Daily exposure to a touchscreen-paradigm and associated food restriction evokes an increase in adrenocortical and neural activity in mice

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ABSTRACT

The translational assessment of mechanisms underlying cognitive functions using touchscreen-based approaches for rodents is growing in popularity. In these paradigms, daily training is usually accompanied by extended food restriction to maintain animals' motivation to respond for rewards. Here, we show a transient elevation in stress hormone levels due to food restriction and touchscreen training, with subsequent adaptation effects, in fecal corticosterone metabolite concentrations, indicating effective coping in response to physical and psychological stressors. Corticosterone concentrations of experienced but training-deprived mice revealed a potential anticipation of task exposure, indicating a possible temporary environmental enrichment-like effect caused by cognitive challenge. Furthermore, the analyses of immediate early gene (IEG) immunoreactivity in the hippocampus revealed alterations in Arc, c-Fos and zif268 expression immediately following training. In addition, BDNF expression was altered as a function of satiation state during food restriction. These findings suggest that standard protocols for touchscreen-based training induce changes in hippocampal neuronal activity related to satiation and learning that should be considered when using this paradigm.

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Introduction

The use of touchscreen-based technologies is a state-of-the-art and convenient way to investigate learning and cognition in rodents (Talpos and Steckler, 2013), and enables the examination of a variety of behaviors with high validity (Horner et al., 2013; Mar et al., 2013). Although growing in popularity, little is known of the psychological and physiological impact of the procedure. Daily training and testing in the boxes is usually accompanied by food restriction to maintain motivation for responding. However, the extent to which daily training and/or food restriction influences stress levels and induces neuronal activity, and thus may impact performance of touch-screen tasks, is unknown.

One way to measure stress in rodents is the analysis of fecal corticosterone metabolites (FCM) (Touma et al., 2004, 2003). This well-established and non-invasive technique allows repeated investigation of corticosterone concentration in subjects during the performance of behavioral tasks, and may provide insight into different stages of experimental progression. In terms of the relatively recent use of touchscreen based approaches, FCM analysis may also help to address some potentially important considerations regarding this task, including the impact of food restriction on stress levels, and whether habituation effects occur with repeated exposure or stress responses. To be aware of the neuroendocrine changes is of relevance for scientist, because these changes modify (i) learning and memory and (ii) the action of drugs.

The expression of immediate early genes (IEG) can also be an indicator of the psychological and physiological impact of a behavioral procedure. IEGs are the first group of genes to be expressed following synaptic activation. They are known to be involved in synaptic plasticity and memory consolidation processes, but also to be influenced by stressful conditions, including hunger (Guzowski, 2002), and even after pathophysiological brain conditions such as epilepsy and ischemia (Gass and Herdegen, 1995; Kiessling and Gass, 1993, 1994). Some IEGs, such as c-Fos and zif-268, act as regulatory transcription factors, while others, including activity-regulated cytoskeleton-associated protein (Arc)

and brain-derived neurotrophic factor, function as effector molecules (Guzowski, 2002).

Here, we examined whether continuous food restriction and daily touchscreen-based training influenced stress levels and induced neuronal activity. In order to address these questions, we examined FCM concentrations in different treatment groups over the course of a standard touchscreen-based experiment (Richter et al., 2014). Furthermore, following completion of the experiment, we examined the effects of food restriction and touchscreen training on immediate early genes (IEG) in the hippocampus in an effort to discriminate learning-induced effects on IEG mRNA expression from stress effects mediated by food restriction.

Material and methods

All experiments complied with regulations covering animal experimentation within the EU (European Communities Council Directive 2010/63/EU), and were approved by German animal welfare authorities (Regierungspräsidium Karlsruhe).

Animals and housing

Forty male C57BL/6N mice (Charles River, Sulzfeld, Germany), 11-weeks old at the start of experiments served as subjects. Mice were single-housed in conventional macrolon cages (Type II, $26 \times 20 \times 14$ cm) with sawdust (RehofixMK-2000; Rettenmaier & Söhne, Rosenberg, Germany), nesting material, and ad libitum food and water prior to food restriction. Single housing was chosen, since we have previously shown that under standard maintenance conditions (i.e. no enrichment) single-housing is less stressful for male mice than group housing (Chourbaji et al., 2008). The colony room was maintained at a temperature of $23 \pm 2\ °C$, a relative humidity of $50 \pm 5\%$, and a reversed 12 h light-dark schedule with the lights off at 7 am (Chourbaji et al., 2012; Lima-Ojeda et al., 2013). Experiments were conducted during the dark period, the active phase of the mice.

Treatment

Mice were separated into three treatment groups. 1) The "handling" group ($n = 8$) was exposed to daily handling and assessment of body weight, and served as non-food-restricted controls to evaluate the effect of food restriction. 2) The "food restricted" group ($n = 8$) received daily handling and weight assessment, and was additionally food restricted to 85–90% of their initial body weight. 3) The "touchscreen trained" group ($n = 24$) was subjected to daily handling, weight assessment and food restriction, and was additionally trained using a touchscreen system.

Handling was performed without physical restrain and followed the cup handling protocol as described in Hurst and West (2010): mice were scooped up and allowed to walk freely over the handler's open hands. This method can minimize a widespread source of anxiety in laboratory mice.

