplitude of this session, the animals were distributed into two groups with balanced mean baseline startle amplitudes.

Day 2 (relief conditioning session): Half of the animals were injected with the vehicle and the other half with5 µg/0.3 µl AMN082 [cf. 16]. AMN082 (A6605, Sigma-Aldrich, Germany) was dissolved in saline containing 0.1% methanol [23]. The injections were performed with a speed of 0.2 µl/min using a microinjection pump (CMA/100, Microdialysis AB, Stockholm, Sweden) and the cannulas were left in place for 1 additional minute. Ten minutes later, the animals were put into the startle chambers. After 5 minutes acclimation time, 15 electric stimuli followed by a light stimulus were delivered to the animals (fixed inter-stimulus interval: 3 s from onset electric stimulus to onset light stimulus). The inter-trial interval (electric stimulus onset to next electric stimulus onset) was pseudo-randomized and varied between 30 - 100 seconds.

Day 3 (retention test): The animals were put into the startle chamber. After 5 minutes of acclimation, 10 startle stimuli were presented to habituate the animals followed by 20 startle stimuli, 10 of them without the light CS and 10 of them upon presentation of the light CS. The order of the trials with and without the light CS was pseudo-randomized.

2.4.2. Experiment 2: Effects of AMN082 Injections on Expression of Relief Memory

Day 1 and 2: Thirteen animals were used for this experiment. The baseline and relief conditioning sessions were identical to those of experiment 1 except that no injections were performed before or during the relief conditioning session.

Day 3: Half of the animals were injected with the vehicle and the other half with 5 µg/0.3 µl AMN082. The animals were put into the chambers immediately after injection and a retention test was run.

Day 4: The animals were re-conditioned with relief conditioning protocol from day 2.

Day 5: The test of day 3 was repeated. However, animals that received injections of saline on day 3 were now injected with AMN082, and vice versa.

2.4.3. Experiment 3: Effects of AMN082 Injections on Locomotor Reactivity to Electric Stimuli

Nine animals were used for this experiment. They were injected with either saline or 5 µg/0.3 µl AMN082. Then, they were put into the startle chambers. After an acclimation time of 5 min, five electric stimuli with increasing intensities (0.0 mA, 0.1 mA, 0.2 mA, 0.3 mA, 0.4 mA) were administered with an inter-stimulus interval of 30 s. Two days later, the same procedure was repeated. However, rats that received saline on the day before now received injections of AMN082, and vice versa.

2.5. Histology

At the end of the behavioral experiments, the animals were sacrificed by CO2. The brains were removed and put into 30% sucrose 4% formalin solution for fixation. The brains were sectioned by a cryostat in 60 µm slices and the sections were Nissl-stained with cresyl violet. Then, injection sites were localized under a light microscope and plotted onto plates taken from a rat brain atlas [20].

2.6. Data Analysis

For each animal the mean response to the electric stimuli, the mean startle amplitudes with and without the light CS (peak amplitudes within the 100 ms after the startle stimulus onset), and their difference were calculated. Since all the data were normally distributed (D'Agostino & Pearson omnibus normality test), means and standard error of the means (SEM) were shown in the figures and parametric statistical tests were used for analysis (Prism 6.0, GraphPad Software Inc., La Jolla, CA, USA). The statistical threshold was set to $p < 0.05$.

To statistically analyze the effects of the AMN082 injections into the nucleus accumbens, analyses of variances (ANOVA) were performed using trial type (experiments 1+2: startle alone, CS-startle; experiment 3: stimulus

