<https://helda.helsinki.fi>

Combination of fluoxetine and extinction treatments forms a unique synaptic protein profile that correlates with long-term fear reduction in adult mice

Popova, Dina

2014-07

Popova , D , Agustsdottir , A , Lindholm , J , Mazulis , W , Akamine , Y , Castren , E & Karpova , N N 2014 , ' Combination of fluoxetine and extinction treatments forms a unique synaptic protein profile that correlates with long-term fear reduction in adult mice ' , European Neuropsychopharmacology , vol. 24 , no. 7 , pp. 1162-1174 . <https://doi.org/10.1016/j.euroneuro.2014.04.002>

<http://hdl.handle.net/10138/158061>

<https://doi.org/10.1016/j.euroneuro.2014.04.002>

acceptedVersion

Downloaded from Helda, University of Helsinki institutional repository.

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.

Combination of fluoxetine and extinction treatments forms a unique synaptic proteins profile that correlates with long-term fear reduction in adult mice

European Neuropsychopharmacology (2014) 24, 1162–1174

<http://dx.doi.org/10.1016/j.euroneuro.2014.04.002>

Dina Popova, Arna Ágústsdóttir, Jesse Lindholm, Ulams Mazulis, Yumiko Akamine, Eero Castrén, Nina N. Karpova *.

Neuroscience Center, University of Helsinki, P.O. Box 56, 00790 Helsinki, Finland
Tel: +358919157629; Fax: +358919157620

* To whom correspondence should be addressed: Nina N. Karpova, E-mail:
nina.karpova@helsinki.fi or nina.karpova@gmail.com

KEYWORDS

Fluoxetine; Fear conditioning; Extinction; Neuronal plasticity; Inhibitory and excitatory transmissions; Presynaptic and postsynaptic protein markers

Abstract

Abstract: The antidepressant fluoxetine induces synaptic plasticity in the visual and fear networks and promotes the structural remodeling of neuronal circuits, which is critical for experience-dependent plasticity in response to an environmental stimulus. We recently demonstrated that chronic fluoxetine administration together with extinction training in adult mice reduced fear in a context-independent manner. Fear conditioning and extinction alter excitatory and inhibitory transmissions within the fear circuitry. In this study, we investigated whether fluoxetine, extinction or their combination produced distinct long-lasting changes in the synaptic protein profile in the amygdala, hippocampus and prefrontal cortex of conditioned mice. We determined that extinction induced synaptophysin expression and down-regulated the GluA1:GluA2 ratio throughout the fear network in water- and fluoxetine-treated mice, suggesting a common fluoxetine-independent mechanism for increased synaptic transmission and re-arrangement of AMPA-receptors by extinction training. In contrast to common changes, the presynaptic vesicular neurotransmitter transporters VGAT and Vglut1 were upregulated after extinction in water- and fluoxetine-treated mice, respectively. The cortical levels of the GABA transporter Gat1 were reduced in high-freezing water-drinking mice, suggesting a maladaptive increase of GABA spillover at cortical inhibitory synapses. Fear conditioning decreased, and extinction induced the expression of GABA-receptor alpha1 and alpha2 subunits in water- and fluoxetine-treated mice, respectively. Only a combination of fluoxetine with extinction enhanced GluN2A expression in the amygdala and hippocampus, emphasizing the role of this NMDA-receptor subunit in the successful erasure of fear memories. Our finding provides novel data that may become helpful in developing beneficial pharmacological fear-reducing treatment strategies.

1. Introduction

Fear conditioning and extinction paradigm is a widely accepted model for anxiety disorders and cognitive behavioral therapies. Although the model does not cover all symptoms of anxiety disorders, the model might help to examine the efficacy of extinction training in transgenic animals or in combination with pharmacological compounds. The extinction-enhancing treatments that have beneficial effects on the long-term reduction of the expression of pathological fear memory in different contexts are essential for anti-anxiety therapies. Several studies have aimed to understand the neurobiological processes that mediate the facilitation of extinction. Accumulating evidence implicates the amygdala, the hippocampus and the infralimbic and prelimbic areas of the medial prefrontal cortex in the expression of fear responses (Quirk *et al.*, 2010). Because both fear acquisition and extinction alter synaptic transmission in these brain areas at excitatory and inhibitory synapses (Ehrlich *et al.*, 2009; Lange *et al.*, 2012), dissecting the synaptic mechanisms of fear reduction through the enhancement of extinction memories is necessary.

Pharmacological agents that potentiate or inhibit the function of proteins involved in synaptic transmission represent one of the most valuable tools for elucidating the molecular mechanisms of extinction facilitation. Over the past decade, D-cycloserine, a partial agonist of the N-methyl-D-aspartate receptor (NMDAR) has been consistently shown to enhance fear extinction learning and reduce several, although not all, anxiety symptoms (Walker *et al.*, 2002; Mao *et al.*, 2006; Guastella *et al.*, 2007). Another important group of compounds are benzodiazepines, agonists of the gamma-aminobutyric acid A receptors (GABA(A)Rs) that have recently been shown to reduce fear potentiated startle responses (Smith *et al.*, 2012). However, the beneficial effect of benzodiazepines seems to be context-dependent, thus reducing their clinical value (Harris and Westbrook, 2001; Otto *et al.*, 2005). Acute treatment with diazepam before extinction training could prevent extinction retention in a shuttle avoidance task potentially due to the induction of anterograde amnesia (Pereira *et al.*, 1989). Another limitation of benzodiazepines is the development of dependence in case of chronic treatment (Wolf and Griffiths, 1991). Finally, a critical function of the antidepressant group of pharmacological compounds, in particular serotonin-selective reuptake inhibitors (SSRIs) in synaptic plasticity and fear memory is currently under intensive investigation. Not all SSRIs were found to have the potential for a beneficial outcome on the combined treatment (Burghardt *et al.*, 2013). The diverse effects of SSRIs are thought to be a result of their different affinity to the serotonin transporter (Owens and Nemeroff, 1998) and selectivity in potentiating various neurotransmitters (Goodnick and Goldstein, 1998). The SSRI fluoxetine has consistently been shown to facilitate extinction learning and retention and long-term context-independent fear reduction in rodents (Karpova

et al., 2011; Camp *et al.*, 2012) and has been shown to significantly reduce fear reinstatement even after discontinuation of fluoxetine exposure (Deschaux *et al.*, 2011). However, the synaptic mechanisms of these effects are largely unstudied.

