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Short communication

## Appetitive odor-cue conditioning attenuates the acoustic startle response in rats

Miriam Schneider<sup>\*</sup>, Rainer Spanagel

*Central Institute of Mental Health (ZI), Department of Psychopharmacology, J5, 68159 Mannheim, Germany*

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### Abstract

We here show that a neutral odor previously paired with a positive emotional context is an effective stimulus for attenuating the acoustic startle response (ASR) in rats. Olfactory cues can, therefore, be effectively used in the startle probe procedure for appetitive conditioning. This cue-induced reduction in ASR is not related to attentional alterations or a more general arousal by odor presentation, the conditioned olfactory cue rather elicits a pleasant emotional state during which the ASR is inhibited. This odor conditioned “pleasure” attenuation of the startle response might, therefore, provide a new effective operational measure for the hedonic aspects of reward.

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The aim to establish a valid psychophysiological measure of emotional states in laboratory animals is still an ongoing search of utmost importance in the field of behavioral neuroscience. In this context, the acoustic startle reflex, a defensive response to a sudden loud noise, has received increasing attention since it was shown that the magnitude of the acoustic startle response (ASR) can be modulated in humans by the organism's ongoing motivational state [19]. This finding indicated the startle probe procedure as a very effective means for assessing emotions. In particular, the fear-potentiated startle paradigm, the augmentation of the ASR in an aversive state, has been used to investigate the pharmacological and neuronal basics of fear in both humans and rats (e.g., [1,2,8,12]). However, it was found in humans that the amplitude of the ASR is not only increased during states of fear and anxiety but also decreases if elicited in a pleasant emotional context [9,19]. This latter phenomenon, termed “pleasure”-attenuated startle (PAS), has also been shown in rats [17]. Here, the ASR was reduced when tested in the presence of light, which had been previously paired with a natural reward (sucrose solution and casein pellets). A similar conditioning procedure in rats was used in a study by Steidl et al. [18], where brain-stimulation reward (BSR) was used for conditioning. BSR

was associated with the presence of light and was also found in this study to attenuate the ASR. A different but also very interesting approach to induce PAS in rats, by using positive familiar olfactory cues, was done by Richardson and Defina [15]. In this study, preweanling rats were tested for the ASR in the presence of a highly preferred olfactory stimulus—soiled homecage bedding material. However, this olfactory stimulus did not attenuate the startle magnitude. The authors suggested that this result might be due to functional immaturity of the neural circuits involved in emotional modulations of the ASR in preweanling animals. Despite the negative finding on PAS, this study was, however, the first approach to use olfactory cues for startle testing in rats. In humans, unconditioned effects of various odors have already been tested on the startle response [3,4,11]. Here, it was shown that unpleasant odors potentiated the ASR, however, only one study was able to find a reduction in ASR after presentation of a pleasant odor [4].

An enormous body of work does exist on startle testing in rats using either auditory, visual stimuli, or even tactile cues, whereas olfactory stimuli, to which rats are especially responsive have been kind of neglected [15]. For rodents, which are mainly active during twilight and in the night, olfaction is the most important sensory system and is used to recognize and locate conspecifics, objects, predators, and prey in their environment [10,13,20]. Therefore, it is not surprising that shortly after the first non-successful attempt to use olfactory stimuli for

<sup>\*</sup> Corresponding author. Tel.: +49 621 17036269; fax: +49 621 17036255.  
E-mail address: [miriam.schneider@zi-mannheim.de](mailto:miriam.schneider@zi-mannheim.de) (M. Schneider).

appetitive emotional startle modulations in infant rats, Richardson et al. [16] managed to demonstrate that an olfactory cue, that had been paired with a food-shock, can be effectively used to potentiate the startle response in adult rats. These findings were extended by Paschal and Davis [13,14] using a very elegant design. The authors managed to build an olfactory apparatus that could deliver discrete olfactory cues during startle testing and were, therefore, able to involve randomized presentations of a startle stimulus given in the presence versus the absence of the conditioned-cue, during one test session.

