Stress, emotional learning and AMPA receptors: from behavior to molecule

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Chapter III

Blocking Mineralocorticoid Receptors facilitates Reduction of Contextual Fear

(In preparation)

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Abstract

**Background**: Mineralocorticoid receptors (MRs) bind corticosteroids and are important for appraisal and response selection to emotional events. Blocking MRs during contextual fear learning impairs the expression of fear on the next day. Given that memories can become temporarily labile upon re-exposure to the conditioned stimulus (such as context or tone cue), we tested whether MR blockade during re-exposure also affects subsequent expression of fear.

**Methods**: Mice were trained in contextual or tone cue fear conditioning paradigms, by pairing mild foot shocks with a particular context or tone respectively. Twenty-four hours after training, context-conditioned animals were re-exposed to the context for 3 or 30 minutes (day 2); tone-conditioned animals were placed in a different context and re-exposed to one or six tones. Twenty-four hours (day 3) and one month later, freezing behavior to the aversive context / tone was scored again. MR blockade was achieved by giving spironolactone subcutaneously one hour before re-exposure on day 2.

**Results**: Spironolactone administered prior to brief context re-exposure potently reduced subsequent freezing behavior, lasting until day 3 but not one month later. Administration of spironolactone without retrieval of the context or after re-exposure on day 2 did not reduce freezing on day 3. Re-exposure to the context for 30 minutes on day 2 did not result in extinction learning during the re-exposure session, but freezing was largely reduced on day 3. This effect was not further enhanced by spironolactone. Administration of spironolactone prior to cue re-exposure on day 2 did not affect freezing behavior.

**Conclusions**: Blocking MRs prior to retrieval of contextual fear information strongly reduces freezing one day but not one month later.
Introduction

Memories for emotionally arousing and stressful events are generally well retained (McGaugh, 2000). An extreme example occurs in posttraumatic stress disorder (PTSD), which is characterized by vivid re-experience of traumatic events (Bremner et al., 2008; Cukor et al., 2009; Gersons and Carlier, 1992; Yehuda, 2002). Many clinical trials, often based on fundamental studies in rodents, have sought to identify methods of ameliorating the distressing symptoms. These methods include pharmacological approaches, but so far the evidence for effectiveness of such therapies has remained inconclusive (Cukor et al., 2009). Another class of treatment involves cognitive-behavioral therapy, which is based on principles of extinction learning (Foa et al., 1999). Extinction learning - a paradigm in which a conditioned stimulus (CS) is repeatedly retrieved in the absence of an unconditioned stimulus (US) – has proven to be effective in reducing acquired fear responses. However, the progressive reduction in the expression of fear after extinction learning is not permanent, because extinction does not directly modify the existing fear memory. Rather, extinction leads to the formation of a new memory that inhibits the activation of the initial trace (Ji and Maren, 2007; Myers and Davis, 2002). The efficacy of this inhibition appears to depend on spatial, sensory, and temporal variables (Bouton, 2004; Ji and Maren, 2007; Myers and Davis, 2002; Nader et al., 2000b).

Interestingly, studies over the past decade have shown that stored memories are rendered labile after being retrieved, and require de novo protein synthesis for reconsolidation (Nader et al., 2000b). Reconsolidation has been demonstrated in various tasks and species (Eisenberg et al., 2003; Nader et al., 2000a; Sangha et al., 2003), including humans (Kindt et al., 2009). The notion that stored memories can be turned into a labile state has opened new avenues to reduce excessive fears more permanently than the traditional extinction procedure (Monfils et al., 2009; Schiller et al., 2010),
including pharmacological intervention during re-exposure (Soeter and Kindt, 2010).

Corticosteroid hormones are released in high amounts from the adrenal glands upon exposure to fearful events. Here we examined whether targeting corticosteroid receptors is effective to modulate fearful memories during this labile phase. Corticosteroid hormones readily cross the blood brain barrier and bind to high affinity mineralocorticoid receptors (MRs) and lower affinity glucocorticoid receptors (GRs). GRs are known to be important for consolidation of fearful information (Oitzl and de Kloet, 1992; Roozendaal and McGaugh, 1996a, b, 1997a, b; Sandi and Rose, 1994b), while MRs seem critical for the appraisal of stressful information and response selection (Bitran et al., 1998; Oitzl and de Kloet, 1992; Sandi and Rose, 1994b). Recently, we reported that blocking MRs in rodents during acquisition of contextual fear conditioning effectively reduces the expression of fear one day later (Zhou et al., 2010b). In the current study we examined whether blocking MRs during re-exposure to the context or tone cue can also reduce fearful memories.