The trafficking of the α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPA_Rs) is critically involved in the plasticity of excitatory synapses and the GluA1-dependent synaptic removal of GluA2-containing calcium-permeable AMPA_Rs during extinction was shown to promote long-term depression in the lateral amygdala and fear memory erasure (Kim *et al.*, 2007; Clem and Huganir, 2010). The GluA1-subunit of calcium-impermeable AMPA_Rs has been recently found to be up-regulated in the basolateral amygdala shortly after the spontaneous fear recovery and context-dependent fear renewal tests (Xue *et al.*, 2014). The key markers of the GABAergic system (different GABA(A)_R subunits, GABA plasma membrane transporter-1 Gat1 and gephyrin) are dynamically regulated in the amygdala nuclei by the fear conditioning and/or extinction: the mRNA expression analysis revealed that the GABAergic markers were primarily down-regulated right after fear conditioning and up-regulated following fear extinction training (Chhatwal *et al.*, 2005; Heldt and Ressler, 2007). Accordingly, the surface expression of the GABA(A)_R β 3 and γ 2 subunits was decreased by the conditioning and increased by the extinction in the lateral amygdala (Lin *et al.*, 2009). However, limited data exist on the fear conditioning/extinction-induced synaptic protein expression outside the amygdala and on the long-lasting effects of the accelerated extinction on synaptic protein changes associated with efficient fear erasure. Such data are of particular importance for developing effective pharmacological strategies in those clinical cases when the immediate behavioral or pharmacological therapies are not applicable.

We have recently shown that chronic fluoxetine applied before and during fear extinction training had a long-term effect on extinction acceleration and fear erasure in adult mice (Karpova *et al.*, 2011). The aim of the present study was to use the same protocol for simultaneous analysis of the fear responses and of the synaptic proteins profile permissive for long-term fear reduction in mice. We applied an immunohistochemical approach to provide spatial specificity of protein expression by outlining the brain areas critical for fear memory: the prelimbic and infralimbic cortices, the basolateral, lateral and central amygdala nuclei and the CA1 region of hippocampus. We evaluated how the combination of fluoxetine treatment and extinction training affected long-term expression of the markers of excitatory and inhibitory transmission: the presynaptic synaptophysin Syp, vesicular glutamate transporter Vglut1, vesicular GABA and glycine transporter VGAT, GABA transporter Gat1, and the postsynaptic major GABA(A)_R subunits α 1 and α 2, postsynaptic density protein PSD95, as well as the major components of glutamate receptors (NMDA_Rs subunit GluN2A

and AMPARs subunits GluA1 and GluA2). Our results provide evidence that a combination of chronic fluoxetine and extinction training forms unique long-lasting synaptic protein patterns in the fear network of conditioned mice compared with either treatment alone.

2. Experimental Procedures

2.1. Animals

Adult male mice C57Bl/6JRcc.Hsd (Harlan, Netherlands) were housed individually for 7 days prior to fear conditioning and were 3 months old at the time of fear conditioning. Mice were kept under a 12 h light/dark cycle (light on at 6 am). Food and water were available *ad libitum*. All animal procedures were done according to the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the experimental Animal Ethical Committee of Southern Finland.

2.2. Drug treatment

Non-invasive approach of drug administration was used. Mice received fluoxetine (kind gift from Dr. Jukka Sallinen, Orion Pharma, Helsinki, Finland) via drinking water in light-protected tubes. Solutions were prepared fresh every day. Fluoxetine was dissolved in tap water at a concentration of 0.08 mg/ml. With this concentration, the fluid consumption level was not affected (Fig. S1) and the mean fluoxetine dosing was 10.26 ± 0.13 mg/kg per day. This protocol of chronic fluoxetine administration to adult mice was reported to results in clinically relevant plasma fluoxetine levels (Dulawa *et al.*, 2004; Rantamaki *et al.*, 2007) and to effectively produce behavioral alterations in depression- and anxiety-like behaviors (Karpova *et al.*, 2009; Karpova *et al.*, 2011).

2.3. Fear conditioning

Fear conditioning took place in the context A (a transparent Plexiglas chamber with metal grids that was cleaned before each session with 70 % ethanol). Freezing behavior was measured with an automatic infrared beam detection system which was placed on the sides of the fear conditioning chamber of (TSE Systems GmbH, Germany). The mouse was considered to be frozen only if it was not moving for at least 3 s, and the measure was expressed as a percentage of time spent freezing. Every mouse was handled in the experimental room for 5-10 minutes during each of the 3 days prior to fear conditioning. On the day of fear acquisition, mice were exposed to context A for 2 minutes and conditioned using 5 pairings of the CS (Conditioned Stimulus; total duration 30 s, 1 Hz, white noise, 80

dB) with the US (Unconditioned Stimulus; 1 s foot-shock 0.6 mA, inter-trial interval: 20-120 s). The US co-terminated with the CS. The freezing level during the first CS, preceding the first US, was taken as the baseline freezing during CS. Mice were then divided into four groups (two extinction, FC+WAT+EXT and FC+FLX+EXT, and two no-extinction, FC+WAT and FC+FLX, groups) with equal levels of freezing, two receiving fluoxetine in their drinking water until the end of the experiment and the other two receiving tap water. For the control group CTRL, mice were subjected to the same fear conditioning experimental protocol except that the CS was not followed by the US (non-conditioned, only context + CS exposure group); then, the CTRL mice received tap water. Two weeks after the fear conditioning day, the mice from the CTRL and both no-extinction groups (n=6 per group) were sacrificed for subsequent immunohistochemical analysis.

2.4. Fear extinction training, spontaneous recovery and fear renewal

Two-day fear extinction training took place 2 weeks after fear conditioning in the context B (a black non-transparent Plexiglas chamber with a planar floor that was cleaned before each session with 70% 2-propanol). Freezing behavior was measured as described above. On the first and second extinction days, conditioned mice received 12 presentations of the CS (total duration 30 s, 1 Hz, white noise, 80 dB, inter-trial interval: 20-60 s). One week after extinction, extinguished mice (n=6 per group) were sacrificed for subsequent immunohistochemical analysis. In parallel, additional mice (n=25 per each extinction group) were tested 7 days after extinction in context B and context A, respectively, using 4 presentations of the CS (inter-trial interval: 20-60 s) and were further used for the Pearson's correlation analysis of context-dependent spontaneous recovery and fear renewal.

2.5. Tissue processing for immunohistochemistry

Animals were deeply anesthetized with CO₂ and were transcardially fixed with phosphate buffered saline (PBS, pH 7.4) followed by chilled 4% paraformaldehyde (PFA) in PBS. Brains were removed from the skull after perfusion, post fixed in 4% PFA overnight at +4°C, cryoprotected in 30% sucrose, embedded in O.C.T. compound (Tissue-Tek, Sakura, Netherlands) and kept at -80°C until sectioning. Coronal sections (25 µm thick) were performed with a Leica CM3050 S cryostat (Germany). Sections were collected and stored in a cryoprotective solution (30% glycerol, 30% ethylene glycol in PBS) at -20°C until use.

