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Active place avoidance is no more stressful than unreinforced exploration of a familiar environment

Edith Lesburguères¹, Fraser T. Sparks¹, Kally C. O'Reilly¹, and André A. Fenton^{1,2,*}

¹Center for Neural Science, New York University, New York

²Department of Physiology, SUNY Downstate Medical Center, Brooklyn, NY

Abstract

Training in the active place avoidance task changes hippocampus synaptic function, the dynamics of hippocampus local field potentials, place cell discharge, and active place avoidance memory is maintained by persistent PKM ζ activity. The extent to which these changes reflect memory processes and/or stress responses is unknown. We designed a study to assess stress within the active place avoidance task by measuring serum corticosterone (CORT) at different stages of training. CORT levels did not differ between trained mice that learned to avoid the location of the mild foot shock, and untrained no-shock controls exposed to the same environment for the same amount of time. Yoked mice, that received unavoidable shocks in the same time sequence as the trained mice, had significantly higher CORT levels than mice in the trained and no-shock groups after the first trial. This increase in CORT disappeared by the fourth trial the following day, and levels of CORT for all groups matched that of home cage controls. The data demonstrate that place avoidance training is no more stressful than experiencing a familiar environment. We conclude that changes in neural function as a result of active place avoidance training are likely to reflect learning and memory processes rather than stress.

Keywords

Learning; memory; navigation; spatial cognition; stress

Introduction

How the brain stores information is a fundamental, unanswered question that has been most productively assessed by manipulating and studying changes in neurobiological variables related to conditioned behavior. In these experiments, learning is typically motivated by aversive or appetitive conditioning, both of which may induce a stress response that can confound the specificity of the learning or memory-related findings (Korz and Frey, 2003; Ahmed et al., 2006). For example, while water maze and conditioned fear tasks are popular and effective for inducing memories that can persist for weeks to months, they are also explicitly stressful (Harrison et al., 2009). Whereas object exploration tasks that are based on an unconditioned preference for novelty appear to be less stressful, these unreinforced

* Please address correspondence to: André A. Fenton, Center for Neural Science, New York University, 4 Washington Place, New York, NY 10003, afenton@nyu.edu, Phone: 212-992-6573, Fax: 212-995-4011.

memories are typically assessed after minutes to hours (Poucet, 1989; Mumby et al., 2002), limiting their utility for investigating the neurobiology of long-term memory, although memory across a day was recently demonstrated (Tsokas et al., 2016).

In an attempt to circumvent some of these issues, we developed an active place avoidance paradigm (see Stuchlik et al., 2013 for an historical review) to investigate the mechanisms of memory (Bures et al., 1997). Place avoidance tasks are rapidly learned and sensitive to hippocampus dysfunction (Cimadevilla et al., 2000; Cimadevilla et al., 2001b). The experience of mild foot shock in a particular location induces place avoidance memories that require persistent hippocampal LTP and causes changes in hippocampal synaptic network function that persist for at least a month (Cimadevilla et al., 2001b; Pastalkova et al., 2006; Burghardt et al., 2012; Kheirbek et al., 2013; Park et al., 2015; Pavlowsky et al., 2016; Radwan et al., 2016). Because stress is known to modify synaptic plasticity and memory-related biochemical signaling (Korz and Frey, 2003; Ahmed et al., 2006), it is of substantial importance to determine to what extent place avoidance training is stressful.

A total of 40 adult male mice (20 C57BL/6 and 20 with mixed genetic background of C57BL/6 and 129 evenly distributed across groups) were used, and all procedures were in accordance with protocols approved by the New York University Animal Welfare Committee that followed NIH guidelines for the care and use of animals in research.

During pretraining, all mice were familiarized to the rotating arena for 10 minutes of unreinforced exploration (Fig. 1A). Training to actively avoid the location of shock started on the following day. The mice were reinforced to avoid a constant current shock (0.2mA, 60 Hz, 500 ms) that was delivered whenever the mouse was detected in a 60° shock zone that was stationary in the room, despite the rotation of the arena (Cimadevilla et al., 2001a). To assess stress induced by the place avoidance training, we measured the CORT levels of independent groups of trained animals at two different time points of the protocol. The first time point was after the first training trial, during which the experience of shock was novel and thus the receipt of shock was the most frequent of the training protocol (1-trial trained group, n=5). In a second group of mice, CORT was measured at the second time point, 24 h later, after a fourth training trial (4-trials trained group, n=5). This is a time when place avoidance behavior is asymptotic (Fig. 1A). Note that on the first training day, the 4-trials trained group received 3 consecutive trials separated by a 90-minute intertrial interval during which the mice were returned to their home cage. For CORT assessment, the 4-trials trained group was given an additional fourth training trial on the following day to match the 1-trial trained group's time of the day.