Materials and Methods

Animals

Male C57/BL6 mice (6-8 weeks old, Harlan, The Netherlands) were housed (2 mice per cage) for at least one week after arrival. All animals were kept on a light/dark cycle of 12 h (lights on at 8 a.m.; humidity 55 % ± 15; room temperature kept at 22 °C ± 2) and food and water were given without restriction. The experiments (training, memory retrieval and testing) were performed between 8:30-11:30 a.m. and approved by the local Animal Ethics Committee of the University of Amsterdam.

Drugs and treatments

The mineralocorticoid receptor antagonist spironolactone (Sigma, 50mg/kg)
or vehicle (VEH, propylene glycol) were injected subcutaneously one hour before the retrieval of contextual or tone-cued information (i.e. twenty three hours after training) or immediately after retrieval. The dosage and drug delivery route were chosen based on previous studies in which MR mediated effects were effectively blocked by the administration of MR antagonist (Herman and Spencer, 1998; Kumar et al., 2007; Pugh et al., 1997a).

**Contextual Fear Conditioning**

Animals were trained in a fear conditioning chamber (Context A, W x L x H: 30 cm x 24 cm x 26 cm) that contained a grid floor with 37 stainless steel rods and was connected to a shock generator and sound generator (Med-Farm LION-ELD) developed in-house. During training, one animal at one time was placed into context A. After three minutes of free exploration, three foot shocks (2 seconds, 0.4 mA) were delivered with an interval of 90 seconds. Sixty seconds after the end of the last foot shock, the mouse was placed back into its home cage. Freezing behavior, defined as no body movements except those related to respiration, was determined every 2 seconds throughout the experiment (Zhou et al., 2010b; Zhou et al., 2009). Twenty three hours later (on day 2), mice were injected subcutaneously with either spironolactone or VEH and returned to their homecages. One hour later, one animal at one time was placed in context A for either 3 min. or 30 min. without receiving any foot shock, presumably initiating reconsolidation and extinction respectively based on previous studies (Eisenberg et al., 2003; Pedreira and Maldonado, 2003; Suzuki et al., 2004). Freezing behavior was scored throughout these periods. Twenty four hours later (day 3; 48 hours after training) one animal at a time was placed in context A for 3 minutes without receiving any foot shock and freezing behavior was scored. At 30 days after training one mouse at a time was returned to the conditioning chamber (context A) for 3 minutes to examine spontaneous
recovery of freezing behavior.

In separate experiments, animals received an injection with spironolactone or VEH at twenty three hours after training without retrieval of contextual information. In a third series, spironolactone or VEH were injected to animals immediately after retrieval of information. Memory retention was examined in both experiments one day (day 3) and one month later.

**Tone-cued Fear Conditioning**

All animals were handled for three days and placed for 20 minutes /day in context B which had the same size as context A, but different contextual background (odor, texture and color). During training, one animal at a time was placed into context A. After three minutes of free exploration, the animal was exposed to a tone (100 dB, 2.8 kHz) that lasted for 30 seconds and co-terminated with a mild foot shock (2 seconds, 0.4 mA, Zhou et al., 2010). Thirty seconds later, the animal was placed back in its home cage. Freezing behavior was scored throughout the experiment. Twenty three hours later (day 2), animals were injected subcutaneously with either spironolactone or VEH and returned to the home cage. One hour later, one animal at a time was placed in context B. After 3 minutes of free exploration, the animal was exposed to one tone for 30 seconds (presumably initiating reconsolidation) or to 6 tones repetitively with an inter-tone interval of 3 minutes (presumably initiating extinction) without exposure to any foot shock (8). Thirty seconds after the last tone, the animal was returned to its home cage. Freezing behavior was scored throughout the experiment. Twenty four hours later (on day 3, i.e. 48 hours after training) as well as thirty days after training, the animals were placed back in context B; after three minutes of free exploration, animals were exposed to one tone (100 dB, 2.8 kHz) for 30 sec. Thirty seconds later, mice were returned to their home cages.
Statistical Analysis

Freezing behavior is expressed as percentage of freezing time versus total testing time. All results are presented as mean ± SEM. Data was analyzed with repeated measures ANOVA, with time as a repeated measure and drug treatment as between-subject factor. Significance over time was followed up by within-subject contrasts or unpaired t-test. P values smaller than 0.05 were considered to be significantly different.