2.6. Fluorescent immunohistochemistry

Immunostaining was performed using free-floating brain sections. After washing with PBS to remove the cryoprotective solution, the sections were incubated in a blocking reagent

consisting of 5% goat serum (Vector Laboratories, UK), 3% bovine serum albumin (Sigma-Aldrich, Finland) and 0.4% Triton X-100 (Sigma-Aldrich, Finland) in PBS to prevent nonspecific binding of antibodies. Before blocking, an antigen retrieval step including an incubation in 0.1% pepsin (Sigma-Aldrich, Finland) in 5 mM HCl for 10 minutes at room temperature, was performed to increase the binding of the primary antibodies for GABA(A)R α 1, GABA(A)R α 2 and Vglut1. Sections were incubated with one of the primary antibodies (Table S1) in PBS containing 0.4% Triton X-100 (PBST) overnight at +4°C. Then, sections were washed in PBST and incubated with the appropriate secondary antibodies (Molecular probes, Invitrogen, Espoo, Finland) for 1 hour at room temperature. Finally, sections were mounted on slides and covered with Prolong®Gold anti-fade reagent with DAPI (Molecular Probes, Invitrogen, Espoo, Finland). Controls “No primary antibody” were included in all immunostaining experiments.

2.7. Image acquisition and processing

Quantitative evaluation of immunostainings was performed by an investigator blind to the treatment groups; all slides were coded until the analyses were finished. The following brain regions were analyzed: the prelimbic (PL) and infralimbic (IL) zones of the medial prefrontal cortex, basolateral (BLA), lateral (LA) and central (CEA) nuclei of the amygdala, CA1 strata oriens (OR), radiatum (RAD), lacunosum-moleculare (LM) and pyramidal layer (PYR) of the hippocampus (Fig. S1). The images were obtained by the Imager M.1 fluorescent microscope (Zeiss, Germany) using AxioVision software. A minimum of 5 sections per brain area/per animal, as well as control sections “No primary antibody”, were imaged using the same microscope and camera settings for all slides within each immunostaining experiment. DAPI nuclear staining helped to locate and focus on the correct brain areas on the control sections “No primary antibody”. Image processing was performed with ImageJ software. To estimate the difference in expression of synaptic markers, brain regions were delineated (Fig. S2) and mean optical densities were measured. The mean optical densities of the controls “No primary antibody” were subtracted from obtained values for every brain area in all immunostaining experiments. The resulting values of optical densities were used for a Pearson’s correlation analysis, whereas results in Figure 3 and Table S2 are expressed as the percentage of the control group CTRL.

2.8. Statistical analysis

The values reported in the Tables S2 and S3 and the Figures 1, 3, 5 and S1 represent the means $\pm$ SEM. Analyses of the behavioral tests were performed using repeated measures ANOVA. Statistical analyses of proteins levels from four fear conditioned groups were performed using a two-way ANOVA with a post hoc Fisher’s PLSD test. For the protein

levels comparison between the control group CTRL and one of the other groups, Student's unpaired two-tailed t-test was used. The bivariate Pearson's correlation and linear regression analyses were performed using Microsoft Excel 2010 and SPSS Statistical packages. A P-value of < 0.05 was considered statistically significant.

3. Results

3.1. Extinction rate correlates with later fear responses

A two-day fear extinction paradigm (Fig. 1A) has been shown to be effective in the analysis of the efficacy of different combinative anti-fear treatments and genotype-specific extinction rate in mice (Karpova *et al.*, 2011; Camp *et al.*, 2012). Moreover, during the first day of extinction a difference between the control and treatment groups was not always observed. However, facilitation of extinction was clearly detected on the second day of training, reflecting successful treatment-enhanced extinction retention both in rats and mice (Santini *et al.*, 2001; Karpova *et al.*, 2011; Camp *et al.*, 2012). Here we first investigated whether the facilitation of fear extinction could be detected in experimental groups with a small number of animals ($n=6$ per group) subjected to further immunohistochemical analysis. Chronic fluoxetine treatment after fear acquisition did not significantly affect the freezing levels during the extinction day 1 ($F_{1,10}=3.60$, $P=0.087$); however, on the second day of extinction fluoxetine-treated mice froze significantly less than water-drinking mice ($F_{1,10}=5.23$, $P=0.045$) (Fig. 1B).

Figure 1. Extinction training is effective in combination with the chronic fluoxetine treatment after fear conditioning. (A) Experimental protocol. “Fear Cond.” – fear conditioning. Black arrows show the time points for samples collection for immunohistochemical analysis of indicated treatment groups. Additional mice from the extinguished groups continued behavioral testing until the tests for spontaneous recovery and fear renewal for further Pearson’s correlation analysis (shown on the Fig. 2). (B) Behavioral analysis of the mice subjected for immunohistochemistry ($n=6$ per group). Fluoxetine facilitated extinction training in the Extinction Context B. N.S. – not significant. $*P < 0.05$ after repeated measures ANOVA.

Next, we aimed to analyze if the mouse freezing levels during the extinction days 1 or 2 has a long-term predictive validity for its freezing during the tests for spontaneous recovery, SR, or fear renewal, FR. We propose that if significant correlations exist 1) between the extinction freezing levels and SR/FR responses, and 2) between the synaptic proteins expression and extinction freezing levels, then the amount of synaptic proteins in fear-extinguished animals may have a predictive potential for their SR/FR responses. To better calculate the first correlation, we analyzed additional large groups of animals in both SR and FR tests ($n=25$ mice per group). We found that the magnitude of both SR and FR fear responses were positively correlated with the averaged (per CS) freezing levels of FC+WAT+EXT mice on both the extinction days 1 and 2, with the higher correlation coefficients for the extinction day 2 (Fig. 2). Although fluoxetine-treated animals exhibited a similar trend, the correlation between their freezing values during extinction and SR or FR tasks was not always significant (Fig. 2), possibly because of considerable amount of non-freezing animals during SR and FR. Together, these behavioral results prompted us to first perform a conventional comparison of the mean group levels of synaptic protein expression and then perform a Pearson’s correlation analysis of protein levels vs. freezing levels on the extinction day 2 in FC+WAT+EXT mice.