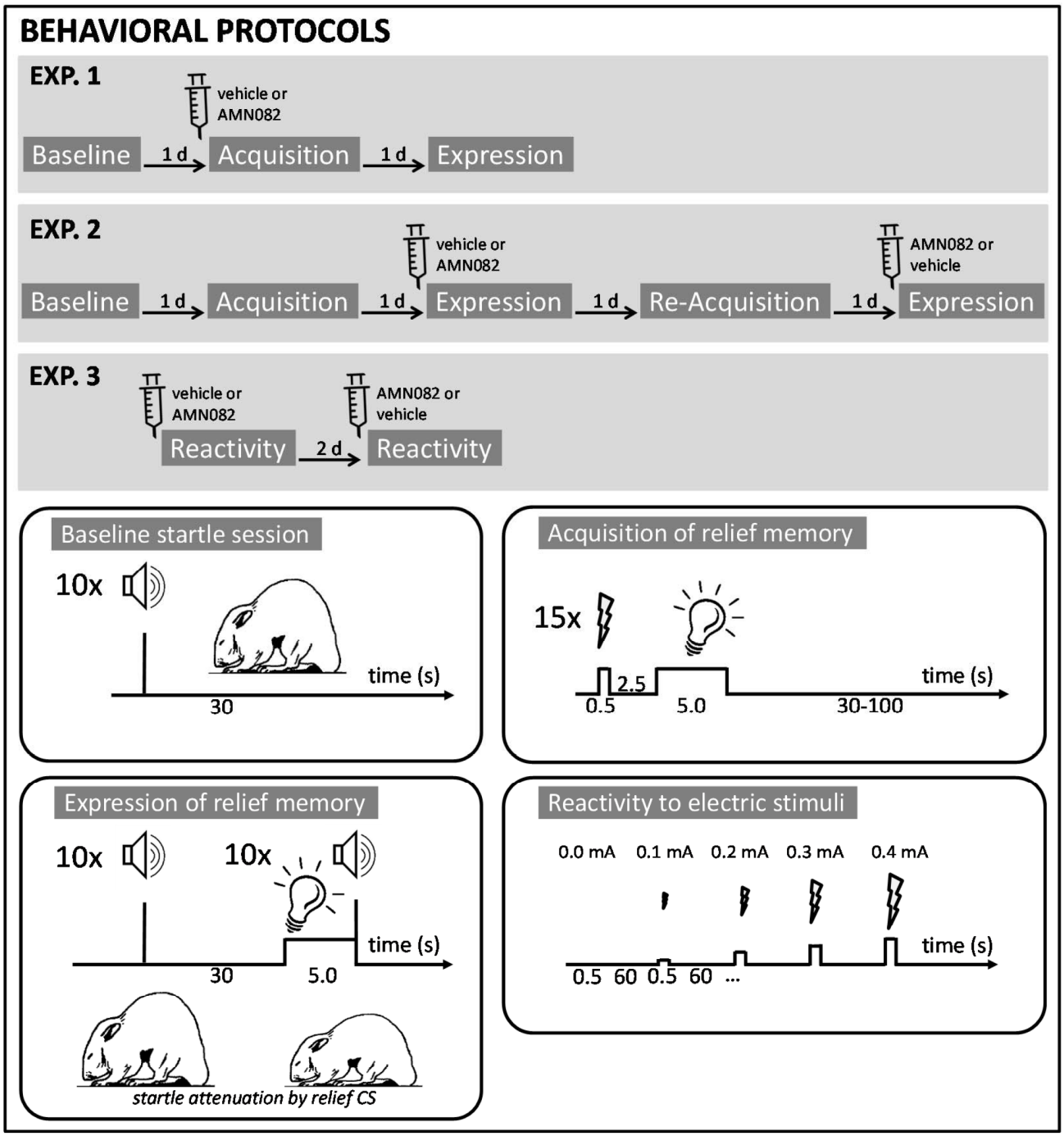

Fig. (1). Behavioral protocols of the present study. Upper panels depict the treatment schedule and the test sessions performed. Lower panels give detailed information on the different test sessions.

intensity) as within-subject factor and treatment (vehicle, AMN082) as between-subject (experiment 1) or within-subject (experiments 2+3) factor.

3. RESULTS

3.1. Histology

The injection sites of the present study are shown in Fig. (**2**). In total, nine animals had to be excluded because of misplaced injections (septum, caudate putamen) or lesions mechanically induced by the cannulas. Three further animals were excluded because they express no or only minimal (amplitude < 10) startle responses, most probably due to damage of the ear drums during the stereotaxic surgery.