With the present study we aimed to establish PAS in rats using unfamiliar olfactory cues instead of light in an appetitive classical conditioning procedure and to further validate the use of this promising paradigm for assessing hedonic aspects of reward. Therefore, animals were trained to associate a neutral odor with a highly preferred natural reward (sweetened condensed milk) and were then tested for their ASR in the presence of the conditioned olfactory cue.

For this study fifty-one naïve adult Wistar rats (Harlan Winkelmann, Borcheln, Germany) weighing 280–320 g were used. The animals were group housed under standard conditions in Macrolon cages (Eurostandard type IV) on a 12 h light–dark schedule (lights on 7:00–19:00). They had free access to tap water and were kept on a mild food restriction schedule of 13g/animal/day during behavioral testing. The experiments were done in accordance with the ethical guidelines for the care and use of laboratory animals, and were approved by the local animal care committee (Karlsruhe, Germany).

Startle testing occurred in a startle chamber (SR-LAB; San Diego Instruments, San Diego, USA). A white noise pulse was used as the startle stimulus, with an intensity of 105 dB sound pressure level (SPL) and duration of 40 ms. An acclimatization time of 5 min, during which the rats received no stimulus except the background noise (65 dB), was followed by the presentation of 5 initial startle stimuli. The test program consisted of 30 startle pulses with an intertrial interval randomized between 10 and 20 s. All animals were tested four times for their ASR in the presence of an odor-cue (orange, essential oils, Primavera Life, Sulzberg, Germany), once before (ASR baseline) and again 24 h, 7 and 14 days after 5 days of odor-reward association training. The odor (30  $\mu$ l) was provided in a Petri dish that was placed in the box during habituation. PAS was calculated as mean percent decrease over baseline ASR amplitude [ $100 - (100 \times \text{mean ASR2 amplitude} / \text{mean ASR1 (baseline) amplitude})$ ].

Animals assigned to odor-reward paired conditions were trained on 5 consecutive days to associate an orange odor with a reward (sweetened condensed milk, SCM, Nestle AG, Frankfurt, Germany). During odor training, which lasted 90 min in total, rats were placed in single cages (Eurostandard type III) and experienced 3 odor-reward presentations at random time points after a habituation period of 10 min to the novel environment. Odor-reward presentations involved the introduction of the odor followed 5 s later by the presentation of the bottle containing the reward. The odor (orange, 15  $\mu$ l) was supplied in a small Petri dish containing a piece of filter paper that was attached in the middle of the wire lid, 2 cm beneath the aperture

of the drinking bottle. After free access to the reward for 5 min the odor and the SCM were removed. Control animals underwent a sham-training procedure where they received 3 random SCM presentations without odor presentation. Exposure to the odor took place for controls in a non-associative manner in the homecage 5 h after/before SCM access.

Two additional control experiments were performed for a behavioral validation of the PAS paradigm. Two separate groups of animals were trained/sham-trained in an identical manner as animals used for the PAS experiment. Those animals were tested in a t-maze task 24 h after the last training session and on the next day for prepulse inhibition (PPI) in the presence of the odor. In addition, an odor-naïve control group underwent the same behavioral tests.

A t-maze preference task was used to assess the strength of the odor-reward association of trained, sham-trained and odor-naïve animals. Two odors were presented during this test, the conditioned odor (orange) and a neutral odor (eucalyptus) in the two opposite arms of the t-maze. The time spent in the odor-paired arms during a 10 min period and the number of arm entries were recorded. Percentage of time spent in the arm paired with the conditioned odor (CS; orange) was calculated ( $\text{CS arm time} / (\text{CS} + \text{neutral arm time}) \times 100$ ). The total number of arm entries was used as an index of general activity.

