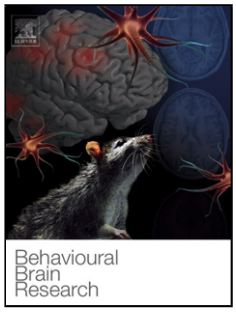

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Highlights

- We investigated a judgement bias test for mice
- BALB/c mice revealed a negative judgement bias under red and white light testing conditions
- 129P3 mice did not differentiate between the odour cues in the judgement bias test
- c-Fos expression levels in distinct brain areas strain-dependently differed in response to the ambiguous cues
- The here presented test might be of use to investigate emotional states via an assessment of judgement bias in mice

A test to identify judgement bias in mice

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Key words: judgement bias; odor conditioning; anxiety; behavior; BALB/c mice; 129P3 mice

Abstract

Emotional states are known to affect cognitive processes. For example highly anxious individuals interpret ambiguous stimuli more negatively than low anxious people, an effect called negative judgement bias. Recently, the measurement of judgement bias has been used to try and indicate emotional states in animals. In the present experiment a potential test for judgement bias in mice was examined. Mice were trained with two distinct odour cues (vanilla or apple) predicting either a palatable or an unpalatable almond piece. Subsequently their reaction to mixtures of both odours, the ambiguous stimuli, was investigated. Mice of the BALB/cJ and 129P3/J inbred mouse strains (high initial anxiety and low initial anxiety phenotypes respectively) were tested. While BALB/cJ mice showed odour association learning and showed intermediate reactions to the ambiguous cues, 129P3/J mice did not discriminate between the cues. Additionally BALB/cJ mice that were tested under more aversive white light conditions revealed a higher latency to approach the almond piece than mice tested under less aversive red light conditions. The ambiguous stimulus however was interpreted as negative under both test conditions. Brain c-Fos expression levels (a marker for neuronal activity) differed between the BALB/c/J and 129P3/J in the lateral amygdala and the prelimbic cortex, indicating differences in ambiguous information processing between the strains. The behavioural results suggest that the present judgement bias test might be used to assess emotional states in at least BALB/c mice, however further research on both behaviour and on the involved brain mechanisms is necessary to confirm this idea.

1. Introduction

In humans it is well known that emotional states influence cognitive processes, an effect that is referred to as cognitive bias [1]. People that are in a negative affective state reveal a better memory of negative events, tend to focus their attention on the occurrence of negative events, and interpret ambiguous stimuli more negatively (negative judgement or interpretation bias) [2-6]. People suffering from anxiety disorders and/or depression have a more negative judgement bias than healthy controls [7].Based on the knowledge mentioned above, a negative judgement bias is understood as an indicator of a negative affective state [6, 8-10].

In animals a measurement of judgement bias is of additional value next to existing behavioural and physiological indicators of emotions, since the measurement of judgement biases includes the cognitive component of emotions and could be used as indicator of emotional valence [11]. Recently, the phenomenon of judgement bias has been investigated in several animal species, some being aimed at welfare assessment while others are more interested in judgement bias in animal models of human affective disorders [12-23]. Judgement biases in animals are measured by testing their behavioural response to an ambiguous stimulus after performing a conditioning procedure in which two different stimuli (of the same sensory modality) are associated with either reward or lower-value reward/punishment. For example, a tone of 2 kHz predicts a food reward and a tone of 4 kHz predicts an aversive white noise, in a test session the reaction of the animals to tones of 2, 3.5 and 4 kHz is investigated [13, 21] by comparing this with the reaction to the positive and negative associated cues.

Anxiety seems to influence judgement biases in animals like it does in humans [24], causing a more negative interpretation of ambiguous stimuli [16, 22, 25]. One way to manipulate state anxiety in laboratory rats is to alter light conditions

during testing: Rats are nocturnal and testing under bright light conditions increases state anxiety (=anxiety at a specific moment in time) [26-28]. Notably, rats that are trained under low light conditions and tested under high light conditions show a more negative judgement bias than rats trained under high light conditions and tested under low light conditions, implying that state anxiety can alter judgement biases in rats like in humans [25]. Interestingly, recent results also demonstrate that dogs suffering from separation anxiety and stereotyping starlings have a more negative bias [22, 29], suggesting that high trait anxiety (=general anxiety trait) may affect judgement bias in animals. This notion elicits the question whether judgement bias may in turn represent a potential read-out parameter for affective states in animals.

The aim of the present study was, firstly, to investigate if judgement bias can be measured in mice and, secondly, if judgement bias would be affected by state or trait anxiety respectively. As different strains of mice are frequently used as animal models of (pathological) anxiety and are often subject of transgenic studies, it seems of high interest to investigate judgement bias in this species. Recently anxiety-related behaviour in two inbred mouse strains, BALB/cJ (BALB/c) and 129P3/J (129P3) was evaluated in our lab and it appeared that BALB/c mice behave highly anxious when initially exposed to a test environment, but show a rapid habituation over time, while 129P3 mice are initially less anxious but do not habituate to the testing environment [30, 31]. Previously, BALB/c mice have been suggested to represent a phenotype of trait anxiety because they show high state anxiety in multiple testing situations [30, 32-34]. Thus to our first aim we performed the test in these previously characterised 129P3 and BALB/c mice (experiment 1) expecting a more negative judgement of the initially highly anxious BALB/c mice. To elucidate effects of state anxiety, BALB/c mice in addition were tested under different test conditions (red or white light, experiment 2), expecting a more negative judgement of the mice tested under

white light conditions. An additional 3rd experiment evaluated the odour perception abilities of 129P3 mice. Next to the behavioural tests on judgement bias, brain area's known to be relevant for emotional processes involved in judgement bias, i.e. the prelimbic cortex [35], lateral septum [36, 37] and amygdala [38, 39] , were analyzed for c-Fos expression, a marker for neuronal activity.

In mice, no procedure has been performed yet that focuses on the effects of anxiety on judgement bias. Thus, in the present study a conditioning procedure was used in which the animals were trained to associate odours with either a positive or a negative experience and their reaction to an ambiguous stimulus (mixture of both odours) was subsequently investigated.

2. Materials and Methods

2.1. Ethical note

The protocols of the experiments were peer reviewed by the scientific committee of our department and approved by the local Animal Experiments Committee. Further the animal experiments followed the "Principles of laboratory animal care" and refer to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council 2003). For more details see supplementary material.

2.2. Animals and general housing conditions

Husbandry procedures and animal experiments were performed by well-trained members of the laboratory. The experiment on judgement bias (experiment 1) was performed with 50 naive male BALB/cJ (BALB/c) mice and 50 male 129P3/J (129P3) mice. The light effect experiment (experiment 2) was performed with 84 naive male BALB/c mice. An additional odour perception experiment (experiment 3) was performed with 6 naive male 129P3 mice. All mice were obtained from The Jackson Laboratory (Bar Harbour, Maine, USA) and were 6-8 weeks old at arrival. The animals were housed individually at the animal laboratory of the Netherlands Vaccine Institute (Bilthoven, The Netherlands), in a temperature (22±2 °C) and humidity (45%-50%) controlled room under a 12/12h reversed light/dark cycle (lights on at 6 pm and off at 6 am). Training and behavioural testing was performed in the same room. Mice chow (CRM, Expanded, Special Diets Services Witham, England) and tap water were available *ad libitum*.

During the two-week pre-experimental period the person that performed the actual experiment handled and weighed the mice regularly. All mice were kept in Eurostandard type 3 macrolon cages (40 x 26 x 20 cm) with standard cage bedding (Aspen chips), a plastic shelter (Mouse House Techniplast®) and tissue

(Kleenex® Facial Tissue Kimberly-Clark) as enrichment. The testing equipment had already been installed in the room before the animals arrived.

2.3. Behavioural testing

All testing was performed with odours as conditioned stimuli, considering the ability of mice to discriminate even slight differences between odours [40]; this ability is also found in individuals of the BALB/c and the 129S1/SvImj sub-strain [41]. Odour mixtures have been used before in a judgement bias experiment with honeybees [17]. Both visual and auditory stimuli were excluded, since specific inbred strains (including the 129P3 strain) have been shown to posses a mutation (*Cdh23*^{ahl}) that causes hearing loss within three months of age [42], moreover the albino BALB/c mice tend to be visually impaired, which makes visual stimuli less suitable. Testing was performed in the home cage of the animals to avoid unwanted environmental stress, potentially induced by testing in a novel environment [43, 43, 44].