Food restriction

Bodyweight of all mice was assessed daily prior to start of touchscreen-based experiments. After an initial food deprivation overnight mice received 2.0 g to 3.3 g food on the first day of restriction. Henceforth, when the current individual bodyweight was lower (or higher) than intended (87.5% of initial bodyweight) the amount of food was increased (or decreased) relative to the previous portion to maintain 85 to 90% of initial bodyweight. Only the touchscreen trained group received sweet condensed milk (SCM; "Milchmädchen" diluted 1:4 in tap water). After each correct response 7 µl of SCM was delivered as a reward during training. Dependent on the number of

trials set for the different training phases this ranged from 30 to 50 servings per day.

Touchscreen procedure and the assessed paradigm STABFLEX test

For the "touchscreen trained" group, training started one week after food restriction onset. Mice were trained on the STABFLEX paradigm, a test used to assess cognitive flexibility and stability in a cue-location association task (Richter et al., 2014). The position of a visual cue on the central screen indicated which responsive fields - left or right from the cue - had to be touched in order to receive the reward. Procedures of this paradigm are divided into three phases: habituation, training and testing. During the habituation phase the mice were accustomed to the mouse touchscreen chambers (Model 80614-20, Campden Instruments Ltd., Loughborough, Leics., UK) made of black Perspex walls chambers (height 19 cm, width 24 respectively 6 cm, depth 17 cm) equipped with a touch-sensitive screen divided into 3 touching zones, a movement detection system consisting of several light beams, a signaling 3-W house light, and a tone generator. The duration of exposure in the boxes was increased stepwise, from initially 10 min to maximal 40 min at the end of habituation. Additionally, they learned to touch the screen on the implemented touch fields in order trigger reward delivery, the association of reward-indicating tone, a signal for incorrect responses and initiation of trials. SCM was delivered as a reward in a food well (height 2 cm, width 2 cm, depth 2 cm) attached to an externally-placed feeder opposite the screen. Progression within the paradigm was based on individual performance and therefore individual learning speed. When phase-dependent progression criteria were reached by the individual mouse it commenced training in the following training step. In mean the habituation lasted approximately two weeks. In the subsequent training phase the individuals acquired the cue-position task until they reached the learning criterion of at least 80% correct responses on two consecutive days with termination after 40 trials or a maximal duration of 60 min. In mean training lasted 9 weeks with minimum 29 and maximum 79 days. All mice were subsequently tested for another 2 weeks in the test phase.

Sampling of feces for fecal corticosterone metabolites analysis

In order to measure FCM concentrations for stress monitoring, fecal samples were collected. Samples were collected at five monitoring time points (Fig. 1): during ad libitum feeding (2 weeks prior to training onset $= FCM_1$), during the food restriction phase (-1 week to training onset $= FCM_2$) and three times during the training phase. In week 2 after touchscreen procedure onset ($= FCM_3$) all mice are in the habituation phase (23 of them in the same program 'punish incorrect'). Four weeks later ($= FCM_4$) almost all mice have further progressed into the training phase. The last samples were taken 10 weeks after onset ($= FCM_5$), when some mice had already progressed to the 'STABFLEX test' while others still remained in the 'final training' and the 'cue-position training'. Since the some mice already completed the 'STABFLEX test' before the next scheduled sampling date, we decided to end sampling.

Directly after the daily treatment, handling or touchscreen-exposure, the mice were introduced into clean cages and feces voided in a 2 h period were collected. When the collected amount was insufficient for analysis, the collection interval was prolonged up to one additional hour. Due to this sampling schedule, the samples do not depict the adrenocortical state of the mice during treatment, as Touma et al. (2003) showed that peak FCMs can be found 4 to 6 h after exposure to a stressor during the dark phase of the diurnal rhythm. We collected earlier samples in order to determine a more general impact rather than the acute effect of the procedures on the mice.
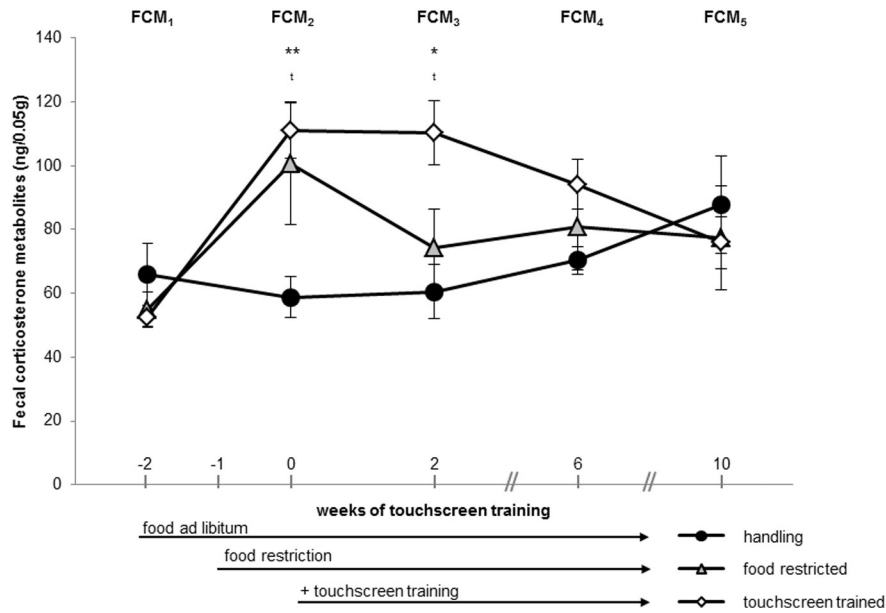
Fig. 1. Food restriction and exposure to touchscreen-based training led to an elevation in fecal corticosterone metabolite (FCM) concentrations relative to handling controls. Data are expressed as means ± SEM. FCM_2 and FCM_3: handling vs. touchscreen-trained group $p^{**} < 0.01$, $p^* < 0.05$; FCM_2: handling vs. food restricted $p^t = 0.07$; FCM_3: food restricted vs. touchscreen trained $p^t = 0.07$.