Two control groups were used to assess the stress levels induced by either the exploration of the rotating arena alone or the exploration associated with the delivery of inescapable foot shocks, at both time points (Fig. 1A). The untrained groups (n = 5 per time point) were exposed to identical environmental conditions as the trained groups, except foot shocks were never delivered. The yoked groups (n = 5 per time point) were also exposed to the rotating arena but received foot shocks in random locations. The number of foot shocks and the time series that they were delivered were the same as for the corresponding trained mouse to which the mice were individually yoked. Animal location during arena exploration and

behavioral assessment of place avoidance (i.e., time to first entrance and number of entrances to the shock zone) were recorded using a PC-controlled video tracking system (Tracker, Bio-Signal group, Acton, MA) and analyzed using TrackAnalysis (Bio-Signal group, Acton, MA). Immediately after the first or fourth trial, the mice were taken to an adjacent procedure room, anesthetized by 3.5% isoflurane and decapitated to collect trunk blood. Blood collection was completed 3–5 minutes after the last behavioral trial.

Two additional control groups were used to estimate the CORT baseline and a strong CORT response to stress. The CORT levels of the different experimental groups were compared to the baseline levels measured in a home cage control group ($n = 5$) and to the elevated CORT level induced by an explicitly stressful experience (predator stress group, $n = 5$). Mice in the home cage control group were handled just like all animals and taken directly from their home cage to collect trunk blood. The stressful experience was exposure to a rat predator (Blanchard et al., 2001; Amaral et al., 2010). Each mouse in the predator stress group was placed in a small wire cage and exposed to two adult male rats for 10 minutes; immediately thereafter blood was collected for CORT analysis. All blood collection was conducted between 08:00 a.m. and 12:00 p.m. to avoid the circadian rise in CORT levels. Plasma CORT concentrations were measured by immunoassay (AssayPro, AssayMax) using triplicates for standards, controls and samples. The intra-assay and inter-assay coefficients of variation were both $< 4\%$.

Results

The trained mice rapidly learned to actively avoid the shock zone, as evidenced by a reduction in the number of entrances into the shock zone, the number of shocks that they received, and an increased latency to enter the shock zone (Fig. 1B, C). Two-way group $\times$ trial ANOVAs confirmed significant effects of the groups, trials and interactions (see Figure caption for details). Tukey-Kramer post-hoc tests confirmed that these effects were due to the trained mice expressing active place avoidance by decreasing the number of entrances and increasing the entrance latency into the shock zone, whereas the performance of the two behavioral control groups were equivalent and did not change across training.

Next, we measured the CORT levels for baseline and the elevated CORT response to the rat predator exposure. Predator stress increased CORT by 2–3 times the levels of the home cage control mice, (Fig. 1D; $t_9 = 5.21$, $p < 0.01$). We then evaluated the effects of place avoidance training, arena exploration and unavoidable shocks on the CORT levels after the first and fourth trials. Two-way group $\times$ trial ANOVA revealed significant effects of the groups and trials (see Figure caption). As shown in Fig. 1E, CORT was elevated in the yoked group relative to the others, but only on Trial 1, as might be expected given that the shocks are inescapable and substantially more prevalent on Trial 1 (approx. 10 shocks) than on Trial 4 (approx. 1 shock; Fig. 1B). One-tailed post-hoc t tests confirmed that CORT levels are elevated in the 1-trial yoked group relative to the other groups and that CORT levels on trial 4 were indistinguishable amongst the groups. One-tailed Dunnett's tests relative to the home cage controls confirmed elevated CORT levels in all of the 1-trial groups, but not in any of the 4-trials groups, suggesting that stress reduces with the experience of each type of training on the rotating arena. We were therefore curious to assess the Trial 1 elevations in

CORT compared to predator stress. After Trial 1, whereas CORT levels were significantly lower in the trained and untrained groups relative to the predator stress, CORT levels in the yoked group were indistinguishable from the mice exposed to predator stress. The levels of stress did not significantly correlate with the number of foot shocks that were received, when the groups were pooled ($df=18$, $r^2 = 0.16$), or when they were considered separately according to the training experience or the time point of the CORT assessment (range of r^2 values 0.005–0.37).

Discussion

We found that relative to home cage controls, the earliest stage of the place avoidance training induces moderately and transiently elevated CORT levels that are indistinguishable from exploration of the environment with no shock. The CORT levels were higher in the trained and untrained animals after the first trial compared to the home cage controls, but not after the fourth trial. While this pattern of observations suggests that initial place avoidance training is stressful, the evidence from the untrained controls and direct comparison with the predator stress group do not fully support this interpretation. The untrained controls are crucial for interpreting the data and are the most important comparison group. This is particularly true when trying to distinguish between a CORT increase due to place avoidance training *per se* and the change in CORT due to the mere experience of exploring an open field. Exploring an open field is the foundation of the active place avoidance assay, and almost all studies of spatial cognition in rodents. Importantly, we found that CORT levels in the trained and untrained groups could at no time be distinguished. Importantly, we cannot attribute the lack of difference between the trained and untrained groups to a failure of the CORT assay or a ceiling effect because we observed up to 3-fold increases in CORT relative to the home cage controls in the predator stress group, demonstrating efficacy of the assay. Because the peak CORT increase occurs 5 minutes after a 10-min exposure to a rat predator (Amaral et al., 2010), it is unlikely that the 3–5 min time point for taking the blood samples was too soon for detecting a strong CORT response in the positive control group or after a training trial, although we cannot exclude the possibility that numerical differences in the CORT increase would have been observed had we taken the blood samples at a different time point, or had we sampled another biochemical marker of stress. Additionally, we observed a strong CORT response in blood samples taken 3–5 min after the yoked experience of the 1-trial group, supporting the assertion that 3–5 min after the first training trial is sufficient to observe a strong CORT response in the 1-trial trained group as well. This elevation in CORT response in the 1-trial yoked group was indistinguishable from what was observed in the predator stress group. Importantly, soon after and during training is a relevant time point for many experimental designs that endeavor to assess mechanisms of memory using electrophysiological approaches (Whitlock et al., 2006; Park et al., 2015; Pavlowsky et al., 2016), although interest in molecular mechanisms could drive interest in later time points (Hsieh et al., 2016; Tsokas et al., 2016).