Pieces of almond (approximately 0.05g) were used as rewards; mice eat these readily even if they are fed *ad libitum* (see for example [45]). The odour stimuli were vanilla and apple (Micro-Plus, Stadtoldendorf, Germany), dissolved in distilled water (0.05%), since mice are attracted by those (e.g. [45]). Both odours were dissolved in a low concentration because the stock solution is highly concentrated and similar concentrations were used before. Odour mixtures for the test sessions were made with the 0.05% solutions, mixing them in the required proportions (see below and table 1).

2.3.1. Experiment 1 and 2: Judgement bias test

2.3.1.1. Apparatus

Experiment 1: During training and test trials almond pieces were presented on a small petri dish (∅ 5.5 cm). The odours were spread on a filter paper (∅ 5.5 cm)

in an amount of 0.1 ml per odour that was positioned in the petri dish [46]. The petri dish with the filter paper was covered by a lid with several holes to let the odours diffuse through the top (see figure 1 A). From now on this dish will be called the odour cup.

Experiment 2: During training and testing almond pieces were presented in an odour apparatus that consisted of a grey PVC cylinder (∅ 3.0 cm, height 3.0 cm) that could be fastened on a transparent Perspex plate (20.0 cm x 9.5 cm), see figure 1 B. From now on this apparatus will be called the odour cylinder. The odour cylinders are similar to those used in the modified hole board and suitable for mice (see for example [30]). Odours (0.05 ml) were spread on a filter paper (∅ 3.2 cm) that fitted underneath the cylinder.

2.3.1.2. Training and testing

Animals were trained and tested when being most active between 9.00 and 13.00. Mice were habituated to eating the piece of almond (30 mg), by offering it with tweezers in the home cage on days 14, 15 and 16 after arrival. On day 19 after arrival the training procedure started. During all trials the home-cage was placed on a table in front of a video camera (placed on the side of the odour cup) that was connected to a dvd-recorder (Panasonic). First of all, the enrichment and water bottle were removed from the home cage. A trial started with placing the odour cup in the home cage (see figure 1). The training trials were terminated when the almond piece was eaten. Test trials lasted 5 minutes.

During training in a positive (POS) trial the odour cup or cylinder was presented with a normal tasting almond piece and in a negative (NEG) trial the odour cup or cylinder was presented with a bitter tasting almond piece. Almond pieces were made bitter by dipping them in a 180 mMol odourless quinine solution (Sigma-Aldrich) and drying them overnight. Half of the mice from one group received the normal tasting almond piece paired with vanilla and the bitter almond piece

paired with apple, and the other half the other way around. In the test trials all odours were presented with a normal tasting almond piece. Learning effects were investigated by statistically comparing latencies to eat the almond piece in the POS trials with that in the NEG trials, a statistical significant difference indicated that the animals had learned the association (on the group level).

Experiment 1: BALB/cJ and 129P3/J mice were trained similarly. For testing, animals of both strains were randomly assigned to five groups (n=10 per group) and the separate groups were tested on their reaction to their group-specific odour concentration. In total all mice received 4 training trials (3 POS trials and 1 NEG trial) over 4 days, one trial per day. The separate groups were either tested (1 trial) on the POS, MIX 1 (85% POS-15% NEG), MIX 2 (50% POS-50% NEG), MIX 3 (15% POS-85% NEG) or the NEG stimulus on the 5th day depending on their experimental group (see table 1 for an overview of the groups). All mice in this experiment were trained and tested in the dark (red light). Animals eating the whole almond piece in the NEG sessions were removed from the analysis (in total 2 129P3 and 6 BALB/c mice), assuming that the bitter taste of the almond was not experienced as being negative by these animals. We therefore assumed that the NEG stimulus could not be considered being 'negative' in these cases.

Experiment 2: BALB/c mice were trained similarly, but different groups (6 groups, n=14 per group) were tested on their reaction to different odour concentrations either in the dark (red light) or in the light (white light, provided by a desk lamp of approximately 120 lux, directed on the animal to be tested). All training was performed in the dark. In total the mice received 8 training trials (5 POS and 3 NEG trials) over 4 consecutive days (two trials per day). The first training day consisted of two POS trials, the other training days of one POS and one NEG trial in a random order. The inter trail interval was approximately 2 hours. Animals were either tested (1 trial) on the POS, MIX (50% POS-50% NEG) or NEG

stimulus on day 5 depending on their experimental group (more details on the treatments per group can be found in table 1).

2.3.1.3. Justification present design

Initially in our first experiment a one-trial learning procedure was applied (one NEG trial) in order to minimize the number of aversive trials, since we have previously found that mice from the 129P3/J strain have difficulties to habituate to a mildly aversive environment [30, 30, 47]. As we continued with BALB/c in experiment 2, some extra trials were added to insure that the animals learned the odour associations. From literature it is known that mice are able to learn odour associations relatively quick [46], which was the reason to choose for the present design. A disadvantage of the one-trial learning procedure (experiment 1) is that it is not possible to make a learning curve for individual mice. However, a comparison between the POS and NEG groups in the test session will reveal whether there is a learning effect on the group level. Since inbred strains of mice were used we did not expect major differences.

In contrast to other studies on cognitive bias, we were interested in investigating neuronal activation in the brain by looking at c-Fos expression. This was only possible if separate groups of mice were exposed to the positive, ambiguous and negative stimulus in the test trial (between-animal design).

2.3.2 Experiment 3: odour perception in 129P3/J mice

Due to the results of experiment 1 an additional experiment was designed to investigate whether the lack of discrimination between the different odours in the test session of experiment 1 in 129P3/J mice (no differences in latencies to eat the almond piece between the groups) was due to a deficiency in odour perception or discrimination.

129P3 mice showed a rapidly decreasing latency to eat the almond piece over trials in experiment 1 indicating that they learned to make the positive association between the odour cup and the almond rapidly. Therefore in this third experiment again a conditioning paradigm was used, but now in such a way that we could draw conclusions on the olfactory capabilities of 129P3 mice.

2.3.2.1. Odour apparatus and almond presentation

The odour apparatus consisted of three grey PVC cylinders (∅ 3.0 cm, height 3.0 cm) that could be fastened equispaced from each other on a transparent Perspex plate (20.0 cm x 9.5 cm), see figure 1c. A trial was initiated by putting the odour apparatus (see 2.3.1) in the home cage. Behaviour during testing was recorded via a camera that was placed above the test set-up. Again odours (0.05 ml) were spread on a filter paper (∅ 3.2 cm) that fitted underneath the open cylinders of the apparatus. One of the cylinders was marked with 0.05% apple odour, another with 0.05% vanilla odour and the remaining cylinder was not marked. The almond was coupled to one of the odours and presented in the corresponding cylinder: Half of the mice could obtain the piece of almond in the vanilla scented cylinder and the other half in the apple scented cylinder. The correct cylinder (the one containing the almond piece) was presented randomly at one of the three locations over trials. To make sure the mice could not identify the correct odour cup by the scent of the almond itself also the other cylinders contained an almond that the mice were unable to obtain (it was contained under a round piece of wire mesh). A total of 30 trials per mouse was performed, 6 trials per day during 5 consecutive days. A trial started with placing the odour apparatus in the home cage and ended after the almond piece was eaten.

2.4. Behaviours scored

Behaviour during the training and the test trials of experiment 1 was scored afterwards from the video material using the computer program "The Observer"

version 5.0 (Noldus b.v., Wageningen, the Netherlands). Behaviour in experiment 2 and 3 was scored live with the same computer programme. Behaviours were scored in a continuous way, i.e. all-occurrence recording of the behaviours of interest. The following behavioural parameters were measured:

Experiments 1 and 2: Latency until eating the almond piece was used as indicator of odour cues judgement (i.e. low latency with a positive interpretation and a higher latency with a negative interpretation). Other measures included exploration (sniffing) of odour cup/cylinder (latency and duration), picking up almond piece (latency), locomotor activity: line crossings between front and back (latency and total number), general exploration: rearing (latency and total number), grooming (latency, total duration and total number).
Experiment 3: head dipping in the correct cylinder was recorded as a correct response, head dipping in the incorrect and unscented cylinder as an incorrect response. Other behaviours that were recorded were exploration (sniffing) of odour cup, head dip (latency and total number), general exploration: rearing (latency, total duration and total number).

2.5. Euthanasia, brain removal and c-Fos analysis

All mice were decapitated two-and-a-half hours after the test session, in a separate room adjacent to the experimental room. Immediately after decapitation the brains of the mice (experiment 1 and 2) were removed and frozen in liquid (-80 °C) 2-methylbutane which was cooled with dry ice and stored at −80◦C. A c-Fos immunohistochemistry was performed only on the brains of experiment 1 to get a general impression of the emotion related brain areas involved in the present test. Brains of experiment 2 are stored and might be further analyzed in the future.