Behavioral measures in touchscreen proceedings

Interpretation of the relationship of stress response and IEG expression with learning requires behavioral measures of performance. Since the progression status within the paradigm varies between the time points of FCM sampling, program-specific parameters were necessary for performance estimation.

At FCM_3 the majority of mice (n = 22) were trained in the 'punish incorrect' program of the habituation phase. The novelty in this program is the introduction of punishment. The mouse were requested to touch a displayed cross on one of the lateral fields. Correct responses were rewarded with SCM delivery, while touches on the central field did not induce any effects. Touches on the other lateral field however were "punished" with a 5 s-timeout with corresponding house light illumination. Parameters used to estimate learning performance were the percentage of correct responses of the day previous to sampling and a classification according to the days needed to fulfill the progression criterion of at least 85% correct on two consecutive days. Class one included mice which fulfilled the criterion the day before sampling, class two those which reached criterion within the next three days and class three all subjects which needed longer.

Almost all mice (n = 22) were trained on the 'cue-position training' at the fecal sampling time point FCM_4. Here, the fundamental association of the reward-indicating position of the presented cue and the correct response side was established. For details see Richter et al. (2014). Assessed behavioral measures on acute performance were the percentage of correct responses and number of correction trials accomplished on the day previous to sampling. A more general estimation of learning pace was the number of days spent on the current program at sampling date, with quick learners progressing sooner and therefore displaying more days in the current step.

The distribution of mice in different phases was more diverse at the last sampling (FCM_5) and included some animals in the STABFLEX test (n = 5), some in the 'cue-position training with distractor' (n = 5) and 13 mice still remaining in the 'cue position training' (assessed in almost all mice on FCM_4). In the 'cue position training with distractor' a further condition was introduced by presentation of a second distractive cue with lower intensity on the central screen and additionally an ambiguous condition in the 'STABFLEX test' (for further details see Richter et al. (2014)). Again percentage of correct responses and number of correction trials accomplished on the day previous to sampling were assessed as estimates of acute performance.

Processing of samples and measurement of fecal corticosterone metabolite concentration

Fecal samples were extracted with methanol using a standard protocol (Palme et al., 2013). Briefly, an aliquot (50 mg) of each well homogenized, dried fecal sample was mixed with 1 ml 80% methanol, shaken for 30 min and subsequently centrifuged for 10 min at 2500g. FCM were analyzed using a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA) as previously described (Touma et al., 2003, 2004).

Preparation of hippocampal tissue and analysis of mRNA levels

Mice were killed by decapitation either 90 min or 24 h after the last food supply or termination of the behavioral task. Hippocampal tissue was dissected onto ice, immediately frozen with dry ice and stored at −80 °C for further processing. The different time schedules allowed investigation of the acute effects of training on molecular activity markers or to depict the molecular state at the expected daily behavioral procedure onset time. Sample preparation and RTqPCR were performed as described in Luoni et al. (2014). Relative target gene expression was calculated according to the 2(−Delta Delta C(T)) method (Livak and Schmittgen, 2001), and all the data were expressed and analyzed as a percentage of mRNA levels in the handling group.

Serum corticosterone measurement

Corresponding corticosterone concentrations to mRNA levels were analyzed in trunk blood serum samples by commercial radioimmunoassay (MP Biomedicals, Eschwege, Germany) as previously described (Ridder et al., 2005).

Statistical analysis

Statistical analyses were carried out using IBM SPSS Statistics 20. As FCM data were not normally distributed, these measures were analyzed using non-parametric Kruskal-Wallis-H tests to investigate effects between treatment groups for FCM and serum corticosterone analysis. Pairwise comparisons were corrected using the Holm-Bonferroni p-value adjustment. Wilcoxon tests were used to investigate changes in FCM concentrations between two sampling dates. Friedman tests were used to analyze effects over time. Alterations in hippocampal mRNA expression were evaluated using a one-way ANOVA followed by Post hoc tests using the Holm-Bonferroni method. The relationship of learning and stress responses were tested with two-tailed correlation analysis using Spearman's rank correlation coefficient. Differences were considered to be significant at $p \leq 0.05$. Effect size estimations are given by eta squared for variance analyses and Cohen's d for pairwise analyses.

Results

Fecal corticosterone metabolites concentrations increased in food restricted and training exposed mice

Feces were sampled during specific phases of the touchscreen protocol: before experimental onset (FCM_1), during food restriction only (FCM_2), and during progressive stages of training (FCM_3, FCM_4, FCM_5) (Fig. 1). Initial FCM concentrations did not show significant differences between treatment groups ($H(2) = 2.17$, $p = 0.33$, $\eta^2 = 0.06$). After the onset of food restriction, FCM_2 concentrations increased in both food restricted groups as compared to the control group ($H(2) = 9.95$, $p < 0.01$, $\eta^2 = 0.26$; handling vs. food restricted $p = 0.07$, $d = 0.94$, handling vs. touchscreen-trained $p < 0.01$, $d = 1.27$, food restricted vs. touchscreen-trained $p = 0.54$, $d = 0.24$). Moreover, FCM concentrations after the onset of the training phase (FCM_3) also revealed significant alterations between the treatment groups ($H(2) = 10.356$, $p < 0.01$, $\eta^2 = 0.27$; handling vs. food restricted $p = 0.48$, $d = 0.33$, handling vs. touchscreen-trained $p = 0.01$, $d = 1.05$, food restricted vs. touchscreen-trained $p = 0.07$, $d = 0.79$). In contrast, later samples, at 6 and 10 weeks following the beginning of training, did not show any differences between the three groups (FCM_4: $H(2) = 2.01$, $p = 0.36$, $\eta^2 = 0.05$; FCM_5: $H(2) = 0.60$, $p = 0.73$, $\eta^2 = 0.02$).