3.2. Synaptic molecular correlates of facilitated extinction

3.2.1. Synaptophysin

Synaptophysin, Syp, is a presynaptic vesicles membrane protein important for synaptic transmission in neuronal circuits involved in learning and memory. We found that chronic fluoxetine did not alter Syp expression after fear conditioning; in contrast, extinction training induced Syp expression in almost all brain areas independently of fluoxetine treatment (Fig. 3 and Table S2). Interestingly, we found no significant Pearson’s correlation between the individual freezing levels on the extinction day 2 vs. Syp expression in the FC+WAT+EXT group ($r < |0.55|$, $P > 0.25$), which suggests that the expression levels of this marker of presynaptic transmission should not be used as a predictive parameter for fear response.

We next determined whether the observed changes in Syp expression levels were correlated with the expression of presynaptic markers for excitatory and inhibitory transmissions Vglut1 and VGAT.

Figure 2. Freezing responses during extinction positively correlate with the later fear responses as found by a Pearson's analysis between the individual freezing levels of FC+WAT+EXT (open circles, solid line) and FC+FLX+EXT (black circles, dash line) mice on the extinction days 1 or 2, EXT d1 or d2, and spontaneous recovery, SR, and fear renewal, FR, levels (n=25 per group).

3.2.2. Vglut1

Fluoxetine treatment did not affect the levels of Vglut1 expression, which was significantly reduced by fear conditioning in the hippocampus (Fig. 3 and Table S2). In contrast, the effect of fear extinction on Vglut1 levels was fluoxetine-dependent. In almost all brain areas of water-drinking animals extinction further decreased Vglut1 expression, whereas fluoxetine combined with extinction prevented the decrease in Vglut1 levels and significantly up-regulated its levels in the lateral and central amygdala of the FC+FLX+EXT mice (Fig. 3). The freezing levels on the extinction day 2 were negatively correlated with Vglut1 expression

in the CA1 pyramidal layer in the FC+WAT+EXT mice ($r=-0.95$, $P=0.004$), suggesting that increased hippocampal Vglut1 expression after extinction might be associated with fear inhibition.

Figure 3. Long-term effect of fear conditioning, chronic fluoxetine and fear extinction on the expression of synaptic markers in the infralimbic (IL) and prelimbic (PL) cortex, basolateral (BLA), lateral (LA) and central amygdala (CEA) areas and hippocampal strata oriens (OR), pyramidal (PYR), radiatum (RAD) and lacunosum-molecular (LM) layers. OD – optical density. Post hoc analysis: * $P < 0.05$ vs. CTRL group (dash line at 100% level); # $P < 0.05$ fluoxetine vs. respective water group - FC+FLX vs. FC+WAT and FC+FLX+EXT vs. FC+WAT+EXT; \$ $P < 0.05$ extinction vs. respective no-extinction group - FC+WAT+EXT vs. FC+WAT and FC+FLX+EXT vs. FC+FLX ($n=6$ per group).

3.2.3. VGAT

The expression levels of VGAT were affected by neither fear conditioning nor extinction in fluoxetine-treated animals (Fig. 3 and Table S2). However, VGAT was significantly down-regulated by fear conditioning in the prelimbic cortex, basolateral amygdala, CA1 pyramidal and radiatum areas of FC+WAT mice. Extinction training significantly increased VGAT levels in the FC+WAT+EXT group compared with the FC+WAT group in all brain areas except for the central amygdala (Fig. 3). Individual VGAT levels were not correlated with freezing levels on the extinction day 2 in FC+WAT+EXT mice ($r < |0.53|$, $P > 0.28$).

We suggested that the extinction-induced Syp expression might correlate with enhanced GABA-ergic or glutamatergic presynaptic transmission in the water or fluoxetine treated animals, respectively. To further test this hypothesis, we measured the Pearson's correlation coefficients between the individual levels of Syp and Vglut1 and between Syp and VGAT in both extinguished groups of animals. Unexpectedly, there was no correlation between increased Syp expression vs. either Vglut1 (FC+WAT+EXT: $r < |0.67|$, $P > 0.14$; FC+FLX+EXT: $r < |0.78|$, $P > 0.067$) or VGAT (FC+WAT+EXT: $r < |0.38|$, $P > 0.45$; FC+FLX+EXT: $r < |0.45|$, $P > 0.37$) levels in any of the brain areas analyzed, suggesting that the extinction-induced up-regulation of these presynaptic proteins was independent of each other.

3.2.4. Vesicular neurotransmitters transporters along fear extinction circuitry

We recently showed that chronic fluoxetine combined with extinction training reduced fear response in a context-independent manner (Karpova *et al.*, 2011). In the extinction context, the infralimbic cortex has been suggested to be activated by the hippocampus and, in turn, activates the basolateral amygdala interneurons, which inhibit central amygdala neurons and the fear response, whereas in the conditioned context no activation of the infralimbic cortex by the hippocampus and, consequently, no fear inhibition occurs (Fig. 4; (Quirk and Mueller, 2008). The individual mouse Vglut1 levels in the CA1, infralimbic cortex and basolateral amygdala and the VGAT levels in the central amygdala may at least partially reflect activated or inhibited states of these brain areas. We calculated the correlation coefficients between the connections $CA1^{Vglut1} - IL^{Vglut1}$, $IL^{Vglut1} - BLA^{Vglut1}$ and $BLA^{Vglut1} - CEA^{VGAT}$ in extinguished animals and found a strong positive correlation for every connection in only fluoxetine-treated mice (Fig. 4). This result might suggest that the combined fluoxetine + extinction treatment could reinforce the fear extinction circuitry sufficiently enough to be active in both extinction and conditioned contexts.

Figure 4. Protein levels correlations between connections CA1^{VGLUT1} – IL^{VGLUT1}, IL^{VGLUT1} – BLA^{VGLUT1} and BLA^{VGLUT1} - CEA^{VGAT} in the fear extinction circuitry of extinguished groups of mice ($n=6$ per group).

3.2.5. Gat1

The GABA plasma membrane transporter Gat1 localizes to both neuronal and glial cells near synapses and has been shown to restrict GABA spillover at cortical inhibitory synapses (Gonzalez-Burgos *et al.*, 2009). We found no significant regulation of Gat1 levels by chronic fluoxetine and/or extinction (Fig. 3 and Table S2). However, we observed a strong negative correlation between the freezing levels on the extinction day 2 and Gat1 expression in the infralimbic ($r=-0.94$, $P=0.005$) and prelimbic cortices ($r=-0.88$, $P=0.021$) of FC+WAT+EXT animals. These results suggest that, in the absence of antidepressant treatment, higher fear response after fear extinction may be associated with reduced GAT1 expression.

3.2.6. Post-synaptic components of GABA-ergic transmission

Here, we aimed to analyze the expression patterns of two major GABA(A)R subunits, $\alpha 1$ and $\alpha 2$, the agonists of which have been associated with sedative and anxiolytic activities, respectively (Atack, 2003).