Experiment 1: Coronal sections were cut (20 μm) and mounted on Menzel SuperFrost Plus slides (Menzel GmbH & Co, Braunschweig, Germany) and stored at −20◦C. For the immunohistochemical detection of c-Fos, rabbit anti-c-Fos (SC-52 Santa Cruz Biotechnology) was used. During the staining procedure the sections were rinsed several times after every step in 0.01 M phosphate-buffered saline (PBS) (pH 7.4).

First, the sections were dehydrated. Endogenous peroxidase was blocked by treatment with H_2O_2(0.1%) for 30 min. Sections were pre-incubated with 5% normal donkey serum (NDS) and 1% bovine serum albumin (BSA) in PBS (PBS-BSA 1% + NDS 5%) for 30 min before the rabbit anti-c-Fos incubation (1:1500 in PBS-BSA 1% + NDS 5%, 4◦C, 24 h). Negative controls, used to control for aspecific binding of the Biotin SP conjugate (Jackson ImmunoResearch Laboratories, Inc., PA, USA), were incubated with the PBS-BSA 1% + NDS 5% solution. Next, the sections were incubated with the donkey–anti-rabbit IgG Biotin SP conjugate (1:400 in PBS-BSA 1% + NDS5%) for 45 min. Subsequently, the sections were incubated with avidin horseradish peroxidase solution (1:400 in PBS-BSA 1%+ NDS 5% VECTASTAIN® ELITE ABC, Brunswich Chemie, Amsterdam) for 60 min and pre-incubated with diaminobenzidine tetrahydrochloride (DAB) solution containing nickel sulphate. For visualization of bound peroxidase complexes, the substrate H_2O_2 (30%, 1:2000) was added to the DAB solution and incubated for 5 min. Afterwards the sections were dehydrated in alcohol and cover slipped.

2.5.1. Image quantification

The images of brain sections were projected (10× magnification) and digitalized using an Olympus BX 51 microscope (Olympus,Tokyo, Japan) with a high-resolution digital camera interfaced with a computer. The following brain regions that have been implicated to be involved in anxiety [48-50] (numbers correspond

with the Bregma location) were investigated: prelimbic cortex (1.78), lateral septum (0.86) and the amygdala (basolateral nucleus and central nucleus, -1.58). The anatomical localization was aided by use of adjacent Nissl stained sections and the illustrations in a stereotaxic atlas [51]. For each region at least two overt landmarks were used. For quantitative analysis of c-Fos positive cells, the program LEICA QWIN (image processing and analysis software, Cambridge, UK) was used. Left and right hemispheres were analyzed in one section separately and averaged for each animal and calculated for stained neurons per square millimeter.

2.6. Corticosterone

In experiment 2 blood samples were collected via tail vein incision to determine the influence of testing on plasma corticosterone (pCORT) levels of the animals in the different groups, i.e. if indeed testing under white light was more stressful for the animals. Only pCORT from experiment 2 was analyzed because no differences in stress levels were expected in experiment 1. Basal blood samples were taken 5 days before testing (BASAL) and another sample half an hour after testing (POST-TEST). All blood sampling took place in a separate room adjacent to the experimental room under red light conditions to not disturb the other animals. To prevent any influence of handling and blood sampling on pCORT, the procedures were done as fast as possible with a maximum of 3 minutes. A small blood sample was collected (±50 µl) and stored in pre-chilled Microvette tubes (CB300, Sarstedt, Numbrecht, Germany) containing lithium heparin. Blood samples were centrifuged (10 min at 20,000×g, 4 ◦C) and stored at−20 ◦C until measurement. pCORT levels were measured by radioimmunoassay (RIA) according to the protocol of the supplier with an ImmuChemTM Double Antibody Corticosterone kit for rats and mice (MPI Biochemicals, Amsterdam, The Netherlands).

2.7. Statistics

Statistical analyses were performed with the statistical program SPSS for Windows (version 16.0, SPSS.Inc.,IL, USA). Continuous data (latencies, durations and number of c-Fos positive cells/mm^2) were presented as means with a standard error of the mean (SEM) as index of variance. Discrete data (numbers of occurrence) were presented as Median with the Inter Quartile Range (IQR) as index of variance. The Kolmogorov–Smirnov one-sample test was used to check Gaussianity of the continuous data. Group analyses using the Kolmogorov–Smirnov one-sample test showed a non-parametric distribution of several continuous parameters. These parameters, as well as the total numerical parameters, were either rank transformed [52] or log transformed (continuous data). The (transformed) data from the experiment were subsequently analyzed with a 2-way ANOVA with group and strain as factors. Another possibility is to perform a multiple regression analysis and using the odour concentration as a continuous variable. This analysis was performed and confirmed the significant effects found by applying the 2-way ANOVA (results not shown). Comparisons within and between the groups in the acquisition phase were done with a repeated measurements ANOVA using group and strain as between subject factors (experiment 1) and trial as within subject factor, differences between positive and negative trials (experiment 2) were assessed with a paired t-test. Post-hoc testing was done using a Dunn-Ŝidák correction. pCORT data was represented as delta scores (POST test values – BASAL values) + SEM as we were interested in the change of the pCORT levels caused by testing to get an indication of state-anxiety induced pCORT. A one-way ANOVA was performed to investigate condition effects. Between condition effects were further investigated by performing a t-test on the separate groups (POS, MIX, NEG) using condition as an independent variable (α was corrected with Dunn- Ŝidák). The choice data in experiment 3 were analyzed with a one sample t-test on the percentage of correct choices for each day against performance on chance level (33.33%). The other data (latencies, duration and numbers over trials) in this experiment was

analyzed with a repeated measurement ANOVA, number data were ranked transformed prior to analysis.

3. Results

3.1. Behaviour

A summary of the behavioural data of all experiments can be found in the supplementary material. Behaviour related to the measurement of judgement bias and odour discrimination will be described in more detail in this section.

3.1.1. Experiment 1

3.1.1.1. Training

Mice from both strains became significantly faster in picking up the almond piece over training trials (trial effect: $F_{(1,89)}$= 10.089, p=0.000) and an overall strain difference was found in the latency to eat the almond piece in the training trials (129P3 mice were faster than BALB/c mice; strain effect: $F_{(1,89)}$= 7.373, p=0.008). No strain*trial interaction effect in the training was found ($F_{(3, 89)}$=109.720, p=0.561), data not shown. In total 2 129P3 and 6 BALB/c mice ate the whole bitter almond piece in the NEG trial and were excluded from the test session data.

3.1.1.2. Test

In the test session the different groups of 129P3 mice showed comparable latencies to eat the almond piece (POS: 8.75 ± 2.1, MIX1: 7.6 ± 1.4, MIX2: 6.3 ± 1.1 MIX3: 6.3 ± 1.6 and NEG: 7.1 ± 1.4 seconds respectively), whereas this latency increased in BALB/c mice when the concentration of the negative odour in the odour mix increased (POS: 10.3 ±3.7, MIX1: 23.2 ± 7.4, MIX2: 25.0 ± 14.9, MIX3: 35.7 ± 18, NEG: 51.1 ± 19 seconds respectively), see figure 2. The 2-way ANOVA did not reveal a group difference ($F_{(4,90)}$= 0.585, p>0.05) , but did reveal a strain difference ($F_{(1,90)}$= 4.552, p= 0.036). No group *strain interaction effect ($F_{4,90)}$= 0.369, p> 0.05) was found. Latencies in the third POS session were significantly lower compared with latencies in the test session (data not shown)

only as a main effect in the BALB/c group (t=-3.109, p<0.005), post-hoc testing revealed no separate group effects (all p>0.025).

3.1.2. Experiment 2

3.1.2.1. Training

The mice showed a decrease in latency to start eating the almond pieces in the positive training trials ($F_{(4)}$= 173.419, p<0.001), and an increase in latencies to start eating the bitter tasting almond pieces in the negative training trials ($F_{(2)}$= 17.882, p<0.001), see figure 3A. In addition, there were significant differences in picking up the almond piece between positive and negative trials on day 2, 3 and 4 (t=-3.900, p<0.001; t= -10.218, p<0.001 and t= -9.686, p<0.001, respectively).