Although FCM concentrations changed significantly between FCM_1 and FCM_2 in both food-restricted groups (two-tailed Wilcoxon: restriction $n = 8$, $p = 0.01$; exposition $n = 24$, $p < 0.01$), a Friedman test revealed a significant alteration from FCM_1 to FCM_5 in the touchscreen-trained group only ($n = 23$, $\chi^2 = 32.27$, $p < 0.01$), with no difference in the food-restricted group ($n = 8$, $\chi^2 = 6.00$, $p = 0.199$). Handling controls did not show significant changes from FCM_1 to FCM_5 (Friedman: $n = 7$, $\chi^2 = 5.14$, $p = 0.27$).

Neural activity in hippocampus is elevated in restricted mice

Hippocampal brain samples were collected either 90 min or 24 h after the completion of the last of the daily protocols in all groups. The 90 min interval gives insight into the acute effects of each manipulation on gene induction, while the 24 h indicates long-term effects prior to the start of the subsequent treatment. In fact, the latter time point may also indicate anticipation of the next treatment. All results are presented relative to handling group mRNA expression levels.

Analyses of IEG gene expression levels revealed significant differences between groups for Arc $F(4,37) = 3.669$, $p = 0.01$, $\eta^2 = 0.31$, BDNF: $F(4,38) = 7.302$, $p < 0.00$, $\eta^2 = 0.46$, c-Fos: $F(4,37) = 3.132$, $p = 0.02$, $\eta^2 = 0.28$ and zif268: $F(4,37) = 4.488$, $p < 0.01$, $\eta^2 = 0.35$ (Fig. 2). Post hoc analyses revealed a significant increase of Arc expression levels in samples of mice sacrificed 90 min after completion of the touchscreen task, relative to controls ($p = 0.04$, $d = 1.13$), and a trend

toward lower Arc expression in touchscreen-exposed mice at the 24 h sampling interval ($p = 0.06$; $d = 0.41$; Fig. 2A).

BDNF mRNA levels were significantly different as a function of treatment and timing (Fig. 2B). While food-restricted mice given food 90 min in advance of tissue sampling displayed unaltered expression levels compared to handling controls, the 24 h food-restricted group ($p = 0.02$, $d = 1.90$) and touchscreen-trained 90 min group ($p < 0.01$, $d = 1.95$) displayed higher levels of BDNF relative to controls, with a trend toward higher levels in the touchscreen-trained 24 h group ($p = 0.08$, $d = 1.18$). Comparisons between the food restricted 90 min group and all other groups except the handling group revealed significantly lower BDNF expression relative to food restricted 24 h ($p = 0.01$, $d = 2.49$), touchscreen-trained 90 min ($p < 0.01$; $d = 2.47$), and touchscreen-trained 24 h ($p = 0.04$; $d = 1.64$). Comparisons of treatment and timing revealed significant alterations in both c-Fos and zif268 expression between the handling and the touchscreen-trained 90 min group (c-Fos: $p = 0.02$, $d = 1.86$, zif268: $p < 0.01$, $d = 1.85$; Fig. 2C and D).

Serum corticosterone concentrations were elevated in touchscreen-trained mice before training

To compare levels of mRNA expression with acute stress, serum samples derived from trunk blood were analyzed. Again, the samples were taken at 90 min or 24 h after the last treatment in all groups.

Corticosterone concentrations differed as a function of treatment ($H(4) = 27.11$, $p < 0.00$; $\eta^2 = 0.70$ Fig. 3). Post-hoc tests showed significantly higher corticosterone in the touchscreen-trained 24 h group relative to the handling group ($p < 0.001$; $d = 5.32$), touchscreen-trained 90 min group ($p < 0.01$, $d = 4.50$) and food restricted 90 min ($p = 0.04$, $d = 5.03$), and a trend toward higher corticosterone relative to the food restricted 24 h group ($p = 0.08$, $d = 4.01$). All other comparisons revealed no significant differences.

Corticosterone concentration correlates with better performance after the initial habituation phase

FCM concentrations of trained mice were compared to behavioral measures at three different stages of training in order to relate task learning to stress levels. During habituation (FCM_3) the FCM concentrations was not different based on the days needed to reach the progression criterion, neither did the FCM concentration correlate to the percentage of correct responses (see Fig. 4). In the other two phases however an association of FCM concentration and learning performance was observed.

During the 'cue-position training' (FCM_4) FCM concentrations correlated to learning related parameters: percentage of correct response $r = -0.469$, $p = 0.028$, number of correction trial $r = -0.517$, $p = 0.014$ and the task-unspecific performance estimate of number of days spent in 'cue-position training' $r = -0.502$, $p = 0.017$. Higher FCM concentrations corresponded with better performance. However, the progression within the paradigm, here apparent by the number of days in 'cue-position training' is not correlated to other learning performance parameters.