3.2. Experiment 1: Effects of AMN082 Injections on Acquisition of Relief Memory

Twenty animals received injections of vehicle (n = 10) or AMN082 (n = 9) before the relief conditioning session. AMN082 injections blocked the acquisition of relief

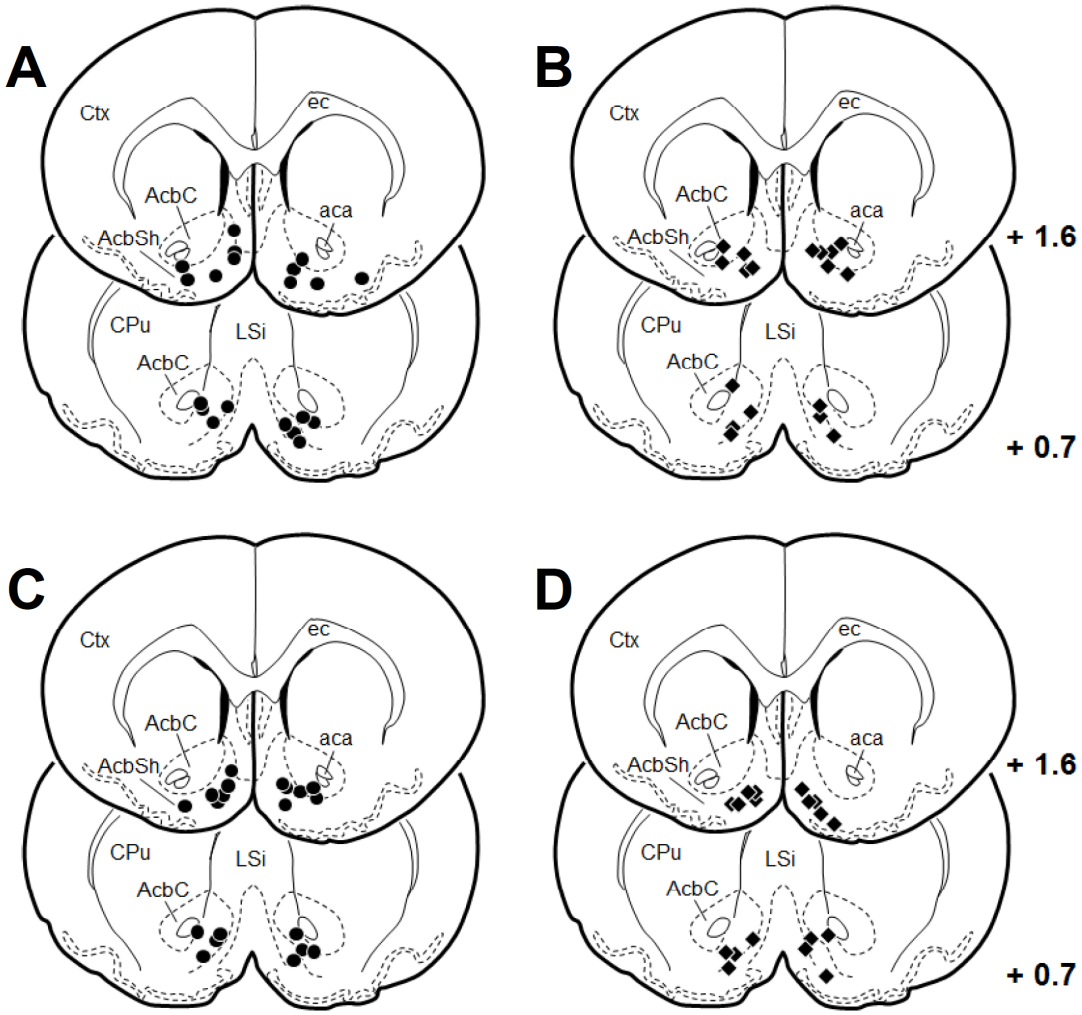

Fig. (2). Reconstruction of the vehicle and AMN082 injections sites into the NAC. (**A**) Vehicle injections before relief conditioning (acquisition). (**B**) AMN082 injections before relief conditioning (acquisition). (**C**) Vehicle and AMN082 injections before the retention test on conditioned relief (expression). (**D**) Vehicle and AMN082 injections before testing on reactivity to electric stimuli. Abbreviations: AcbC, core of the nucleus accumbens; AcbSh, shell of the nucleus accumbens; CPu, caudate putamen; Ctx, cortex; ec, external capsule; LSi, lateral septal nucleus. Values represent the anterior distance to bregma (mm) according Paxinos and Watson [20].

memory, indicated by a significant interaction of trial type and treatment (Fig. (**3A**), ANOVA: $F_{1,17} = 4.33$, $p = 0.03$). There were no main effects of treatment ($F_{1,17} = 0.95$, $p = 0.66$) and trial type ($F_{1,17} = 3.09$, $p = 0.06$). Post-hoc comparison of the startle magnitudes to startle alone and CS-startle trials demonstrated significant startle attenuation by the relief-CS in the vehicle group (Sidak's multiple comparison test: $t = 3.26$, $p < 0.01$) but not in the AMN082-injected animals ($t = 0.26$, n.s.). This analysis is supported by a t-test of the difference scores demonstrating significant effects of AMN082 injections ($t_{17} = 2.45$, $p = 0.03$).