3.1.2.2. Test

The latency to eat the almond piece in the test session is presented in figure 3B. Mice tested under white light conditions showed a higher latency to eat the almond piece than mice tested in the dark (condition effect $F_{(2,78)}$ = 47.293, p< 0.001). Post-hoc testing revealed a significant condition effect when the POS (t= -5.865, p< 0.001) MIX (t=-3.324, p= 0.003) and NEG groups (t= -3.811, p= 0.001) were compared between light conditions, (adjusted α=0.017, Dunn-Ŝidák correction). The two-way ANOVA a showed a trend for differences in latencies to eat the almond piece between the groups ($F_{(2,77)}$= 2.482, p= 0.09), no group*condition interaction effect was found ($F_{(2,77)}$= 0.015,p= 0.985). Mice from the MIX groups showed a similar latency to eat the almond piece when compared with the NEG group from the same condition (dark: t= 0.646, p=0.524; light: t= 0.104, p= 0.918). When the MIX group and POS group within the same condition (dark or light) were compared the latencies to eat the almond piece show a trend to be higher in the MIX groups in both conditions (dark: t= -1.840, p= 0.087; light: t= -1.919, p= 0.075).

3.1.3. Experiment 3

3.1.3.1. Choice

The percentage of trials in which the correct choice was made is presented in figure 4. During the first four days of testing the mice made no difference between the three cylinders; choice for the correct cylinder was not significantly different from chance level (day 1: t= 1.085, p= 0.328; day 2: t= -1.168, p= 0.296; day 3: t= 1.746, p= 0.141; day 4: t= 1.936, p= 0.111). On the last day of testing (day 5) the mice chose on average 58.33% ± 5.69 of the time the correct odour cylinder which was significantly different from chance level (t= 4.392, p= 0.007).

3.2 c-Fos expression experiment 1

Data are presented in figure 5.

3.2.1. Prelimbic cortex

For the c-Fos expression in the prelimbic cortex no general strain ($F_{(1,37)}$=1.538, p=0.223) or group ($F_{(2,37)}$=0.359, p=0.7) effect was found, however the strain*group interaction approached significance ($F_{(2,37)}$=2.945, p=0.065), this was due to the trend for a difference in positive cells between strains in the group exposed to the ambiguous stimulus (t_{11}=-2.091, p=0.061).

3.2.2. Lateral amygdala

In the lateral amygdala a significant difference was found between strains ($F_{(1,40)}$=12.631, p=0.001) and groups ($F_{(2,40)}$=4.010, p=0.026) the strain*group interaction ($F_{(2,40)}$= 2.028, p=0.145) was not significant. There were no differences in c-Fos expression levels in BALB/c mice of the different groups (POS: 10.2 ± 1.5, MIX3: 10.3 ± 1.2 and NEG: 9.1 ± 0.9 cells/mm^2). There were differences between the groups of 129P3 mice (POS: 5.3 ± 1.5, MIX3: 9.7± 1.4 and NEG: 5.0 ± 0.8 cells/mm^2). Post-hoc testing revealed a significant difference

between strains in the POS and NEG groups (t_9=3.323, p= 0.009 and t_{15}=3.408, p=0.004 respectively) and a significant difference between the MIX3 and NEG group (p=0.006) and a trend for a difference between the POS and MIX3 group (p=0.021) in the 129P3 strain (corrected α p<0.0085).

3.2.3. Central amygdala

In the central amygdala no differences were found between strains ($F_{(1,40)}$=0.396, p=0.533) and groups ($F_{(2,40)}$= 0.016, p=0.984), also the strain*group interaction effect was not significant ($F_{(2,40)}$=1.986, p=0.150).

3.2.4. Lateral septum

The expression of c-Fos in the lateral septum was not different between strains ($F_{(1,37)}$=0.377, p=0.543) and groups ($F_{(2,37)}$=0.996, p=0.379) and no significant group*strain interaction effect was found ($F_{(2,37)}$=1.322, p=0.279). A difference between groups could be seen in BALB/c mice (POS: 24.9 $\pm$3.5, MIX3: 14.7 $\pm$ 2.0 and NEG: 24.4 $\pm$ 2.7 cells/mm^2). When tested separately on a group effect these differences indeed appeared to be significant (ANOVA $F_{(2,22)}$= 4.234, p= 0.029).

3.3. pCORT experiment 2

Delta values between BASAL and POST test samples are represented in figure 6. There was a significant difference in delta pCORT values between testing conditions ($F_{(5,47)}$= 1.266, p= 0.046), mice tested under white light had higher delta values than mice tested under red light. No group ($F_{(2,47)}$=0.002, p=0.998) or group*condition ($F_{(2,47)}$= 1.118, p= 0.336) interaction effect was found. Post-hoc testing (corrected α= 0.025) revealed only a trend for a difference between conditions in the MIX group (t= 2.327, p= 0.033) and not between conditions in the POS and NEG groups (POS: t= -0.429, p=0.674; NEG: t= -0.728, p= 0.477). Actual and delta pCORT values of BASAL and POST TEST blood plasma samples can be found in the supplementary material.

4. Discussion

BALB/c mice showed a differentiation between positive and negative stimuli in both judgement bias experiments (experiment 1 and 2), i.e. already after exposure to one negative trial BALB/c mice show increased latencies to eat the almond piece (Fig 3A) and responded with an increased latency to both mixed and negative odour cues in the test trial comparison with the third positive trial. Moreover their response latency seemed to be gradually increased by mixing the positive odour with increasing amounts of the negatively associated odour (Fig.2) in the test, although this effect did not reach statistical significance. Further, when the light conditions were changed during testing towards more aversive white-light conditions (experiment 2), BALB/c mice revealed an increase in response times to all odour cues next to elevated pCORT levels after testing (Fig. 6), together indicating an increase in state anxiety. Notably, response latencies towards the mixed and negative cues were similar under both testing conditions and differed from the positive cue, suggesting that testing under red and white light conditions induced a negative judgement bias in BALB/c mice. We therefore conclude that the present test set-up provides a basis for the investigation of judgement bias effects in mice.

However, in contrast to BALB/c individuals, 129P3 mice did not respond differently to the different odour mixtures. Other studies have shown that BALB/c mice are relatively fast learners in paradigms using odours as conditioned stimuli [53, 54] and have a high odour sensitivity [55] in comparison to other strains. Restivo et al. (2006) hypothesized that this difference in learning capacity could be related to eyesight; in general albino mice (CD1 and BALB/c) had a better ability to learn odour associations than non albino mice (129S2/SvPasCrl, C57/Bl6 and DBA2).

To our knowledge, no data on the olfactory learning capacities of 129P3 mice are available. Yet, the results of our third experiment confirmed that 129P3 mice are

able to discriminate between both odours (Fig. 4). 129P3 mice revealed rapidly decreasing latencies to approach all odour cups after a few training trials in both odour conditioning tasks, showing that 129P3 mice are able to learn the spatial location of a reward, a finding that confirms earlier results of our lab showing that these mice are relatively fast in learning the location of reward in the modified hole board test [30, 47]. However, 129P3 mice did not seem to build any negative association with the odour predictive for the bitter-tasting almond piece readily as indicated by comparable response times to different odours in the test session. It might be hypothesised that 129P3 mice need more trials than BALB/c animals to establish the association with positive and negative cues, respectively. This hypothesis has to be explored in further experiments.

An alternative explanation for the lack of discrimination between the negative and positive odour, respectively, in 129P3 mice may be that 129P3 mice experience the bitter taste of the almond as less aversive than BALB/c mice. However, this explanation seems unlikely because almost all of the mice rejected the bitter tasting almond in the negative trial.

Effects of test conditions (white light vs. red light) on judgement bias

To evaluate whether the test set-up allows for assessing the effects of a more negative emotional state on judgement bias in mice, BALB/c mice were tested under white light in experiment 2, a condition that has previously been shown to increase avoidance behaviour in the same strain [30]. It is remarkable that the latencies to eat the almond piece under dark testing conditions were shorter than the latencies found in experiment 1, which might be explained by the different test set-ups used and the familiarity with the test.

Regardless of this it was hypothesized that testing under more aversive bright light conditions would cause a more negative judgement bias than testing under dark (red light) conditions. Yet, it was found that mice under both dark and light testing conditions showed indications of a negative judgement bias, i.e. the

response latency in BALB/c mice towards the ambiguous and the negative stimulus was identical under both light conditions, while it tended to differ between the ambiguous and the positive stimulus (Fig. 3). A judgement bias by definition is a relative reaction (or "interpretation") to an ambiguous stimulus: if the reaction to the ambiguous stimulus is similar to the negative stimulus, a negative bias is to be concluded while a positive bias is indicated by a comparable reaction to both the positive stimulus [56]. The response profile in BALB/c mice to the different ambiguous stimuli in experiment 1 and 2 was similar to that of previous studies on cognitive bias in rats and sheep, in which the response time to the ambiguous stimulus was higher when the presented ambiguous cue was more similar to the negative cue [12, 13, 23].