Similar to the findings from FCM_4, the FCM concentrations on the last sample time point reveal a relationship with learning parameters. Mice which progressed further within the paradigm had higher FCM concentrations (Spearman's rank $r = 0.529$, $p = 0.009$). This effect is visualized by different color marking in Fig. 4. Oneway ANOVA according to phases revealed significant differences between the concentration of mice in the three different stages $F(2.22) = 4.312$ $p = 0.028$ (Holm-Bonferroni corrected post-Hoc: cue-position vs. test $p = 0.056$; cue-position vs. final training $p = 0.066$). Finally, a trend for correspondence of correct performance and FCM concentrations was observed (Spearman's rank $r = 0.379$, $p = 0.075$).
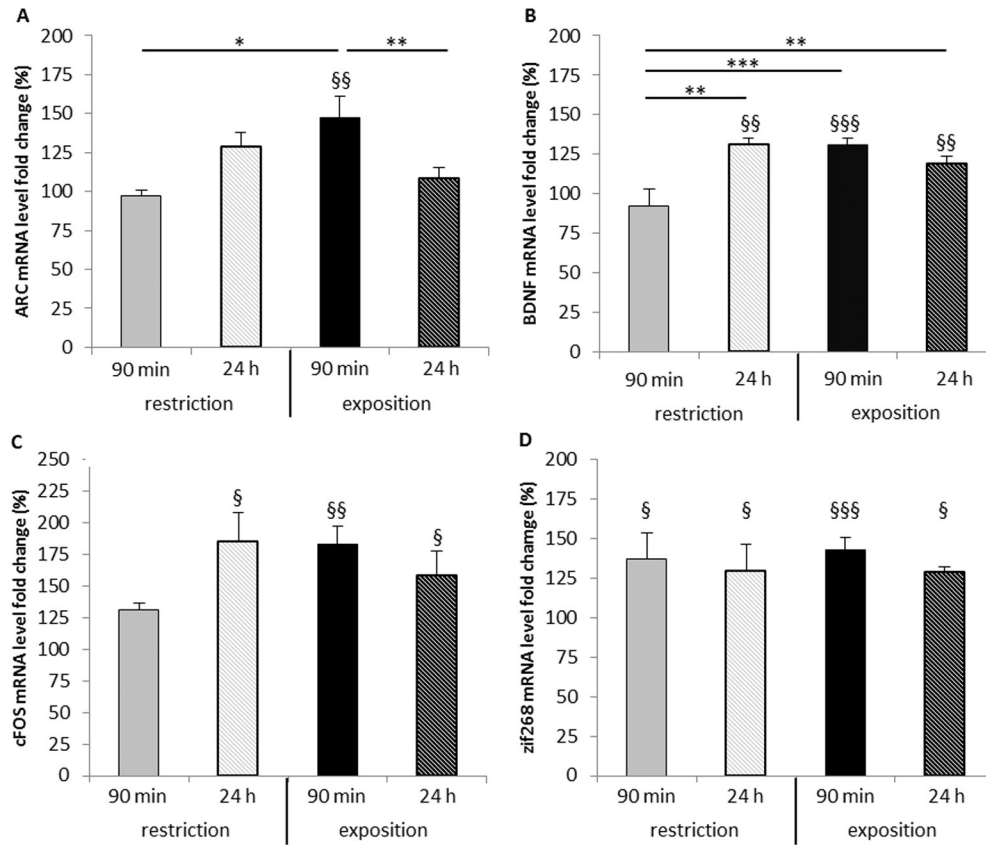
Fig. 2. Hippocampal samples revealed elevated expression of markers for neural activity. A) ARC levels were selectively higher in the touchscreen-trained group 90 min after task performance B BDNF levels were unaltered in satiated food restricted mice, but increased in hungry food-restricted controls; additionally, the touchscreen-trained group displayed higher levels, independent of satiation level and task performance interval, C and D c-Fos and zif268 expression was significantly higher after recent task performance. Data are expressed as mean ± SEM of mRNA level changes relative to handling controls (≙100%). plain bars: 90 min interval, striped bars: 24 h interval; yellow: restriction group, red: exposure group; comparison to handling group (above bar) $p^§ < 0.05$ $p^{§§} < 0.01$, $p^t = 0.06$, comparison to food restricted 90 min $p^* < 0.05$, $p^{**} < 0.011$, comparison touchscreen-trained 90 min vs 24 h: $p^t = 0.08$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Correct performance correlates with higher BDNF and higher c-Fos expression

The IEG expression levels were measured from brain samples taken 90 min or 24 h after the last test performance. IEG expression
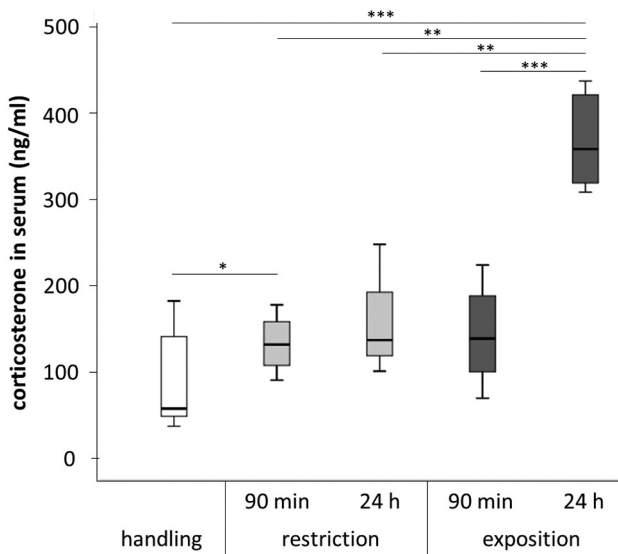

Fig. 3. Serum corticosterone concentrations were significantly higher in touchscreen-trained mice 24 h after last task performance. $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$, $p^t = 0.08$.

levels were not significantly altered between the two interval groups (Fig. 3B and C). When comparing the percentage of correct responses to IEG expression in the 90 min interval no correlation became obvious. However, in the 24 h interval mice with higher levels of BDNF or c-Fos exhibit more correct responses (BDNF vs. % correct: $r = 0.625$ $p = 0.030$ $n = 12$; c-Fos vs. % correct: $r = 0.664$ $p = 0.018$ $n = 12$) (Fig. 5).