3.2.6.1. GABA(A)R $\alpha 1$ (sedative)

The expression of the GABA(A)R $\alpha 1$ subunit was altered by fear conditioning and extinction in water-drinking groups of animals: fear conditioning decreased and extinction training restored GABA(A)R $\alpha 1$ levels in the infralimbic and prelimbic cortex (Fig. 3 and Table S2). In the CA1 pyramidal layer, GABA(A)R $\alpha 1$ expression was down-regulated by fear conditioning and restored by extinction to the levels of the CTRL group in both water and fluoxetine-treated animals (Table S2). Although no significant correlation was found between

GABA(A)R $\alpha 1$ levels and the freezing levels on the extinction day 2 in the FC+WAT+EXT mice ($r < |0.68|$, $P > 0.14$), the main effects of extinction training on the increase in GABA(A)R $\alpha 1$ were significant in the prefrontal cortex, central amygdala and hippocampal CA1 layers (Table S2).

3.2.6.2. GABA(A)R $\alpha 2$ (anxiolytic)

In contrast to GABA(A)R $\alpha 1$, GABA(A)R $\alpha 2$ expression was mainly not affected by fear conditioning or extinction in water-drinking animals. However, chronic fluoxetine after fear conditioning significantly decreased GABA(A)R $\alpha 2$ levels in the prefrontal cortex and CA1 pyramidal layer (Fig. 3 and Table S2). Moreover, extinction training in the FC+FLX+EXT group not only reversed GABA(A)R $\alpha 2$ expression in these brain areas to control levels but also markedly up-regulated GABA(A)R $\alpha 2$ levels in the amygdala (Fig. 3).

Interestingly, despite the finding that fluoxetine treatment combined with extinction increased the expression of the GABA(A)R $\alpha 2$ subunit in several brain regions, its levels in the amygdala of FC+WAT+EXT mice were positively correlated with their freezing levels on the extinction day 2: lateral amygdala ($r = +0.814$, $P = 0.049$) and central amygdala ($r = +0.83$, $P = 0.041$). Taken together, the effects of the fear conditioning-induced stress and extinction training on the expression patterns of GABA(A)R $\alpha 1$ and $\alpha 2$ subunits were dependent on whether the animals received fluoxetine treatment or not.

3.2.7. PSD95, a marker of excitatory post-synaptic transmission

PSD95 is a major scaffolding protein of the excitatory post-synaptic density. Extinction training significantly up-regulated PSD95 levels in the amygdala and, when combined with fluoxetine, in all hippocampal layers (Fig. 3, Table S2). Moreover, PSD95 level in the basolateral amygdala of FC+WAT+EXT mice was negatively correlated with their freezing levels on the extinction day 2 ($r = -0.90$, $P = 0.015$) further suggesting a role of enhanced amygdala PSD95 expression in fear reduction.

3.2.8. Ionotropic glutamate receptors. GluN2A

The NMDAR agonist D-cycloserine has been shown to facilitate the extinction of conditioned fear (Walker *et al.*, 2002). We were specifically interested whether the beneficial effect of chronic fluoxetine on fear extinction was positively associated with the GluN2A subunit of NMDARs, which is abundantly expressed in the adult brain. We found that chronic fluoxetine after fear conditioning had no effect on GluN2A expression (Fig. 3 and Table S2). However, when combined with extinction training, chronic fluoxetine significantly up-regulated GluN2A levels in the amygdala areas and in the CA1 pyramidal layer (Fig. 3). Furthermore, the

GluN2A levels in the CA1 pyramidal layer of the FC+WAT+EXT mice was negatively correlated with their freezing levels on the extinction day 2 ($r=-0.84$, $P=0.036$) supporting the role of enhanced expression of this subunit in successful erasure of fear memories.

3.2.9. Ionotropic glutamate receptors. AMPA receptors

AMPA glutamate receptors play a key role in mediating fast excitatory synaptic transmission. We asked whether extinction of conditioned fear in water- or fluoxetine-treated mice alters the expression patterns of a Ca^{2+} -permeable GluA1 subunit and the most abundant Ca^{2+} -impermeable GluA2 subunit of AMPA receptors, which are thought to mediate different forms of synaptic plasticity.

3.2.9.1. GluA1

We detected significant main effects of extinction training on the reduction of GluA1 expression in only the CA1 hippocampal areas (Fig. 3 and Table S2). Furthermore, fluoxetine treatment further decreased GluA1 expression in the CA1 pyramidal layer ($F_{1,20}=5.26$, $P=0.033$); however, the magnitude of the observed changes was very small.

3.2.9.2. GluA2

In contrast to GluA1, the GluA2 subunit showed opposite and more prominent changes in the fear circuitry. We observed a significant main effect of the extinction factor on GluA2 up-regulation in all brain regions analyzed (Fig. 3 and Table S2). Chronic fluoxetine slightly increased the GluA2 levels in the CA1 pyramidal layer (main effect of fluoxetine treatment $F_{1,20}=4.37$, $P=0.049$). Expression levels of both GluA1 and GluA2 subunits in the FC+WAT+EXT mice were independent of their freezing levels on the extinction day2 (GluA1: $r<|0.79|$, $P>0.062$; GluA2: $r<|0.67|$, $P>0.14$).

3.2.9.3. GluA1:GluA2 ratio

The reduced GluA1:GluA2 ratio in the nucleus accumbens of stress-resilient mice has been suggested to decrease glutamatergic transmission and, thus, to be protective against the deleterious effects of stress (Vialou *et al.*, 2010). We analyzed here how a stress-resilient state of AMPA receptors in fear circuitry was affected by fluoxetine and/or extinction training. Stress induced by fear conditioning increased the GluA1:GluA2 ratio in only the CA1 pyramidal layer in FC+WAT mice (Fig. 5 and Table S3). In contrast, extinction significantly down-regulated the ratio in all brain areas analyzed. Fluoxetine treatment additionally reduced the GluA1:GluA2 ratio in the CA1 pyramidal layer ($F_{1,20}=5.93$, $P=0.024$), although the magnitude of this effect was minor (Fig. 5).

Figure 5. Extinction training decreases the GluA1:GluA2 ratio in the fear circuitry. Post hoc analysis: * $P < 0.05$ vs. CTRL group; # $P < 0.05$ fluoxetine vs. respective water group - FC+FLX vs. FC+WAT and FC+FLX+EXT vs. FC+WAT+EXT; § $P < 0.05$ extinction vs. respective no-extinction group - FC+WAT+EXT vs. FC+WAT and FC+FLX+EXT vs. FC+FLX ($n=6$ per group).