3.3. Experiment 2: Effects of AMN082 Injections on Expression of Relief Memory

Nine animals were conditioned without any treatment and received injections of vehicle and AMN082 before the two retention tests on relief memory. AMN082 injections into the nucleus accumbens did not affect the expression of relief memory (Fig. (**3B**), ANOVA. Interaction trial type x treatment $F_{1,8} < 0.0001$, $p = 0.99$). There was a main effect of trial type ($F_{1,8} = 4.81$, $p = 0.0006$) but not of treatment ($F_{1,8} = 0.72$, $p = 0.66$). Post-hoc comparison of the startle magnitudes to startle alone and CS-startle trials demonstrated significant startle attenuation in both groups ($t = 5.53$, $p < 0.01$ for vehicle, $t = 5.55$, $p < 0.01$ for AMN082).

3.4. Experiment 3: Effects of AMN082 Injections on Locomotor Reactivity to Electric Stimuli

Ten animals were injected with vehicle and AMN082. The effects of these injections on the locomotor response to electric stimuli with increasing intensity were tested in two experimental sessions. AMN082 significantly reduced the locomotor reactivity to the electric stimuli (Fig. (**4**), ANOVA: factor treatment: $F_{1,8} = 5.36$, $p = 0.049$). In addition, there was also an effect of stimulus intensity ($F_{4,32} = 15.03$, $p < 0.0001$) but no interaction between stimulus intensity and treatment ($F_{4,32} = 1.68$, $p = 0.18$). Post-hoc comparisons by Sidak's multiple comparison tests identified a significant

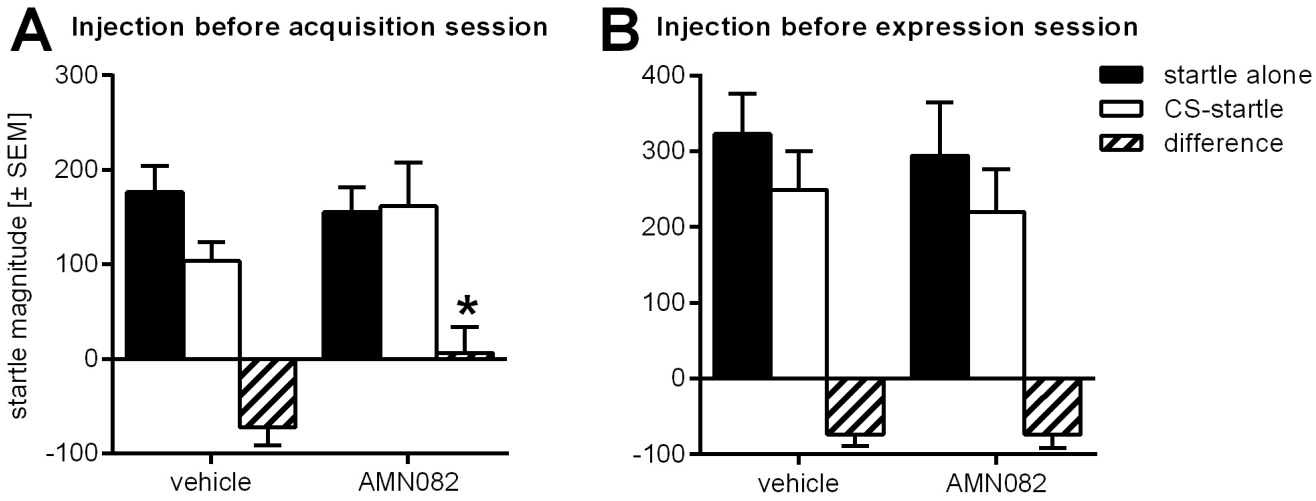

Fig. (3). Effects of intra-accumbal AMN082 injections on the acquisition and expression of relief memory. (A) AMN082 injected before conditioning dose-dependently blocks acquisition of relief memory. (B) AMN082 injected before testing expression of relief memory has no effects. Depicted is the startle magnitude (arbitrary units + SEM) without and with the presence of the relief CS (light stimulus), as well as the difference between these two trial types. * $p < 0.05$ comparison with vehicle.

treatment effect at 0.4 mA ($t = 3.27$, $p < 0.05$) but not at the other stimulus intensities ($ts < 0.89$, n.s.).