Discussion

As the touchscreen system in mice has not been extensively characterized with regard to its potential stress effects, we analyzed alterations in FCMs, IEGs, and serum corticosterone concentrations as a function of different components of a specific touchscreen task and at different time points, including food restriction and performance at different intervals. These findings provide some insight into factors that should be considered with regard to the translational validity of touchscreen-based systems.

Food deprivation leads to fecal corticosterone metabolites elevation and quick adaption

We investigated FCM concentrations in mice exposed to daily handling only, to food restriction and daily handling, or to touchscreen training and food restriction and daily handling, respectively. One week after food restriction onset, both food-restricted groups showed an increase in FCM concentrations compared to handling controls, though this increase only reached significance in the touchscreen group. Interestingly, FCM concentrations of the food restriction group
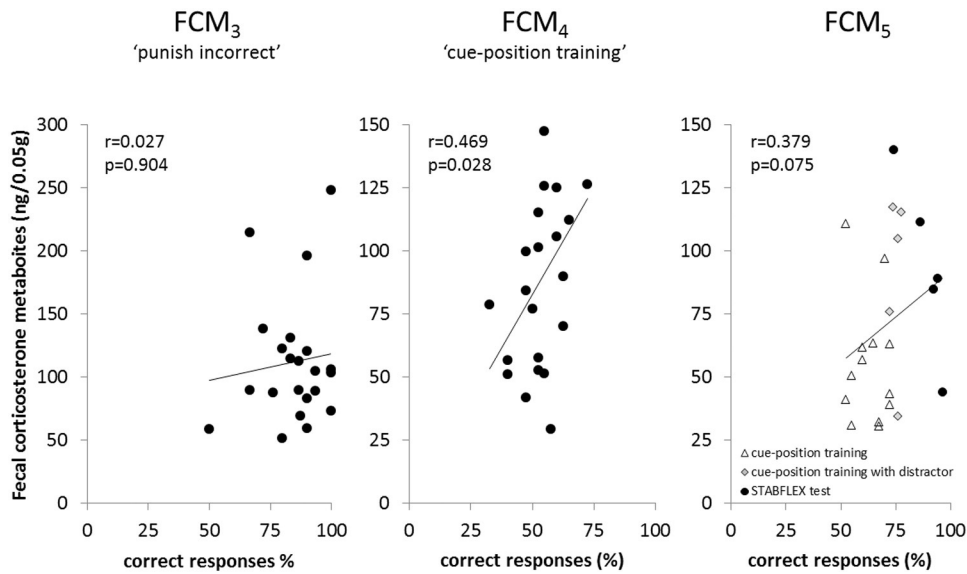
Fig. 4. Correlation of corticosterone concentrations and task performance of touchscreen trained mice during different learning stages of the STABFLEX paradigm. Initial concentrations on FCM3 do not show association of behavioral outcome within the paradigm; while later stages (FCM$_4$ and FCM$_5$) reveal a positive correlation of magnitude of stress response and correct performance. In FCM$_5$ subjects were in the last three stages of the paradigm, with cue-position training being the least and STABFLEX test the most progressed stage. Presented correlations are based on Spearman's rank test.

decreased to baseline at the next measurement two weeks later, indicating an adaptation to the new restricted feeding scheme.

Touchscreen training leads to a sustained fecal corticosterone metabolites increase with later-onset adjustments

FCM concentrations in the touchscreen-trained group remained at similar elevated levels as caused by food restriction alone at the measurement three weeks after onset of food deprivation. This gives clear evidence of a sustained effect of the touchscreen task on HPA axis activity. As the experiment progressed, FCM concentrations in the touchscreen-trained group converged back to those similar to the food-restricted group and handling controls, suggesting an adaptive effect, and were of comparable magnitude to those of food restricted mice.