4. Discussion

In the present study we show that a combination of fluoxetine and extinction treatments forms a specific synaptic landscape permissive for long-term fear extinction facilitation and fear erasure in adult mice. In addition, our immunohistological approach, in parallel with the behavioral assessments of fear-conditioned mice, provides novel findings on the synaptic profiles of more efficient fear reduction by extinction in control water-drinking animals. The key findings are summarized in Table 1.

4.1. Synergic and distinct long-term synaptic modifications induced by fear acquisition in water- and fluoxetine- treated mice

Hippocampal synaptic protein (synapsin and synaptophysin) loss has been described in a mouse model of posttraumatic stress disorder (Herrmann *et al.*, 2012). We found that stress induced by fear conditioning had a long-lasting inhibitory effect on the expression of pre- and postsynaptic components of inhibitory and excitatory transmissions (Syp, GABA(A)R $\alpha 1$ and GluA2 receptors) in the hippocampal CA1 pyramidal layer and in the whole hippocampus (Vglut1) of both water- and fluoxetine-treated conditioned animals. Neuronal excitotoxicity due to GluA2 dysfunction and the subsequent increase in Ca^{2+} -permeability of AMPAR has been associated with motor neurons loss (Van Damme *et al.*, 2002). However, the

hippocampal GluA2 reduction observed in our study was modest in comparison with the reduced Vglut1 expression throughout the brain of the fear conditioned mice regardless of fluoxetine treatment. The expression pattern of Vglut1 suggests that glutamatergic transmission was inhibited at the presynaptic level by fear conditioning, which could mediate anxiety-like symptoms in the absence of extinction training. Indeed, reduced glutamate function due to Vglut1 deficiency (Wojcik *et al.*, 2004) has been linked to impaired LTP, learning and memory (Balschun *et al.*, 2010) and depression- and anxiety-like behaviors (Tordera *et al.*, 2007).

What molecular mechanisms could accelerate fear extinction in fluoxetine-drinking animals? We found that expression levels of pre- and postsynaptic markers of inhibitory transmission were dependent on fluoxetine treatment. The decrease in GABA-receptors function has been specifically implicated in anxiety- and fear-related disorders (Luscher *et al.*, 2011). In contrast, to our knowledge no studies exist that analyze the presynaptic markers of inhibitory transmission in development of fear memories, although a decreased expression of the vesicular GABA and glycine transporter, VGAT, has been recently associated with cold water swim stress-induced anxiety (Schoenfeld *et al.*, 2013). Similarly, in our study, stress induced by fear conditioning reduced VGAT expression but only in water-drinking mice. At the GABA-receptor level, we found that the sedation-associated GABA(A)R $\alpha 1$ subunit was primarily down-regulated in water-drinking mice; in contrast, the anxiolytic GABA(A)R $\alpha 2$ subunit expression was primarily decreased in fluoxetine animals. To date, this is the first study that suggests a role for GABA(A)R $\alpha 2$ in the facilitation of fear extinction by fluoxetine. Consistent with our findings, the distinct role of $\alpha 1$ - and $\alpha 2$ - containing GABA(A)Rs in sensitivity to benzodiazepines treatment and expression of anxiety responses has been revealed using transgenic mice with reduced levels of either subunit (Smith *et al.*, 2012; Koester *et al.*, 2013). These results and our findings expand the previous knowledge about decreased GABA-ergic tones following fear conditioning (Chhatwal *et al.*, 2005; Ehrlich *et al.*, 2009; Mahan and Ressler, 2012) and a GABA-ergic deficit hypothesis of neuropsychiatric disorders (Luscher *et al.*, 2011).

Antidepressant fluoxetine reactivated the ocular dominance plasticity in the adult visual cortex and induced the structural rearrangement of inhibitory circuits providing a mechanism for experience-dependent plasticity in response to visual deprivation (Chen *et al.*, 2011). Our findings on the distinct changes in the inhibitory system produced by fluoxetine after fear conditioning suggest that fluoxetine treatment may promote a permissive remodeling at the synaptic structural level that would facilitate circuit-specific modifications in response to an environmental stimulus such as extinction training.

Table 1 Summary of the key synaptic protein changes induced by each type of treatment following fear conditioning in the prefrontal cortex, amygdala and hippocampus

Treatment	No treatment	Fluoxetine only	Extinction only	Fluoxetine+Extinction
Marker	(FC+WAT) [#]	(FC+FLX)	(FC+WAT+EXT)	(FC+FLX+EXT)
Syp			All brain areas ↑	All brain areas ↑
Components of excitatory transmission				
Vglut1	Hippo ↓	Hippo ↓	All brain areas ↓	Amy ↑ , Hippo ↑ *
PSD95			Amy ↑	Amy, Hippo ↑ ↑
GluN2A				Amy ↑
GluA1:GluA2			All brain areas ↓	PFC, Amy ↓ , Hippo ↓↓
Components of inhibitory transmission				
Gat1			PFC↗	
VGAT	All brain areas ↓		All brain areas ↑	
GABA(A)α1	PFC ↓		PFC ↑ *	Amy ↑
GABA(A)α2		PFC ↓	Amy↘	PFC ↑ *, Amy ↑ ↑

PFC - prefrontal cortex; Amy - amygdala; Hippo - CA1 area of the hippocampus

↓ - decreased, ↓↓ - strongly decreased, ↑ - increased, ↑↑ - strongly increased and ↑* - restored to control level protein expression

PFC↗ and **Amy↘** indicate elevated (↗) or decreased (↘) protein expression in the lower-freezing FC+WAT+EXT mice as determined by a Pearson's correlation analysis of protein levels vs. freezing levels on the extinction day 2

[#] Upper row: the groups are indicated in parentheses

4.2. Long-term synaptic modifications in water- and fluoxetine treated mice in response to extinction training

An increasing amount of studies provide evidence that the extinction of conditioned fear may represent both 1) a depotentiation of synapses that were potentiated after fear conditioning and 2) a new learning while the fear acquisition memories remain intact (Myers and Davis, 2007; Hong *et al.*, 2009). We found that the inhibitory influence of fear acquisition on the expression of several synaptic proteins was primarily neutralized in a week after extinction,

which is consistent with both hypotheses of extinction. Indeed, the reversal of original protein expression might happen at the synapses that were lastingly affected by fear conditioning. Alternatively and/or simultaneously, some long-term extinction-induced synaptic changes may involve new synapses or circuits resulting in restored or up-regulated total protein levels. In line with previous studies on enhanced synaptic densities and transmissions by extinction (Heldt and Ressler, 2007; Lin *et al.*, 2010), we found that extinction training increased total protein levels of Syp, PSD95, the sedation-associated GABA(A)R $\alpha 1$ subunit and the GluA2 receptor, suggesting enhanced excitatory and inhibitory synaptic plasticity in both water- and fluoxetine-treated mice. Moreover, our results suggest that extinction training alone may promote AMPA-receptors re-composition in the fear memory network to more stress-resilient state (Vialou *et al.*, 2010). These data suggest common mechanisms of extinction-induced excitatory and inhibitory plasticity independent of fluoxetine treatment.