Results

Contextual fear conditioning

During training all animals displayed a progressive increase in freezing behavior (repeated measures ANOVA, F_{3, 108}=29.47, P<0.01; data not shown). Importantly, freezing behavior between the groups that were treated with vehicle or spironolactone 23 hours later was comparable. One hour later freezing behavior to the same context was measured (memory retrieval; day 2) and then compared to that measured one day later (memory retention; day 3) or 1 month later. In the first series of experiments we tested the effect of drug treatment prior to a brief (3 minutes) re-exposure to the aversive context (Figure 1). Repeated measures ANOVA showed a main effect of time (F_{2, 32}=38.84, P<0.01). Follow-up contrasts revealed that compared to day 2 - animals displayed significantly less freezing both on day 3 (Figure 1B, P<0.01) and one month later (Figure 1C, P<0.01). We observed a spontaneous recovery of the fear response, as is indicated by more freezing at 1 month than at day 3 (P<0.05). Furthermore, a main effect of treatment (F_{1, 16}=9.92, P<0.01) suggested that mice treated with spironolactone showed less freezing. Planned post-hoc comparisons confirmed that treatment with spironolactone one hour before retrieval of contextual fearful information reduced freezing behavior during the retrieval trial, compared to vehicle treated mice (Figure 1A, t_{16}=3.46, P<0.01) as well as 24 h later, on day 3 (Figure 1B, t_{16}=2.61, P<0.05); however, there was no
significant treatment effect left after 1 month (Figure 1C, P>0.05). There was no significant interaction between time and treatment (F_{2, 32}=0.49, P>0.05). These results show that spironolactone, when applied before retrieval of contextual information reduces fear expression, but this effect disappeared at one month.

**Figure 1. Administration of spironolactone before brief retrieval reduces subsequent contextual fear.** A) Treating mice with spironolactone one hour before brief retrieval (3 minutes) of contextual fearful information (on day 2) reduced freezing behavior during the retrieval trial when compared to vehicle treated mice. B) Twenty four hours later (day 3) both vehicle and spironolactone treated animals displayed significantly less freezing when compared to the retrieval performance. Spironolactone treated mice showed less freezing relative to vehicle treated animals. C) One month later, both vehicle and spironolactone treated mice displayed less freezing when compared to the retrieval performance at 24 hours after training. ## reflects P<0.01 when compared to retrieval by within-subject contrast; * and ** reflect P<0.05 and P<0.01 when compared to vehicle by unpaired t-test (n=9 for both vehicle and spironolactone treatment).

In a second series of experiments spironolactone was applied 23 hours after training, but now in the absence of re-exposure to the fearful
context. No main effect of time ($F_{1, 10}=4.19$, $P>0.05$) or spironolactone treatment ($F_{1, 10}=0.44$, $P>0.05$) was observed, nor a time x treatment interaction ($F_{1,10}=0.26$, $P>0.05$; Figure 2). This suggests that spironolactone is only effective when combined with a labile period induced by retrieval. If spironolactone was administered immediately after re-exposure, there also was no main effect of time ($F_{2, 20}=2.73$, $P>0.05$), treatment ($F_{1, 10}=0.21$, $P>0.05$) nor any interaction between these two parameters ($F_{2, 20}=0.32$, $P>0.05$; Figure 3). This implies that spironolactone is only effective in reducing the expression of contextual fearful memories when administered prior to and not after re-exposure.

**Figure 2. Administration of spironolactone without retrieval does not reduce contextual fear.** Spironolactone was injected 23 hours after training in the absence of re-exposure to the fearful context. No effect of spironolactone on contextual memory retention was found A) twenty four hours (day 3) or B) one month later (repeated measures ANOVA, $P>0.05$) (n=6 per group).

**Figure 3. Administration of spironolactone immediately after brief retrieval**
does not reduce contextual fear. Freezing behavior during brief retrieval was comparable between the two groups A). Treating animals with spironolactone immediately after brief re-exposure did not affect contextual memory retention B) at twenty four hours (day 3) or C) one month after retrieval (repeated measures ANOVA, P>0.05) (n=6 per group).