Stress, fecal corticosterone metabolite concentrations and touchscreen exposure

The assessment of glucocorticoid levels represents a physiological measure that can be used to interpret the emotional state of a subject at a precise time point. It is important to emphasize that different affective states can lead to the same physiological response. For example, fear induction and sexual activity both lead to increased cortisol levels (Paul et al., 2005). Therefore the association of glucocorticoid levels and stress should always be considered carefully. Stress, as a response to internal and external stimuli, enables a subject to cope with demanding situations. According to the 'allostasis concept,' stress is a regulatory process to stabilize a dynamic system to external challenges (Crofton et al., 2015). Stressful conditions might contribute to adaptive processes, leading to resilience toward future stressors, and can thus be
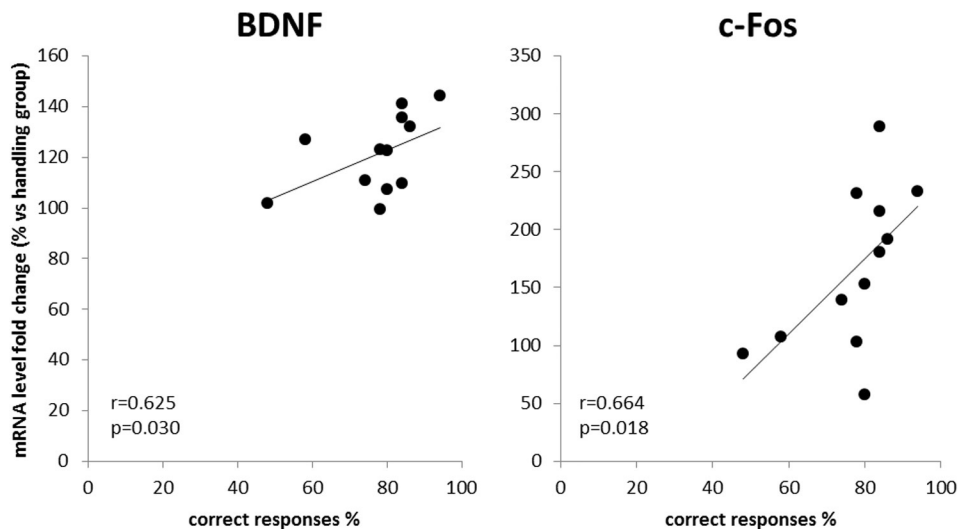

Fig. 5. Correlation of IEG expression levels and task performance of touchscreen trained mice 24 h after last training exposition. Higher IEG expression is related to better performance rated in percentage of correct responses. Presented correlations are based on Spearman's rank test.

considered beneficial. Alternatively, they can lead to maladaptive outcomes, which may be associated with the development of psychiatric disorders (Karatsoreos and McEwen, 2013a, 2013b).

Similar to findings in chronic stress paradigms, daily training in touchscreen-chambers leads to initial elevation of corticosteroids concentrations, which will eventually attenuate (Dienstbier, 1989; Fox et al., 2006; Lyons et al., 2009). In the current study, FCM concentrations were elevated as a consequence of food restriction onset and decreased quickly in the food restricted control group, as the mice adapted to the new feeding scheme. Adaptation toward touchscreen training took longer, but again the adaptive ability of the mice became obvious with decreasing FCM concentrations.

Administration of corticosterone leads to a decrease in neurogenesis (Murray et al., 2008). However, unlike exogenous increase of corticosterone, endogenous corticosterone elevation not necessarily shares this effect. Behavioral activities e.g. voluntary running can lead to higher levels of excreted corticosterone metabolites similar to the touchscreen training evoked effect presented here. As described by Fuss et al. (2010), physical exercise concurrently induces a significant increase of hippocampal neurogenesis and BDNF expression. Other studies showed a link between increased corticosterone concentrations and elevated hippocampal neurogenesis due to learning engagement and environmental enrichment (Kannangara et al., 2009; Leuner et al., 2004). Our results go in line with these findings, as induction of IEG expression was observed due to training (Fig. 2).

Mice learn to cope with the limitation of food and repeated exposure to the touchscreen training. Touchscreen-based training itself could also act similar to the inoculation stress that occurs during environmental enrichment (Crofton et al. (2015)). The animal is repeatedly exposed to a challenging environment, where it learns step-by-step the correct response for the task. This includes the staggered presentation of novel stimuli and may be similar to the novelty experienced in environmental enrichment protocols. Since the daily procedure follows a structured time schedule, the processes are not unpredictable, a feature of many psychiatric animal models, such as learned helplessness and chronic mild stress.

Anticipation of task performance evokes elevated corticosterone concentrations in serum

Increased corticosterone concentrations are likely related to arousal in the touchscreen trained group 24 h after their last performance. According to the daily schedule those mice are presumably expecting the introduction into the testing chamber for the next training session.

Due to the feeding scheme, the mice received their food only after the execution of the behavioral task. This means that both food restricted groups were considered to be hungry due to the long interval of last food supply. However, the food-restricted control group did not display increased corticosterone concentrations at the 24 h time point. Mice adapted to the restrictive feeding scheme beforehand and thus hunger was not the cause for their high corticosterone concentration. It neither was a general training-associated effect. Recently trained mice (90 min group) did not show alterations of corticosterone concentrations. Instead, anticipation of the expected rewards for correct responses during training or even the task itself could account for this excitation. The mice may also benefit from the cognitive challenge provided by the task and environment.

Food restriction and touchscreen exposure influence neuronal activity in the hippocampus

We further investigated the impact of long-term touchscreen-based training and associated food restriction hippocampal markers related to stress and learning, including immediate-early genes with regulatory transcription factor (c-Fos, zif268) and effector (Arc, BDNF) functions. These genes have been demonstrated to be activity-dependent in

hippocampal neurons and play a role in memory consolidation and synaptic plasticity (Guzowski, 2002) and stress (Molteni et al., 2010, 2008). Alterations in the expression of these genes can therefore be evaluated either as stress- and/or learning induced. To distinguish a satiation effect from training effects, we investigated alterations in mRNA level expression in hippocampal samples of the training exposed and food restricted groups relative to handling controls prior to and following food exposure. In addition, acute changes (90 min) were compared to changes mediated after 24 h. Interestingly, mRNA levels of all IEGs investigated revealed task-specific induction in the hippocampus in recently exposed mice (90 min), indicating neuronal activity associated with learning and memory.