Some concerns regarding this apparent negative judgement bias under both testing conditions might be raised. Firstly, most cognitive bias experiments in animals describe a relative negative bias when comparing a negatively manipulated group with an appropriate control group that shows a more positive bias and show no differences in reaction to the positive and negative cues [12, 19, 57]. Here all groups tested under bright light conditions, irrespective of whether they were tested on either a negative or a positive odour, revealed an increase in latency to explore and pick up the almond, indicating a general anxiety-induced behavioural inhibition. In addition, post-testing stress hormone levels (pCORT) were increased in mice that were tested under white light, confirming that testing under these conditions indeed was more stressful for the animals. Although this is in accordance with previous results showing that an aversive environment (such as exposure to novelty or predator odour) causes an inhibition of familiar food intake in mice [58, 59], it is difficult to compare the groups tested under the different light conditions regarding their relative judgment bias. Further, it might be discussed whether results were confounded in that the presentation of a negative associated odour cue itself induced a more negative affective state and whether, thus, the mere presence of this odour in the

mixture inhibited the mice from eating the almond piece. Here, latencies to explore the odour cups and cylinders did not differ between the groups in both experiments 1 and 2 (see supplemental material), indicating that the motivation to search for food at least did not differ between the groups. Others have resolved this problem by using a conditioning paradigm based on expectancy of reward size or value as indication of reward (e.g. [18]). However, for the measurement of anxiety such an approach might be less suitable, since high anxiety is hypothesized to cause an increase in the expectancy of negative events and not a decrease in the expectancy of positive events [7].

Rats show a difference in judgement bias between dim and bright light testing [25]. In contrast with our study these rats were trained under dim light conditions, but tested under bright light conditions or vice versa. It appeared that a shift towards a more aversive test condition induced a negative judgement bias, while shifting towards less aversive conditions resulted in a positive judgement bias. In our experiment, all animals were trained under dim (red) light conditions and tested either under the same or more aversive white light conditions which could explain the difference with the mentioned rat study. A more negative interpretation of ambiguous cues is thought to be related to a more negative affective state, which again can be influenced by current environmental conditions, trait affect and previous experiences [60]. Notably, it has been argued that the BALB/c inbred strain represents a high trait anxiety phenotype [33, 34], which would be in line with a given sensitivity to establish a negative bias under less-aversive and aversive conditions.

c-Fos expression

Despite the apparent lack of discrimination between the different odour stimuli in 129P3 mice (experiment 1), a higher c-Fos expression was found in the lateral nucleus of the amygdala in the group that had been exposed to the ambiguous stimulus in comparison with the groups exposed to the positive or negative

stimulus, respectively. In addition, and similar to the lateral nucleus of the amygdala, a trend for an increase in c-Fos expression was found in the prelimbic cortex in the 129P3 group that was exposed to the ambiguous cue, while no differences were found in BALB/c mice. The connection of this region with the amygdala might explain the similarity in the c-Fos responses in both regions, i.e. the prelimbic cortex projects to the basal part of the lateral amygdaloid nucleus and neurons from this same part also send projections back [61, 62].

Lesion experiments suggest that the amygdaloid nuclei involved in appetitive and aversive learning are functionally similar [63], which may explain why in the present experiment no differences in c-Fos expression were found between the groups exposed to either the positive or the negative cue. However, in combined action with higher order regions such as the prefrontal cortex, the basolateral amygdala is indicated to be involved in this evaluation of ambiguous and uncertain situations [38]. In humans there is some evidence that exposure to uncertainty and ambiguous cues results in an higher amygdala activation [39, 64-67]. In addition some authors have suggested that uncertainty is processed similar to ambiguity since the chance of a forthcoming event in both situations cannot be foreseen [64, 65]. Experimental work has indicated that unpredictability increases c-Fos expression in the mouse lateral amygdala [65] and might thus also be implicated in response to ambiguous cues. Thus, while the increased amygdala and prelimbic activity that was seen in 129P3 mice in response to exposure to the ambiguous cue might indeed seem to indicate that the ambiguity of the cue is processed at the brain level, it remains unclear why these mice were unable to translate process into an appropriate behavioural response.

While in BALB/c mice no differences were found in both the lateral nucleus of the amygdala and the prelimbic cortex, in the lateral septum there appeared to be a

decrease in c-Fos expression in response to the ambiguous cue. The lateral septum is an essential node in integrating cognitive information with emotional information [36]. This area acts as a system that compares known information with actually presented information, which is especially important for the identification of ambiguous cues. A human patient for example with lesions in this region has been reported to reveal problems with judging the valence of novel environmental information [37]. c-Fos expression in the dorsal part of the lateral septum revealed a trend towards reduction in response to ambiguous cue exposure in the BALB/c strain (when the statistical analysis was done separately from 129P3, the difference reached significance), but not in 129P3 animals. It may be hypothesized that this difference in the processing of ambiguous and predictable information between 129P3 and BALB/c mice in the lateral septum may be related to differences in behaviour in the test session. The nature of the difference found on the brain level remains to be investigated, as c-Fos expression as a quantitative measure only can offer a first indication.

Conclusions

In summary, the behavioural data reveal that there is a strain difference in performance in the odour discrimination task intended to measure judgement bias in mice: BALB/c mice discriminate between an odour predicting an almond piece and an odour predicting a bitter tasting almond piece, while 129P3 mice respond very fast to all odour cues presented. BALB/c mice also are more reluctant to eat almond pieces that are presented together with the ambiguous odours and reveal a negative judgement bias both under red and white light conditions. Therefore we conclude that the present test provides a basis for evaluating judgement bias in BALB/c mice. At the brain level, c-Fos expression in the amygdala, prelimbic cortex and lateral septum indicated that there may be strain differences in information processing: while c-Fos expression levels did not differ between positive and negative cue exposure in both strains, exposure to

the ambiguous cue increased c-Fos activity in the lateral nucleus of the amygdala and the prelimbic cortex in 129P3 mice and seemed to decrease c-Fos activity in the lateral septum in BALB/c mice. These results suggest that 129P3 mice may perceive the ambiguous cue as different from the positive and negative cue at the level of the brain, only this perception is not translated into a behavioural response. Notably, exposure to an ambiguous cue affected c-Fos activity in the lateral septum in BALB/c, but not in 129P3 mice. This area is important for linking emotional with cognitive information and it has been shown in other experiments that neuronal activation of this specific area differs between the two strains. Thus the lateral septum might be an important target to investigate in future experiments.

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Figure captions

Figure 1: A diagram of the different odour apparatuses that were used to present the odours in the home cage during training and testing. A) Odour cup that was used in experiment 1 (adapted figure from Schellinck et al. (2001)), line crosses were measured when the animal crosses the grey line in the middle of the cage with all 4 paws. B) Odour cylinder that was used in experiment 2 and C) odour apparatus that was used for the odour discrimination test in experiment 3. One cylinder is marked with a filter paper with vanilla odour, one with apple odour and one is unscented.

Figure 2: Time in seconds (+ SEM) from the start of the trial until the almond piece is eaten in the 3rd POS trial and the test session (all mice tested in the dark) of experiment 1. In the test trial a significant strain difference was found, as well as a significant increase in latency between the start of the trial and picking up the food for the BALB/c strain ($P<0.005$) when compared with the 3rd POS session. A significant difference between the 129P3 and BALB/c strain was found in the test session ($p <0.05$).

Figure 3: Behaviour experiment 2. A) Latencies to eat the almond piece of BALB/c mice (presented as mean ± SEM) on training days. Significant differences were found between POS and NEG trials, $**p< 0.001$. B) Latencies of BALB/c mice (presented as means + SEM) to eat the almond piece in the test session. A significant effect was found for light conditions ($P<0.001$), the group effect for both conditions failed to be significant ($p=0.09$). $*p <0.01$, $**p \leq 0.001$, $t^1= 0.086$, $t^2= 0.075$

Figure 4: Mean percentage of correct hole visited during testing on day 1 till 5 in experiment 3. Choice for the correct odour cylinder was compared with performance on chance level (33%). On day 5 the mice chose the correct odour cylinder more than was expected on chance level, $*p=0.007$.