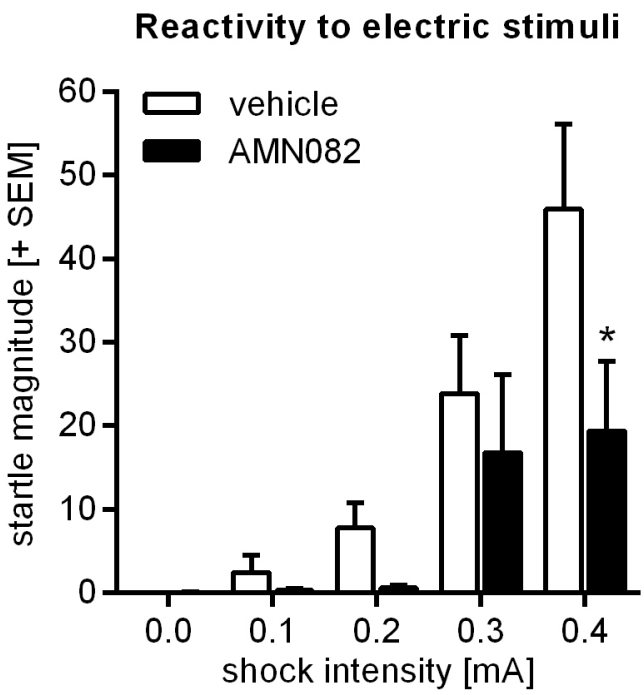

Fig. (4). Effects of intra-accumbal AMN082 injections on the locomotor reactivity to electric stimuli. Shown is the mean locomotor activity (arbitrary units + SEM) during the electric stimuli (500 ms duration, different intensities). AMN082 injections reduced the reactivity to electric stimuli. * $p < 0.05$ comparison with vehicle.

4. DISCUSSION

The aim of the present study was to investigate whether mGluR7 within the nucleus accumbens is involved with the mediation of relief learning. Therefore, we injected the allosteric mGluR7 agonist AMN082 into the nucleus accumbens and tested the effects of these injections on different phases (acquisition, expression) of relief learning and on the locomotor response to electric stimuli, which are used as an unconditioned stimulus in relief learning. Our study demonstrates that AMN082 injections before relief conditioning but not before the retention test on relief memory prevented conditioned relief. Notably, AMN082 injections also affected the locomotor reactivity (flinch response) to the electric stimuli. Since the acoustically-induced startle response was not affected by intra-accumbal AMN082 injections, this strongly suggests that the AMN082 injections interfere with the sensory processing of the electric stimuli.

The present study is focused on relief learning which is the association of a formerly neutral cue with the relief of an aversive stimulus - here an electric stimulus. There are different ways to measure behavioral changes induced by learned relief. In fruit flies, approach behavior to an odor that was paired with the relief from an electric stimulus is observed [6, 7, 24, 25]. However in rodents, enhanced lever pressing for food [26], preference to a relief-associated compartment [27, 28] or an attenuation of the startle response [8, 9] can be measured. The latter paradigm is also used in humans [8, 10, 11]. It is important to note that some authors regard relief learning as a kind of safety learning [29, 30], *i.e.* they argue that relief learning is the learning that the CS predicts a safe period without aversive stimuli. However, recent studies demonstrated that the nucleus accumbens is involved in relief learning [9, 27, 28] but not safety learning [9,31]. This indicates different underlying neural circuitries for relief and safety learning which in turn suggests that relief and safety learning are two distinct learning processes. The fact that the nucleus accumbens is involved in relief but not in safety learning is also consistent with evidence that relief from an aversive stimulus can have rewarding effects [32-34] whereas this is not the case for a safety cue [35].