Placing animals in the stressful training context for 3 minutes is thought to initiate reconsolidation, whereas placement in this context for 30 minutes supposedly promotes extinction (Mamiya et al., 2009; Suzuki et al., 2004). To specifically study the effect of spironolactone treatment on the latter, a separate batch of animals was exposed to the aversive context for 30 minutes, 24 hours after training. Re-exposure for 30 minutes resulted in considerable levels of freezing behavior (Figure 4A). Interestingly, freezing behavior stayed remarkably stable over the entire 30 minutes (within-group effect, F$_{9, 135}=1.28$, P>0.05). Animals treated with spironolactone displayed less freezing during the first 3 minutes of this period (Figure 4A, t$_{15}=2.26$, P<0.05), but this subsequently became indistinguishable from controls. We determined the freezing behavior during the final 3 minutes of the 30-minutes retrieval period and compared it with freezing measured on day 3 and one month later. Repeated measures ANOVA supported a main effect of time (F$_{2, 30}=27.47$, P<0.01). The follow-up contrast analysis showed that compared to the final 3 minutes during retrieval, animals displayed significantly less freezing both one day (Figure 4B, P<0.01) and one month later (Figure 4C, P<0.01), with one month testing yielding higher values than one day after treatment (P<0.01). Contrary to what we found when spironolactone was administered prior to a 3-minutes retrieval session, there was no main effect of treatment when animals were re-exposed to the context for 30 minutes (F$_{1, 15}=0.08$, P>0.05), nor a time x treatment interaction (F$_{2, 30}=0.98$, P>0.05).
Figure 4. Administration of spironolactone before long retrieval does not reduce contextual fear. A) The overall freezing behavior during 30-minute re-exposure was not affected by spironolactone treatment (repeated measures ANOVA, P>0.05) except for the first 3 minutes (unpaired t-test, *P<0.05). B) Twenty four hours and C) one month later, freezing behavior was significantly reduced when compared to the last 3 minutes of freezing behavior of 30-minute retrieval (within-subject contrast, ##P<0.01). No differences were found between the experimental groups (repeated measures ANOVA, P>0.05) (n=9 for vehicle-treated group; n=8 for spironolactone-treated group).

Tone-cue fear conditioning

We next tested if spironolactone modifies the expression of fear when the drug is administered prior to retrieval of tone-cue memories. During training trials in context A, freezing behavior during the tone and immediately after the tone was comparable between the groups that were treated with vehicle or spironolactone 23 hours later (data not shown). One day after training, both vehicle and spironolactone treated mice displayed high levels of freezing in response to one tone in context B (Figure 5A). The analysis on data obtained on day 2, day 3 and one month later indicated a main effect of time (F2, 24=31.56, P<0.01). The follow-up contrast showed a significant reduction in freezing behavior one day (P<0.01) and one month (P<0.01)
after drug treatment, compared to the retrieval session on day 2. Freezing behavior one day after treatment was higher than measured one month later (P<0.01). No main effect of spironolactone was found (F1, 12 =0.26, P>0.05), nor an interaction between time and treatment (F2, 24=0.10, P>0.05).

Repeated exposure to the tones on day 2 decreased freezing behavior over 30 minutes (Figure 6A, within-group effect, F5, 50=4.94, P<0.01), indicating that extinction training was effective on day 2. Spironolactone did not affect freezing behavior during repeated tone exposure (between-group effect, F1, 10=0.94, P>0.05). For comparison with tone-cue freezing one day and one month later, freezing behavior was determined during the last tone in the retrieval session. A main effect of time was discerned (F2, 24=10.84, P<0.01). Follow-up contrast analysis showed that freezing behavior was decreased both one day (P<0.05) and one month (P<0.01) after retrieval on day 2, the former time-point showing more freezing than the latter (P<0.05). No main effect of spironolactone pre-treatment was found (F1, 12=4.65, P>0.05), nor an interaction between time and treatment (F2, 24=0.31, P>0.05).

Figure 5 Administration of spironolactone before retrieval does not affect tone-cue fear memory. A) Twenty four hours after training in context A, both vehicle and spironolactone treated mice displayed high levels of freezing behavior
in response to the tone in context B. No differences were found between vehicle and spironolactone treated animals. B) Twenty four hours (day 3) and C) one month later, both vehicle and spironolactone treated mice displayed significantly less freezing behavior compared to previous retrieval performance (within-subject contrast, ##P<0.01). No differences were found between the experimental groups (repeated measures ANOVA, P>0.05) (n=7 per group).

Taken together, our results suggest that, unlike contextual fear memory, spironolactone given prior to re-exposure to the tone cue has no effect on the subsequent expression of tone-cued fear memory.

A. Day II  
B. Day III  
C. One month

**Figure 6 Administration of spironolactone before extinction learning does not reduce tone-cue fear memory.** A) Repeated exposure to the tone reduced freezing behavior over time (repeated measures ANOVA, & &P<0.01). No difference was found between vehicle and spironolactone treatment during this period (P>0.05). B) Twenty four hours (day 3) and C) one month later, both vehicle and spironolactone treated mice showed reduced freezing behavior compared to the last retrieval trial (within-subject contrast, ##P<0.01). No differences were found between the experimental groups (repeated measures ANOVA, P>0.05) (n=7 per group).