Figure 5: c-Fos expression levels in experiment 1 (expressed as the number of positive cells per mm^2 + SEM) in (a) the prelimbic cortex, (b) the lateral septum the central (c) and lateral (d) amygdala. A trend was found for a group*strain interaction for the prelimbic cortex (p=0.065). In the lateral amygdala a significant strain and group effect was found (p= 0.001 and p=0.026). t=trend p=0.061, ** p<0.01, *p<0.05

Figure 6: Delta pCORT (nmol/l + SEM) levels between BASAL and POST testing plasma samples of experiment 2. t= trend p= 0.034

Table captions

Table 1: experimental groups (experiment 1 and 2), tested with different odour concentrations. In the POS (= positive conditioned stimulus) sessions the almond pieces were presented with one odour (either apple or vanilla, odour 1) and in the NEG (= negative conditioned stimulus) sessions bitter tasting almond pieces presented with the other odour (odour 2).

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Figure 1

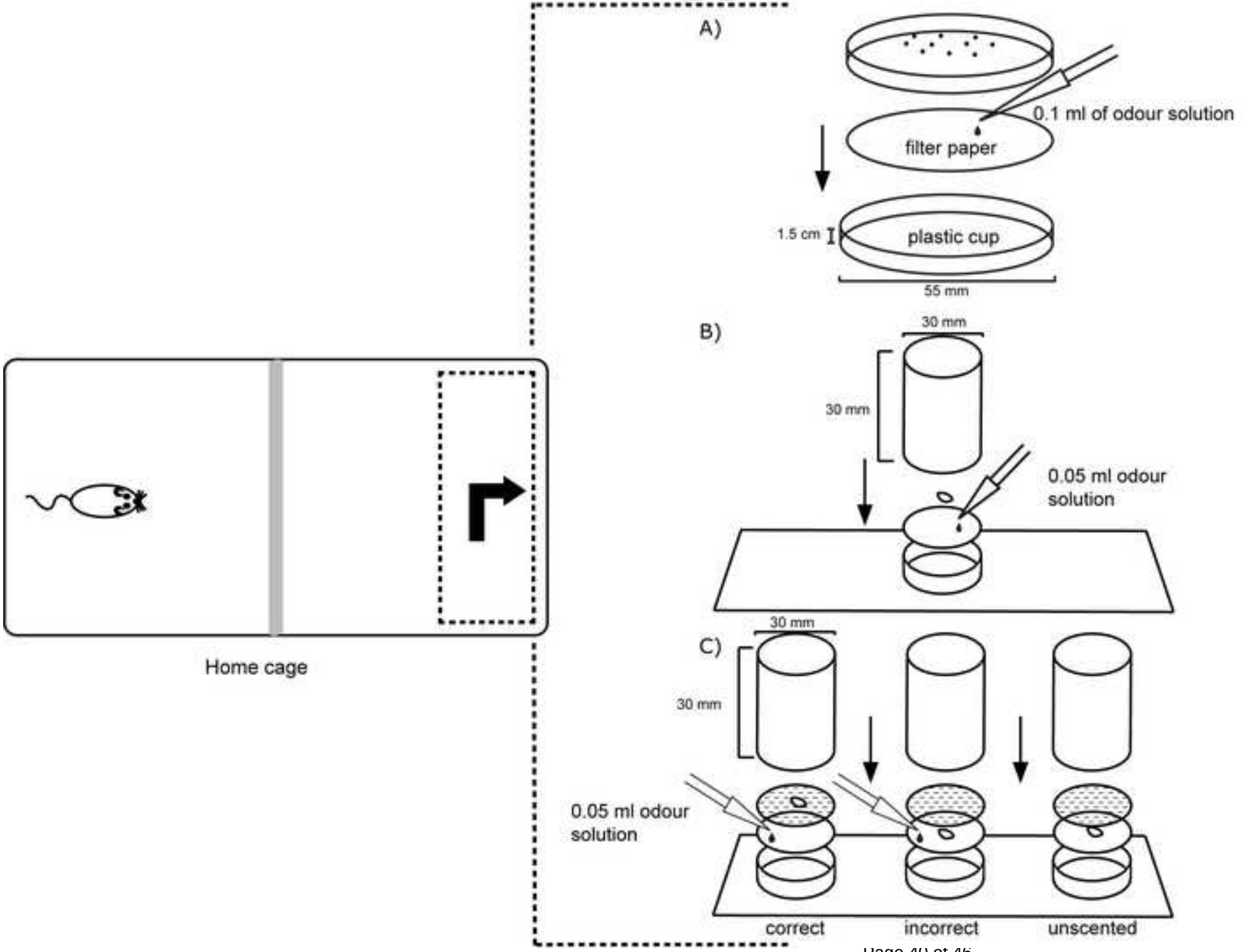

A)
0.1 ml of odour solution
filter paper
1.5 cm
plastic cup
55 mm
B)
30 mm
30 mm
0.05 ml odour solution
C)
30 mm
30 mm
0.05 ml odour solution
correct
incorrect
unscented
Home cage

Figure 2

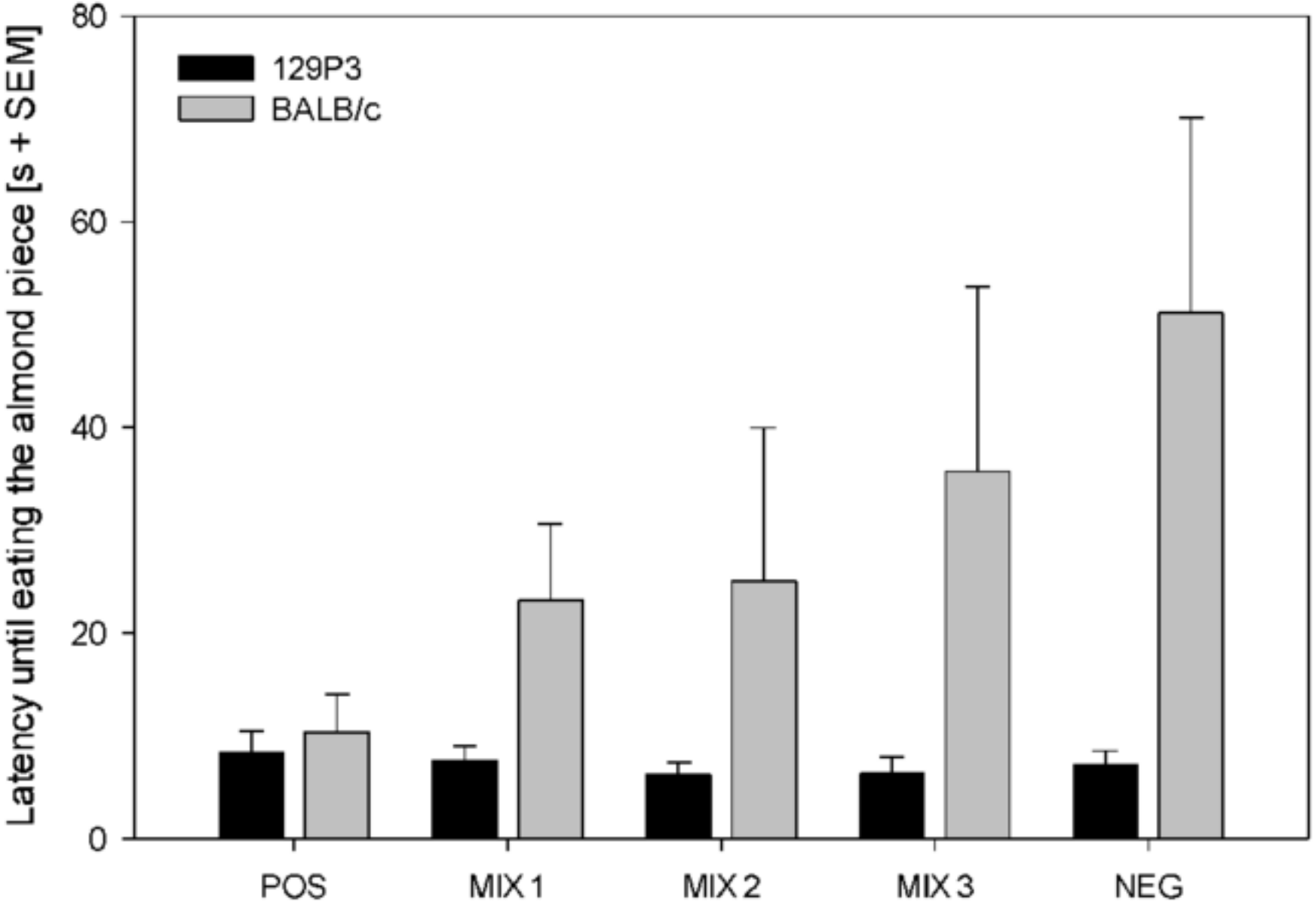

Latency until eating the almond piece [s + SEM]
80
60
40
20
0
129P3
BALB/c
POS
MIX 1
MIX 2
MIX 3
NEG