These observations strongly suggest that despite the use of the mild foot shock, place avoidance training is no more stressful than unreinforced exploration of a familiar environment. Using defecation in rats as an index of stress (Friedman and Ader, 1965), we arrived at a similar conclusion after finding that the shocks for place avoidance training do

not change defecation in the test environment any more than the fact of the rats simply being in the environment (Shen et al., 2010). The present findings indicate that the role of stress is negligible in the outcomes of active place avoidance experiments when comparisons to untrained controls are utilized.

We were motivated to investigate the role of stress in active place avoidance learning because the task is used to identify neurobiological correlates and mechanisms of memory, and like all other memory tasks, the neurobiological changes that are associated with memory formation could also be associated with confounding variables like motivation, sensation, motor activity, and stress, rather than memory itself. Virtually all behavior-based studies of learning and memory must deal with this confound and typically control experiments, such as the use of untrained and yoked controls, are used to account for the impact of confounding factors. However, the impact of confounds like stress is complex, hard to assess, and hard to control because it can interact with the variables of interest (Korz and Frey, 2003; Ahmed et al., 2006; Sajikumar et al., 2007). The present study provides an informative example: given that the CORT levels in the untrained and trained mice could not be distinguished, the more appropriate control for the experience of place avoidance training would be the “no-shock” untrained mice rather than the yoked mice. Despite having the identical physical experience as the trained mice, the yoked mice nonetheless had a different stress response. The only differences between the experiences of the trained and yoked mice are internal cognitive variables, like a sense of agency. While such cognitive variables are important, they are also inaccessible to direct measurement using behavior, but their potential importance has been well-documented as a source of stress that affects mechanisms of memory (Shors et al., 1989; Kim et al., 2007). Stress is a tricky confound to manage in other paradigms such as water maze, contextual fear, and hole board tasks like the Barnes maze that use stressful conditions to motivate the behavior of interest (Harrison et al., 2009; Kiyokawa et al., 2015). The present findings that stress is both minimal and indistinguishable in the trained and the untrained mice are encouraging because active place avoidance provides a robust memory paradigm that does not activate a stress response any greater than free exploration of an open environment, which is itself a staple of spatial learning and memory paradigms.

Although, we found that after asymptotic active place avoidance, the stress response in both the untrained animals and the trained animals was indistinguishable from that of home cage controls, it was nonetheless elevated, but not statistically. These results highlight the importance of using experimental designs with an appropriately stress-matched control group to properly interpret effects of stress on endpoint measures.

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FIGURE 1.

Active place avoidance paradigm, behavioral results and measures of plasma CORT levels.

(A) Schematic of the experimental design. (B) Trained mice learn to avoid the shock zone during the first trial, as indicated by a reduction in the number of entrances into the shock zone on Trial 1 (Group: $F_{2,12} = 7.98$, $p < 0.01$; Trial: $F_{1,12} = 12.25$, $p < 0.01$; Interaction: $F_{2,12} = 12.20$, $p < 0.01$). Mice trained for four trials further reduce the number of entrances into the shock zone (Group: $F_{2,12} = 11.36$, $p < 0.01$; Trial: $F_{4,9} = 21.46$, $p < 0.01$; Interaction: Wilk's lambda $F_{8,18} = 4.94$, $p < 0.01$). The number of shocks, received by both trained and yoked at each trial is indicated. (C) Mice display learning by increasing their latency to enter the shock zone for the first time across training sessions (Group: $F_{2,12} = 13.69$, $p < 0.01$; Trial: $F_{4,9} = 5.90$, $p < 0.05$; Interaction: $F_{8,18} = 2.72$, $p < 0.05$). (D) Predator stress causes elevated levels of plasma CORT (ng/ml) compared to the baseline levels of home cage mice ($t_9 = 5.21$, $p < 0.01$). (E) CORT levels induced by place avoidance training, arena exploration and unavoidable shocks (absolute values (ng/ml) on the left y-axis, and on the right y-axis expressed as percentage relative to the home cage control). (Group: $F_{1,24} = 11.15$, $p < 0.01$; Trial: $F_{2,24} = 4.38$, $p < 0.05$; Interaction: $F_{2,24} = 2.27$, $p > 0.1$). All values are presented as mean $\pm$ SEM.