PPI serves as an operational measure for sensorimotor gating and a deficient PPI is linked to impairments in attentional processing [5]. In order to ensure that the reduction in ASR observed in the present study was related to a conditioned pleasant emotional state and not due to a general enhancement in arousal or distraction, trained, sham-trained and odor-naïve controls were tested for PPI in the presence of the odor-cue. For PPI testing a white noise pulse (105 dB SPL, duration 40 ms) was used as the startle stimulus. Four different white noise intensities (68, 72, 76 and 80 dB SPL, duration 20 ms,) were used as prepulses. An acclimatization time of 5 min to the background noise (65 dB), was followed by the presentation of 5 initial startle stimuli. After this habituation program the test program was started with seven different trial types presented in a pseudorandom order: (1) pulse alone, (2) no stimulus, (3–6) pulse with preceding prepulse (prepulse 68, 72, 76, or 80 dB SPL 100 ms before pulse), (7) prepulse alone (80 dB). A total of 10 presentations of each trial type were given with an intertrial interval randomized between 10 and 20 s. The odor (30  $\mu$ l) was provided in the box during testing in a Petri dish. PPI was calculated as the percent decrease of the ASR magnitude in trials when the startle stimulus was preceded by a prepulse [ $100 \times (\text{mean ASR amplitude on pulse alone trials} - \text{mean ASR amplitude on prepulse-pulse trials}) / \text{mean ASR amplitude on pulse alone trials}$ ].

The effects of pre-training conditions on ASR amplitudes, PAS [%], as well as PPI were evaluated using two-way repeated measure ANOVA, followed by post hoc Tukey *t*-tests for pairwise comparisons. For evaluation of percentage of time spent in the CS paired arm, total arm entries in the t-maze and no stimulus recordings during PPI testing a one-way ANOVA, followed by post hoc Tukey *t*-tests for pairwise comparisons was used. An alpha value of 0.05 was considered to represent a significant effect.

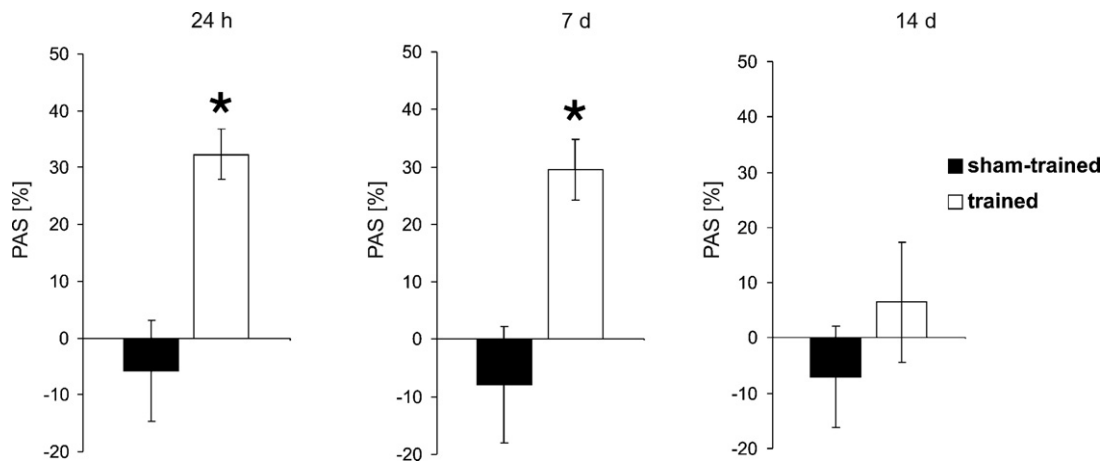


Fig. 1. Odor-induced PAS was tested repeatedly 24 h, 7 and 14 days after training cessation. A significant increase in percentage attenuation of ASR over baseline was seen in trained rats compared to sham-trained controls 24 h ( $p=0.002$ ) and 7 days ( $p=0.003$ ) after training cessation. However, 14 days after training the PAS effect declined and trained animals did not differ anymore significantly from sham-trained controls (trained:  $n=11$ , sham-trained:  $n=14$ ).