Figure 3

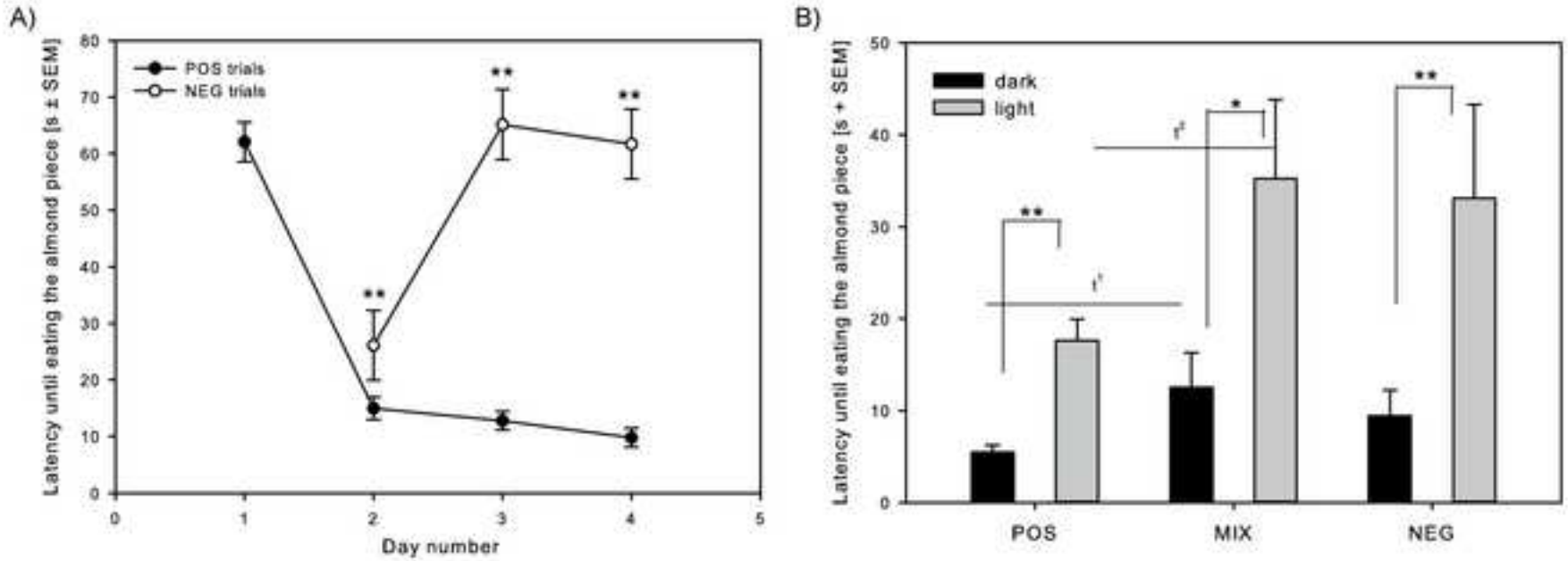

A)
POS trials
NEG trials
Latency until eating the almond piece [s ± SEM]
Day number
B)
dark
light
Latency until eating the almond piece [s + SEM]
POS
MIX
NEG

Figure 4

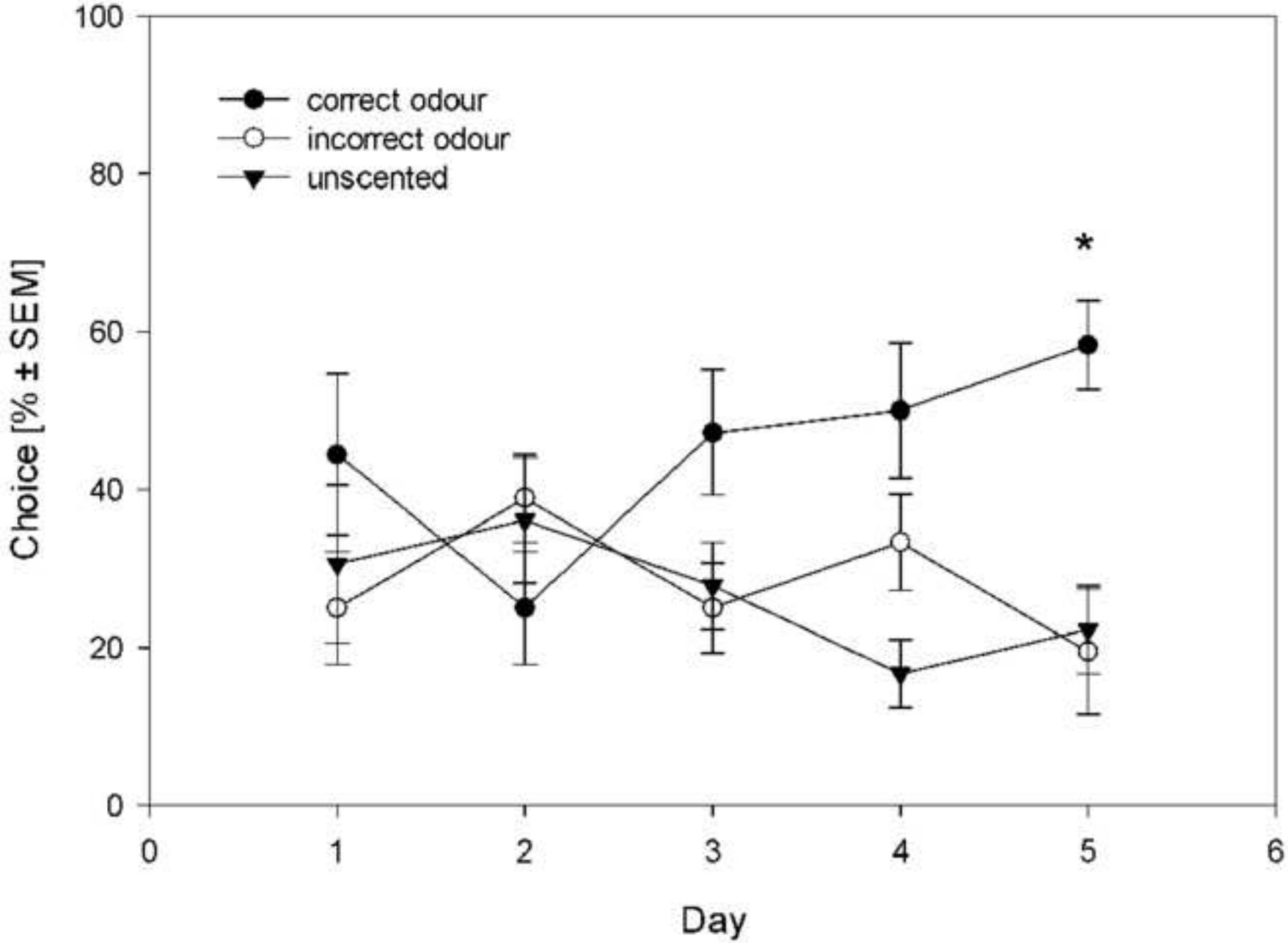

100
80
60
40
20
0
Choice [% ± SEM]
correct odour
incorrect odour
unscented
*
0
1
2
3
4
5
6
Day