How does chronic fluoxetine combined with extinction subsequently reduce fear memories (this study and (Deschaux *et al.*, 2011; Karpova *et al.*, 2011; Camp *et al.*, 2012)? Does the combined treatment 1) enhance synaptic changes produced by extinction in water-treated animals or 2) promote unique alterations in synaptic proteins expression? To support the first hypothesis, we found that consolidation of extinction memories might benefit from the combined treatment by a decrease in GluA1:GluA2 ratio in the CA1 pyramidal layer (see also (Vialou *et al.*, 2010); however, the effect of fluoxetine was minor and seems unlikely to mediate a robust behavioral effect on fear erasure.

In contrast, our data provide more evidence for a unique long-lasting remodeling of synaptic protein expression profiles as a result of the combined treatment. D-cycloserine, an NMDA-receptor partial agonist, has been consistently shown to facilitate extinction (Walker *et al.*, 2002; Lin *et al.*, 2010), which is in agreement with our findings on enhanced GluN2A expression with the combined treatment. In addition, the patterns of the presynaptic markers Vglut1 (decreased) and VGAT (increased) showed that extinction favored towards inhibitory transmission in water-drinking mice and this effect was prevented by fluoxetine. The level of VGAT expression correlates with the release of the inhibitory neurotransmitters GABA, glycine and potentially beta-alanine (Wojcik *et al.*, 2006; Juge *et al.*, 2013), and our data suggest that extinction training potentiated GABA release from presynaptic terminals in the water group. In contrast, increased Vglut1 expression in the amygdala or hippocampal areas of fluoxetine mice implicates enhanced glutamate release in fear reduction by extinction. A plausible explanation of these extinction-induced presynaptic changes could be a potentiation of inhibitory drive onto fear neurons (Ehrlich *et al.*, 2009; Vlachos *et al.*, 2011) in the water group and of excitatory drive onto extinction neurons in the fluoxetine group. In addition, we found strong positive correlations between Vglut1 levels in the fear circuitry

CA1- infralimbic cortex - basolateral amygdala and the VGAT level in the central amygdala specifically in FC+FLX+EXT mice. These data might implicate simultaneous increase of glutamatergic transmission along the fear network in sustained inhibition of the central amygdala neurons after extinction (Quirk and Mueller, 2008) observed in only fluoxetine-treated mice. Because we analyzed only the major brain areas involved in the formation of fear memory, this suggestion needs further experimental validation with more molecular markers and anatomical connections that each brain area forms.

To date, a brain-derived neurotrophic factor, Bdnf, represents the most studied signal transmitter in the fear network. Bdnf-containing inputs from the hippocampal CA1 to the infralimbic cortex mediate the efficacy of fear extinction (Heldt *et al.*, 2007; Peters *et al.*, 2010), and basolateral amygdala Bdnf signaling is important for fear reduction by extinction (Chhatwal *et al.*, 2006; Karpova *et al.*, 2011). Bdnf deficiency weakens hippocampal Bdnf-inputs (Chen *et al.*, 2006) and results in extinction impairment (Soliman *et al.*, 2010) independently of fluoxetine treatment (Karpova *et al.*, 2011). Potential synaptic mechanisms of this impairment might involve Bdnf-dependent control of Vglut1 expression and glutamate release in LTP (Melo *et al.*, 2013) impaired in the hippocampus of Bdnf-deficient mice (Patterson *et al.*, 1996).

A unique feature of the prefrontocortical GABA-ergic transmission has been detected in FC+WAT+EXT mice. Specifically we found a strong negative correlation between Gat1 expression and freezing levels on the second extinction day. The reduced cortical GAT1 levels implicated an increased GABA spillover in the prolongation of IPSP duration, which is critical for timing of GABA-ergic inhibition during network oscillation (Gonzalez-Burgos *et al.*, 2009). Prolonged IPSP may lead to overlaps in time of GABA-ergic inputs from distinct interneurons and thus perturb cell-type specific inhibition during inhibitory learning (Gonzalez-Burgos *et al.*, 2009) such as extinction. Therefore, the reduced cortical Gat1 in higher-freezing mice may predict subsequent higher fear response in water-drinking mice, an effect absent in mice after combined treatment.

A strengthened inhibitory transmission has been shown to play a critical role in fear extinction memories (Chhatwal *et al.*, 2005; Quirk *et al.*, 2010). Consistent with this view, we found that extinction essentially restored conditioning-induced deficits of the GABA(A)R $\alpha 1$ or $\alpha 2$ subunits in the water- or fluoxetine-group, respectively. It's possible that chronic fluoxetine treatment reshapes the fear memory network and promotes structural remodeling of inhibitory circuits (Chen *et al.*, 2011) so the anatomical location of GABA(A)R $\alpha 2$ -containing receptor subtypes functions to facilitate the inhibitory input to the fear-associated neurons during or after extinction. In line with this suggestion, a recent study demonstrated

specific IPSP kinetics expressed by interneurons with different GABA(A)R subunits (Eyre *et al.*, 2012). Moreover, GABA(A)Rs with different α subunits preferentially localize in distinct cellular compartments in cortical and hippocampal neurons (Fritschy and Mohler, 1995; Volk *et al.*, 2002), although to our knowledge no data exist about GABA(A)R $\alpha 1$ and $\alpha 2$ subcellular localization in amygdala nuclei. Recently, the subcellular distribution of key plasticity-related molecules, such as Bdnf, has been shown to play a critical role in learning and memory (Chiaruttini *et al.*, 2009), and further studies should provide better insight into the function of the anatomical location of different GABA(A)R subtypes in fear extinction learning.

4.3. Conclusions

Our data demonstrate that a combination of chronic fluoxetine with extinction training forms a specific synaptic proteins profile associated with an animal's ability to cope with fear during recovery/renewal/reinstatement tasks. Our findings that fluoxetine treatment together with extinction comprises the effects of the NMDAR- and GABA(A)R-potentiating drugs in a subunit- and brain area-specific manner provide a background for further optimization of fear-reducing therapeutic approaches. In this study, we analyzed only a portion of the immense synaptic machinery and in only a few critical brain regions. Future studies should elucidate 1) the synaptic mechanisms of currently used effective pharmacological and non-pharmacological enhancers of fear extinction, 2) whether other SSRIs share a similar potential and mechanisms of extinction facilitation with fluoxetine, 3) how long after discontinuation of fluoxetine exposure the efficacy of the combined treatment persists, and 4) whether human polymorphism in one of the most critical genes described here may underlie a possible failure of the combined therapy in the treatment-resistant patients.