The expression of zif268 and c-Fos was significantly higher in hippocampal samples of training-exposed mice that recently performed the task, relative to handling controls. As mice were expected to learn, consolidate and retrieve the task during training, this effect was anticipated, as c-Fos and zif268 are associated with learning and memory. This finding suggests that at the time point examined, expression was likely mediated by recent learning during training. Arc expression level changes were similar, in that recently trained mice showed higher expression levels relative to handling controls, and thus likely related to learning. IEGs are known to be induced by synaptic activity and to play a role in the maintenance of synaptic potentiation and long-term consolidation of memory (Fleischmann et al., 2003), and thus induction due to recent training appears reasonable. Furthermore, the satiation level of mice did not appear to influence c-Fos, zif268 or ARC expression.

An effect of satiation level was only observed for levels of BDNF, a marker of neurogenesis and learning and previously demonstrated to be coupled to stress-evoked pathways (Calabrese et al., 2014; Cattaneo and Riva, 2015). Satiated mice in the food restricted group did not show alterations in BDNF relative to handling controls, while BDNF levels in non-satiated mice were significantly elevated, indicating increased expression due to hunger. Thus, long-term restriction itself did not lead to a general elevation in BDNF. The elevation in satiated, touchscreen-exposed mice at the 90 min interval suggests training-induced neuronal activation in the hippocampus, as hunger can be excluded as a factor. This learning effect was likely temporary, as expression levels of BDNF in the 24 h touchscreen-trained group did not demonstrate an accumulative effect of training and hunger, and only exhibited a trend toward elevation relative to handling controls. Additionally, the satiated food restriction demonstrated a similar alteration in BDNF expression levels to all other treatment groups, with no cumulative effect demonstrated.

Stress response and learning performance

FCM concentration and data from the touchscreen behavioral task are linked in later phases of the paradigm. This is shown for both non-habituation samples (FCM_4 and FCM_5), where higher corticosterone concentrations corresponded with higher accuracy of responses. The direction of their correspondence is unknown, since both factors influence each other. On one hand higher stress levels could occur due to higher anticipation of the training and therefore lead to better results in more motivated mice, while on the other hand mice showing less adaptation do not benefit from enhanced corticosterone concentrations, as they lead to better performance. Interestingly, the progression within the paradigm measured by days on the training phase is not correlated to other learning performance parameters, e.g. percentage of correct responses or number of correction trials indicating that each mouse has an individual learning pace. However, they all improved performance, with some learning quicker than others. During the habituation the variety of progression was less pronounced. On sampling time point FCM3 almost all animals were in the same phase and classification for behavioral analysis had to be based on the forthcoming into the next phase rather than past differences. All trained mice displayed high prolonged FCM3 concentrations compared to food restricted controls. This could

be considered a ceiling effect of stress response and be a reason for similar performance, since all mice could similarly benefit from high corticosterone concentrations. To some extent both, the stress system adaptation leading to alloyed performance and the active triggering of corticosterone concentrations due to task anticipation might affect learning performance.

Mice with higher BDNF and c-Fos levels might benefit from anticipation evoked serum corticosterone concentration elevation

In order to interpret the relation of IEG expression and task performance, behavioral results of the final test session were compared to changes in gene expression grouped in the two intervals of final training performance (90 min or 24 h). The correctness in performance and IEG expression levels did not differ between the groups even though they displayed significant differences in serum corticosterone concentrations. This finding suggests that more corticosterone does not lead to better performance within the task nor altered expression of IEGs. However, while the 90 min group did not show any relationship between performance measures and IEG expression, the 24 h group revealed higher accuracy in mice with higher BDNF and *c*-Fos expression. This group-specific effect co-occurs with the explicitly high serum corticosterone concentration of mice in the 24 h subgroup. Since corticosterone concentrations themselves did not reveal significant correlations with performance outcome anticipating mice only appear to benefit from high BDNF and c-Fos expression.

Conclusion and outlook

Touchscreen-based behavioral testing is a relatively new technique used to gain insight into fundamental cognitive processes and their underlying mechanisms in rodent animal models.

The reported alterations in the stress system are not necessarily limited to touchscreen-based tasks but could also be relevant for any enduring operant chamber tasks. The presumption that availability of a touchscreen within the chamber triggers these substantial effects might be dispensable. Instead it seems possible that rather the promoted task and the sustained training induce changes in the stress response. An additional control group to check for the effect of chamber exposition without task application could provide evidence for this possibility. We considered thoroughly the implementation of these controls and traded off the relevance of collected data with animal welfare interests and decided to preferably reduce the number of laboratory animals. In comparison to other operant systems animals respond directly to the presented stimulus by touching the screen with their nose. Nosepoking appears to be a more natural exploratory behavior than e.g. pressing levers. For this reason touchscreen-based training could reside to be more intuitive and perhaps even less stressful during the habituation phase.

Here, we aimed to use a general approach to investigate the basic effects of daily exposition and food restriction on the animals. However, the touchscreen setup allows many different paradigms focusing on cognitive functions. Of course, the provided task could also influence stress response and IEG expression. Potentially, tasks used to assess different cognitive domains e.g. the 5-choice serial reaction time task testing attention or the trial-unique task analyzing working memory may lead to different effects on stress response or IEG expression. This task-specificity could reveal impact of individual IEGs on those functions and provide insight to underlying molecular mechanisms.

For motivation purposes, touchscreen-based training is commonly accompanied by food restriction. Here we show a number of changes in learning and stress-related indicators based on protocols associated with touchscreen-based training. These findings are an important beginning to determine whether the demands of touchscreen-based paradigm procedures and associated food restriction are stressors that should be considered when implementing new tasks.

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