Figure 5

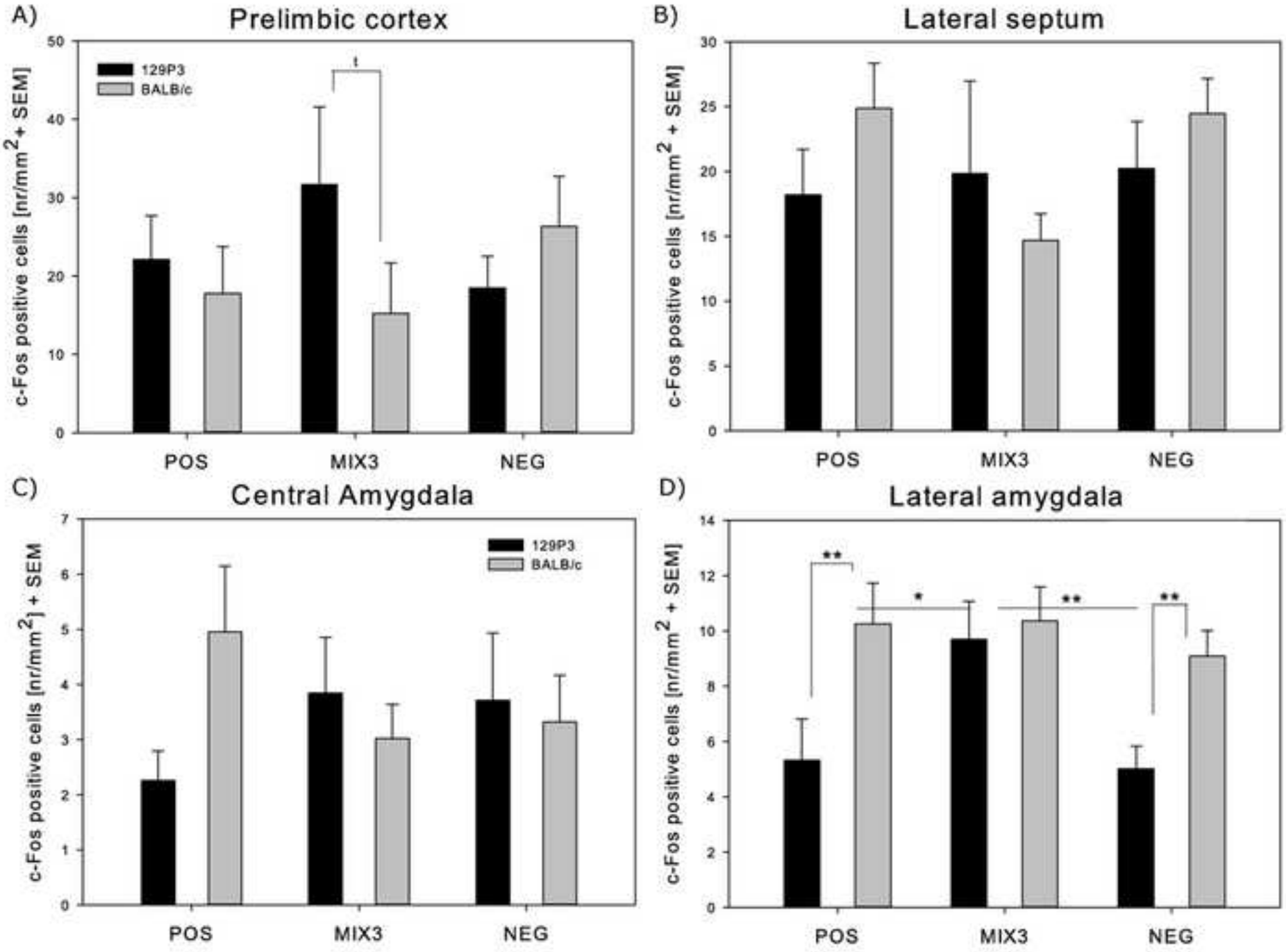
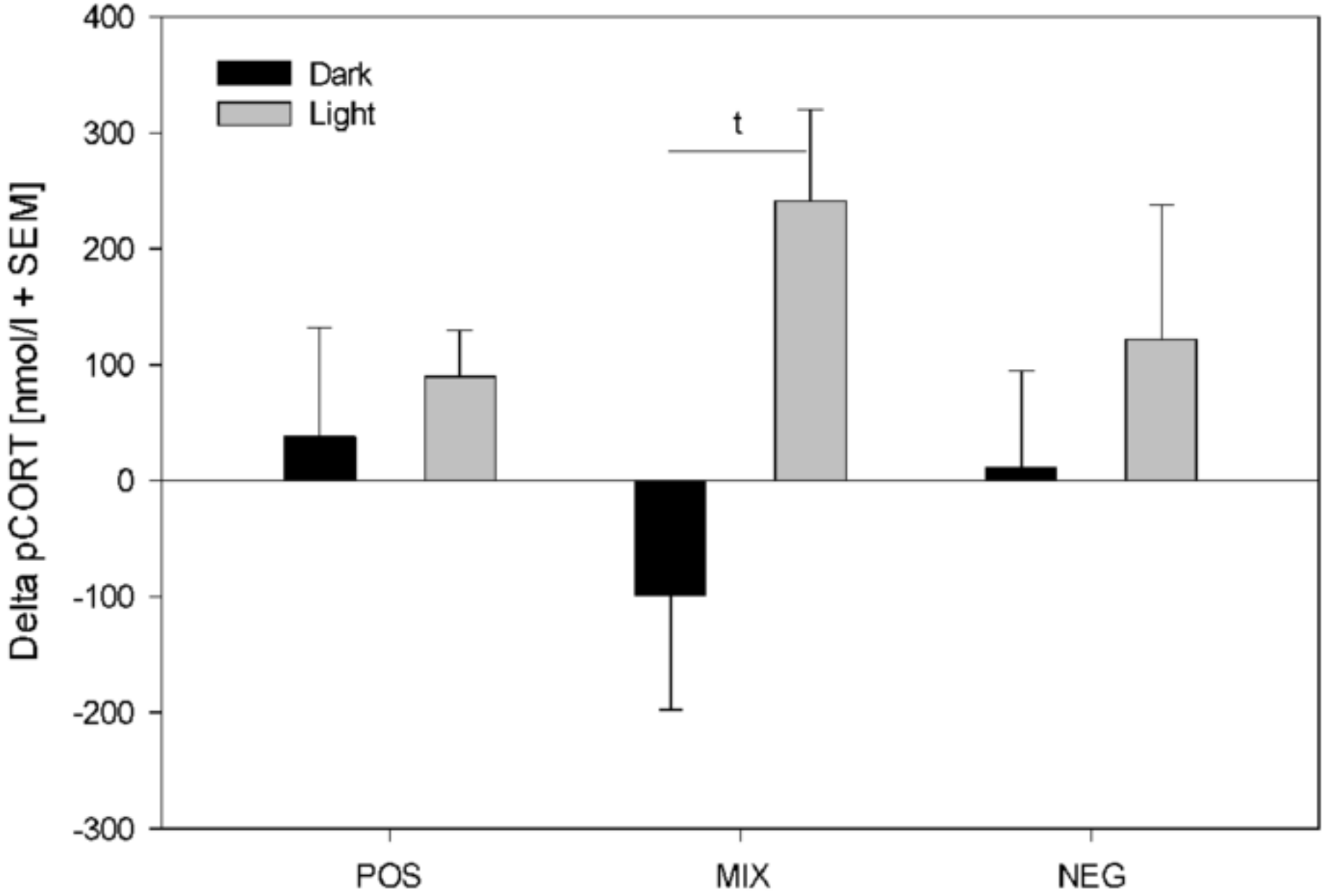

A)
Prelimbic cortex
129P3
BALB/c
t
c-Fos positive cells [nr/mm2 + SEM]
POS
MIX3
NEG
B)
Lateral septum
c-Fos positive cells [nr/mm2 + SEM]
POS
MIX3
NEG
C)
Central Amygdala
129P3
BALB/c
c-Fos positive cells [nr/mm2] + SEM
POS
MIX3
NEG
D)
Lateral amygdala
**
*
**
**
c-Fos positive cells [nr/mm2 + SEM]
POS
MIX3
NEG

Figure 6


400
300
200
100
0
-100
-200
-300
Delta pCORT [nmol/l + SEM]
Dark
Light
t
POS
MIX
NEG

Groups	1	2	**Training** Odour POS, almond piece Odour NEG, bitter tasting almond piece				**Test** Presentation POS, NEG and MIX
Experiment 1: strains							
	BALB/c	**129P3**	Day 1	Day 2	Day 3	Day 4	Day 5
1	POS	POS	POS 1	POS 2	POS 3	NEG 1	POS
2	MIX 1	MIX 1					MIX 1 (85% POS, 15% NEG)
3	MIX 2	MIX 2					MIX 2 (50% POS, 50% NEG)
4	MIX 3	MIX 3					MIX 3 (15% POS, 85% NEG)
5	NEG	NEG					NEG
Experiment 2: light conditions							
	Dark (red light)	**Light** (white light)	Day 1	Day 2	Day 3	Day 4	Day 5
1	POS	POS	POS 1 POS 2	POS 3 NEG 1	POS 4 NEG 2	POS 5 NEG 3	POS
2	MIX	MIX					MIX (50% POS, 50% NEG)
3	NEG	NEG					NEG