**Discussion**

Retrieval of fearful events turns these memories into a labile state (Monfils
et al., 2009; Nader et al., 2000a; Nader et al., 2000b), and protein synthesis is required for these memories to become reconsolidated (Nader et al., 2000a). Here we examined whether targeting MRs during re-exposure affects subsequent expression of fear. Our data show that administration of the MR-antagonist spironolactone shortly before retrieval affects the expression of contextual but not tone-cued fear. Spironolactone was ineffective when administered immediately after retrieval. Extinction learning, evoked by a 30-minutes period of re-exposure to the context in the absence of the US, was not affected by treatment.

The protocol that we used was previously described to promote reconsolidation of fear conditioning (Mamiya et al., 2009; Suzuki et al., 2004). One could question, however, whether information was really reconsolidated or subject to weak extinction due to 3-minute re-exposure on day 2. This would argue for interpretation of re-exposure as a weak form of extinction, but absolute comparison of freezing levels between different cohorts of animals should be done with extreme care. Nevertheless, we cannot exclude that MR blockade promotes a weak form of extinction rather than reconsolidation of earlier learned information. If we targeted extinction learning more specifically, by re-exposing the mice to the adverse context for 30 minutes, treatment did not result in significant effects; yet, freezing one day later was already near-absent under vehicle conditions, so that the absence of treatment effects could be a ‘bottom’ phenomenon. It is important to note, though, that also with the longer exposure time on day 2, freezing on day 3 or one month later by no means was increased by spironolactone treatment, so that the duration of re-exposure is not critical for the direction of the outcome, but only for the amplitude of the effect.

Mineralocorticoid receptors (MRs) have been implicated in appraisal of novel and stressful events and response selection (Oitzl and de Kloet, 1992; Sandi and Rose, 1994b). If blocking MRs upon retrieval affects response selection, we would predict a temporary fear reduction, but a
return of fear at the long-term. On the other hand, if blocking MRs upon retrieval affects reappraisal of the event, a permanent fear reduction would be expected. Our current findings that spironolactone when administered prior to retrieval of information reduces freezing behavior during re-exposure as well as one day later (in the absence of the drug) but not one month later suggests that MRs are involved in response selection rather than re-appraisal of the fearful situation.

By blocking MRs, esp. in the context of elevated endogenous corticosteroid levels, the relative contribution of GRs in behavioral effects might be increased. This is of relevance, since elevated corticosteroid levels are thought to reduce retrieval of information and to promote the extinction of irrelevant information (Brinks et al., 2009; de Kloet et al., 1999). This seems unlikely, though. First, the effects of spironolactone are not restricted to retrieval, because they last at least until the next day. Second, our results do not support the notion that spironolactone treatment affects extinction learning, directly or indirectly. Moreover, spironolactone did not affect fear memory when given after re-exposure, while in all behavioral studies described so far pharmacological intervention with GRs after acquisition of the task (also in extinction paradigms) has been very effective (Roozendaal and McGaugh, 1996a, 1997b; Sandi and Rose, 1994b; Yang et al., 2006). The finding that administration of spironolactone after training was ineffective in reducing the expression of fear also argues against a general non-selective action of the drug. Therefore, the effects of spironolactone are most likely established by interfering with MR-mediated events on hippocampal synaptic transmission (Karst et al., 2005; Olijslagers et al., 2008) or synaptic potentiation, an important cellular model for learning and memory formation (Wiegert et al., 2006).

While administration of spironolactone prior to retrieval reduced the expression of contextual fear memory, tone-cue memories were not significantly changed. This again strongly argues against a non-specific role
of the drug in reducing fearful information. The differential effectiveness of spironolactone in context versus tone-cued memories could be explained by the MR distribution in areas linked to these two types of memories, i.e. the hippocampus and basolateral amygdala respectively. Thus, MRs are highly expressed in all hippocampal fields, whereas MR expression in the basolateral amygdala is much lower (de Kloet et al., 2005). Also, the efficacy of MRs to modulate synaptic transmission is different in amygdala (Karst et al., 2010b) compared to hippocampus (Karst et al., 2005).

In conclusion, previous studies have reported that elevated corticosteroid levels reduce the retrieval of information (de Quervain et al., 1998). This approach has been used to reduce the expression of for example phobic fear (Soravia et al., 2006). Our results demonstrate for the first time that blocking MRs prior to retrieval of contextual fearful information reduces the expression of contextual fear.

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