Odor-reward association training significantly increased the percentage attenuation of the startle response (ANOVA:  $F_{1,46} = 10.2, p < 0.05$ ). In addition, the ANOVA revealed a significant interaction effect for training and test days. As can be seen in Fig. 1, the percent decrease in ASR over baseline in the presence of the odor-cue was significantly higher in animals trained for odor-reward association, compared to sham-trained rats 24 h and 7 days after conditioning. However, 14 days after conditioning the PAS effect declined (ANOVA:  $F_{2,46} = 3.2, p < 0.05$ ).

Furthermore, a direct comparison of baseline ASR and startle amplitudes 24 h after conditioning showed that the ASR was significantly reduced only in rats that underwent the association training in the test condition after conditioning (data not shown) (ANOVA:  $F_{1,26} = 5.1, p < 0.05$ ).

Odor-preference testing of trained, sham-trained and odor-naïve control groups, assessed in a t-maze task, revealed that trained rats showed a significant preference for the compartment with the conditioned odor compared to sham-trained controls and odor-naïve rats that did not show any odor preference (ANOVA:  $F_{2,23} = 13.6, p < 0.05$ ). This result is taken as an evidence that 5 days of training were sufficient to form an adequate odor-reward association and that the conditioned odor was highly preferred in trained animals (Fig. 2A). No differences were seen in total arm entries between all groups (Fig. 2B) (ANOVA:  $F_{2,23} = 0.2, p > 0.05$ ), indicating a similar general activity of all animals, irrespective of pre-training procedure.

No differences were found in PPI between the different groups (ANOVA:  $F_{2,69} = 0.04, p > 0.05$ ), indicating that odor presentation during testing did not induce any alterations in attentional processing in trained animals (Fig. 3). In addition, the recordings during the no stimulus trials were taken as an index for the activity of the animals in the presence of the odor during startle testing. No significant differences were found between the three different training groups regarding their basal locomotor activity during PPI testing (data not shown) (ANOVA:  $F_{2,23} = 0.8, p > 0.05$ ).

We here demonstrate that a neutral odor previously paired with a reward by classical conditioning is an effective stimu-

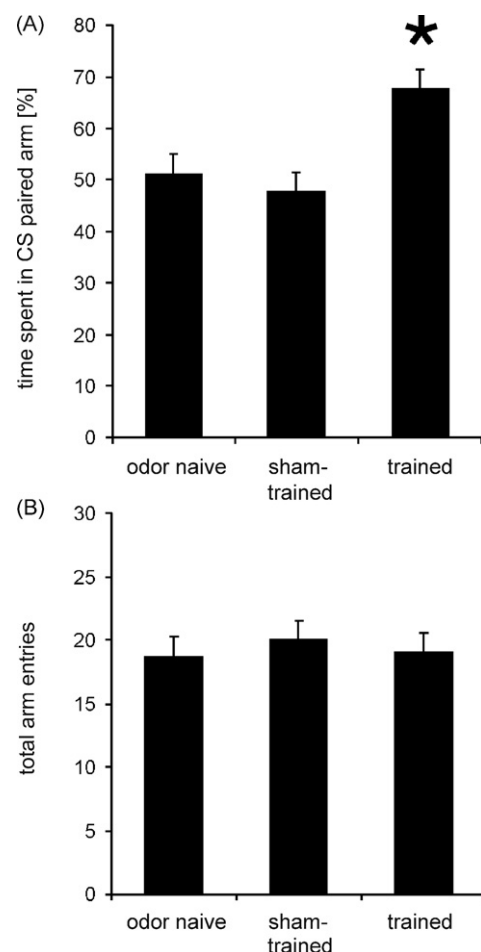


Fig. 2. t-Maze testing for odor-reward association. Odor preference testing in a t-maze revealed that animals trained to associate the odor CS with a reward showed a significant preference for this specific odor compared to sham-trained controls ( $p < 0.001$ ) or odor-naïve rats ( $p = 0.005$ ) (A). Total arm entries did not differ significantly between the groups (B) (trained:  $n=8$ , sham-trained:  $n=12$ , odor-naïve controls:  $n=6$ ).