It is well established that the dopaminergic projection from the ventral tegmental area to the nucleus accumbens is involved in the mediation of reward and reward-related learning [36-39]. By electrophysiological recording of cell activity during and after electric stimuli, Brischoux and colleagues identified a population of neurons within the ventral tegmental area which is excited by the onset and also by the offset (relief) of electric stimuli [40]. The dopaminergic neurons in this brain area are activated by pain relief [27] and project to the shell of the nucleus accumbens [37]. This suggests that relief is mediated by a dopaminergic projection from the ventral tegmental area to the shell of the nucleus accumbens. In fact, as stated above, temporal inactivation of the nucleus accumbens blocks both acquisition and expression of relief learning [8, 9].

Several studies from Xia Li and colleagues demonstrated that systemic and intra-accumbal injections of the allosteric mGluR7 agonist AMN082 inhibits reward processes [16-18, 41, 42]. Furthermore, AMN082 is able to block associative learning as well as underlying long-term potentiation such as Pavlovian fear conditioning and amygdala long-term potentiation [43, 44]. Since relief learning is associative learning and relief has reward-like properties, we hypothesized that accumbal mGluR7may be involved in the acquisition of relief learning. Indeed, the present data demonstrate that intra-accumbal AMN082 blocked acquisition but not expression of conditioned relief. However, the locomotor reactivity to electric stimuli was also strongly reduced by our injections. This effects seems to be specific to the reactivity to foot shocks (flinch response [45]) since both the acoustic startle response (present study, Fig. **3B**) and rotarod performance [17] is not affected by intra-accumbal AMN082 injections. This indicates an anti-nociceptive effect of intra-accumbal AMN082 injections.

This interpretation is supported by literature data demonstrating anti-nociceptive effects of systemic AMN082 application [46, 47]. Such systemic AMN082 effects cannot be mediated by the amygdala since intra-amygdala administration of AMN082 reduces the pain threshold and enhances pain-induced amygdaloid activity [48, 49]. The present data suggest that the nucleus accumbens may mediate the anti-nociceptive effects of systemic AMN082 application.

There is already a multitude of information on AMN082's pharmacological effects within the nucleus accumbens. *Via* microdialysis, Xia Li and colleagues showed that AMN082 application into the nucleus accumbens increases accumbal glutamate release but decreases GABA release whereas dopamine release was not affected [18, 41]. These effects were mGluR7-dependent since they are blockable by the specific mGluR7 antagonist MMPIP [18]. Such a control experiment is useful since Sukoff Rizzo and colleagues [50] showed that AMN082 has also monoaminergic activity, *i.e*. it blocks noradrenaline and serotonin transporters at higher concentrations. In the present study, MMPIP was not co-administered, however, Xia Li' data indicate that the effects observed in the present study are mGluR7-mediated. In further studies, Li and colleagues demonstrated that cocaine-induced glutamate release but not dopamine release was blocked by AMN082 [18]. The authors suggested that the effect on glutamate was secondary to the AMN082 effects on GABA release [41]. On a behavioral level, intra-accumbal AMN082 injections lead to a blockade of cocaine self-administration [17] and cocaine-induced reinstatement of drug-seeking behavior [18].

In the present study, electric foot shocks were used as aversive stimuli whose offset induce the relief that should be associated with a neutral cue. Such electric foot shocks induce an increase of dopamine release within the shell of the nucleus accumbens [51], as well as a short-lasting decrease and later an increase of accumbal glutamate release [52]. Based on these findings and those of Xia Li described above, it is probable that the AMN082 injections of the present study prevent this foot shock induced decrease of glutamate release. Thereby analgesia could be induced which leads to a reduction of the locomotor reactivity to the painful electric stimuli, as it was observed in the present study. This explanation is supported by a number of studies demonstrating that the nucleus accumbens is involved in analgesia and antinociception [*e.g*. 53-55]. A further effect of the AMN082 injection could be that the rewarding feeling of relief was reduced. This could apply for unconditioned relief but not for conditioned relief since our intra-accumbal AMN082 injections had no effects on the expression of conditioned relief. Therefore we think that the antinociceptive effect of AMN082 is responsible for the blockade of relief learning observed in the present study.

Altogether, the present study shows that injections of the allosteric mGluR7 agonist AMN082 into the nucleus accumbens interferes with relief learning. Notably, our AMN082 injections impaired locomotor reactivity to electric stimuli suggesting that accumbal mGluR7 isinvolved in the mediation of analgesia and/or antinociception. The latter effect might be responsible for the blockade of relief learning after AMN082 injections.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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