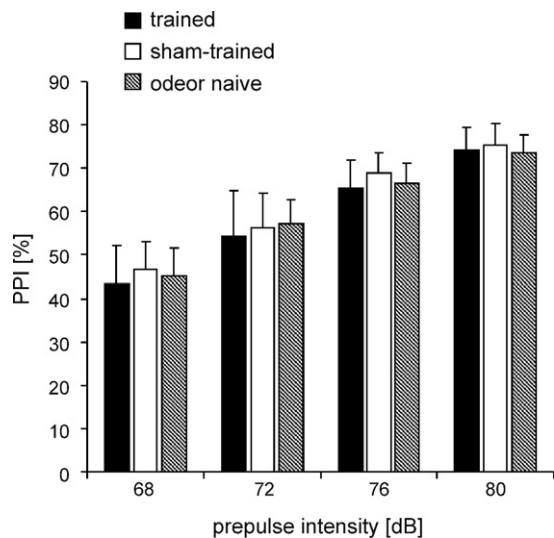


Fig. 3. Effects of odor presentation on PPI in trained, sham-trained and odor-naïve animals. Rats trained to associate the odor-cue with a reward did not differ significantly in their performance in PPI in the presence of odor from sham-trained controls and complete odor-naïve animals (trained:  $n = 8$ , sham-trained:  $n = 12$ , odor-naïve controls:  $n = 6$ ).

lus for attenuating the startle response in rats. PAS does not require instrumental responding to a stimulus, but instead probes the incentive properties of a conditioned-cue by an attenuation of an unconditioned reflex rather than by reinforcing a behavior. Therefore, it has been suggested as a paradigm for the measurement of hedonic states in animals and is suitable for the dissociation of the neural substrates of instrumental motor responding and emotional responding in appetitive motivated behavior [6,17].

Repeated testing of trained animals showed that the PAS effect was still observable 7 days after conditioning before it declined 7 days later. The finding that the expression of PAS can be measured repeatedly might be of importance for further pharmacological studies. Although, the odor was presented without the SCM reward during the two preceding startle sessions 24 h and 7 days after conditioning, it is not likely that the decline in PAS at the 14 days retention interval was mediated by extinction of the odor/reward association. However, further experiments will have to clarify the exact time course of PAS and the involvement of extinction processes in its decline.

The use of sham-trained control groups in the present study demonstrates that PAS was not due to long-term habituation of the ASR as a result of repeated testing. In addition, the attenuation of the ASR after appetitive conditioning was not related to attentional alterations or a more general arousal elicited by odor presentation. Neither locomotor activity nor attentional processing was altered in trained animals in the presence of the odor when compared to sham-trained controls or odor-naïve animals. The lack of locomotor activation by presentation of the conditioned-cue is consistent with earlier findings [17]. We, therefore, conclude that the olfactory cue elicited a pleasant emotional state in animals trained to associate the odor with a reward, during which the ASR was inhibited, rather than affecting other behavioral domains.

Since PAS can be measured in humans as well as in rats, it serves as a cross-species model to measure reward related affect [7]. It has been suggested before that the impact of emotions on ASR modulation is mediated by emotional priming, in which emotions are viewed as action dispositions that prepare the organism to respond to environmental stimuli, ultimately improving survival by inducing approach or avoidance responses [8]. It has, therefore, been proposed that PAS is due to negative motivational priming, whereby a hedonic emotion suppresses or “de-energizes” inappropriate behaviors in a pleasant emotional state like the defensive startle response. The PAS paradigm offers a completely new approach for investigating the neural mechanisms of reward, since it measures the reduction of an aversive reflex, instead of reinforcing or increasing certain behaviors, as can be seen in standard paradigms for assessing reward, such as the conditioned place preference test or self-administration procedures [7,8,17].

The new PAS protocol established in the present study extends this interesting paradigm to the olfactory domain, thereby offering new possibilities in examining positive affective states in rats. With this simple conditioning procedure it might be possible to further evaluate the neuronal bases of various food or non-food rewards. Odor-induced PAS might, therefore, provide a new effective operational measure for assessing different aspects of the hedonic impact of reward.

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