Role of funding source

Funding for this study was provided by the Centre for International Mobility Fellowships program (DP), the ERASMUS Program (UM), Sigrid Jusélius Foundation and the Academy of Finland Center of Excellence program (EC), which had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Contributors

NK designed the study and wrote the protocol. DP, AA, JL, UM, YA and NK conducted the experiments. NK undertook the statistical analysis. DP, EC and NK drafted the manuscript. AA contributed to proof-reading of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

EC is a shareholder of Hermo Pharma, Inc. All other authors declare that they have no conflicts of interest.

Acknowledgments

We thank Dr. Jukka Sallinen, Orion Pharma, for the generous gift of fluoxetine.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.euroneuro.2014.04.002>.

References

- Atack, J.R., 2003. Anxiolytic compounds acting at the GABA(A) receptor benzodiazepine binding site. *Curr Drug Targets CNS Neurol Disord* 2, 213-232.
- Balschun, D., Moechars, D., Callaerts-Vegh, Z., Vermaercke, B., Van Acker, N., Andries, L., D'Hooge, R., 2010. Vesicular glutamate transporter VGLUT1 has a role in hippocampal long-term potentiation and spatial reversal learning. *Cereb Cortex* 20, 684-693.
- Burghardt, N.S., Sigurdsson, T., Gorman, J.M., McEwen, B.S., LeDoux, J.E., 2013. Chronic antidepressant treatment impairs the acquisition of fear extinction. *Biol Psychiatry* 73, 1078-1086.
- Camp, M.C., Macpherson, K.P., Lederle, L., Graybeal, C., Gaburro, S., Debrouse, L.M., Ihne, J.L., Bravo, J.A., O'Connor, R.M., Ciochi, S., Wellman, C.L., Luthi, A., Cryan, J.F., Singewald, N., Holmes, A., 2012. Genetic strain differences in learned fear inhibition associated with variation in neuroendocrine, autonomic, and amygdala dendritic phenotypes. *Neuropsychopharmacology* 37, 1534-1547.
- Chen, J.L., Lin, W.C., Cha, J.W., So, P.T., Kubota, Y., Nedivi, E., 2011. Structural basis for the role of inhibition in facilitating adult brain plasticity. *Nat Neurosci* 14, 587-594.
- Chen, Z.Y., Jing, D., Bath, K.G., Ieraci, A., Khan, T., Siao, C.J., Herrera, D.G., Toth, M., Yang, C., McEwen, B.S., Hempstead, B.L., Lee, F.S., 2006. Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science* 314, 140-143.
- Chhatwal, J.P., Myers, K.M., Ressler, K.J., Davis, M., 2005. Regulation of gephyrin and GABAA receptor binding within the amygdala after fear acquisition and extinction. *J Neurosci* 25, 502-506.
- Chhatwal, J.P., Stanek-Rattiner, L., Davis, M., Ressler, K.J., 2006. Amygdala BDNF signaling is required for consolidation but not encoding of extinction. *Nat Neurosci* 9, 870-872.
- Chiaruttini, C., Vicario, A., Li, Z., Baj, G., Braiuca, P., Wu, Y., Lee, F.S., Gardossi, L., Baraban, J.M., Tongiorgi, E., 2009. Dendritic trafficking of BDNF mRNA is mediated by translin and blocked by the G196A (Val66Met) mutation. *Proc Natl Acad Sci U S A* 106, 16481-16486.
- Clem, R.L., Huganir, R.L., 2010. Calcium-permeable AMPA receptor dynamics mediate fear memory erasure. *Science* 330, 1108-1112.
- Deschaux, O., Spennato, G., Moreau, J.L., Garcia, R., 2011. Chronic treatment with fluoxetine prevents the return of extinguished auditory-cued conditioned fear. *Psychopharmacology (Berl)* 215, 231-237.
- Dulawa, S.C., Holick, K.A., Gundersen, B., Hen, R., 2004. Effects of chronic fluoxetine in animal models of anxiety and depression. *Neuropsychopharmacology* 29, 1321-1330.
- Ehrlich, I., Humeau, Y., Grenier, F., Ciochi, S., Herry, C., Luthi, A., 2009. Amygdala inhibitory circuits and the control of fear memory. *Neuron* 62, 757-771.
- Eyre, M.D., Renzi, M., Farrant, M., Nusser, Z., 2012. Setting the time course of inhibitory synaptic currents by mixing multiple GABA(A) receptor alpha subunit isoforms. *J Neurosci* 32, 5853-5867.
- Fritschy, J.M., Mohler, H., 1995. GABAA-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J Comp Neurol* 359, 154-194.
- Gonzalez-Burgos, G., Rotaru, D.C., Zaitsev, A.V., Povysheva, N.V., Lewis, D.A., 2009. GABA transporter GAT1 prevents spillover at proximal and distal GABA synapses onto primate prefrontal cortex neurons. *J Neurophysiol* 101, 533-547.
- Goodnick, P.J., Goldstein, B.J., 1998. Selective serotonin reuptake inhibitors in affective disorders--I. Basic pharmacology. *J Psychopharmacol* 12, S5-20.

- Guastella, A.J., Lovibond, P.F., Dadds, M.R., Mitchell, P., Richardson, R., 2007. A randomized controlled trial of the effect of D-cycloserine on extinction and fear conditioning in humans. *Behav Res Ther* 45, 663-672.
- Harris, J.A., Westbrook, R.F., 2001. Contextual control over the expression of fear in rats conditioned under a benzodiazepine. *Psychopharmacology (Berl)* 156, 92-97.
- Heldt, S.A., Ressler, K.J., 2007. Training-induced changes in the expression of GABAA-associated genes in the amygdala after the acquisition and extinction of Pavlovian fear. *Eur J Neurosci* 26, 3631-3644.
- Heldt, S.A., Stanek, L., Chhatwal, J.P., Ressler, K.J., 2007. Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. *Mol Psychiatry* 12, 656-670.
- Herrmann, L., Ionescu, I.A., Henes, K., Golub, Y., Wang, N.X., Buell, D.R., Holsboer, F., Wotjak, C.T., Schmidt, U., 2012. Long-lasting hippocampal synaptic protein loss in a mouse model of posttraumatic stress disorder. *PLoS One* 7, e42603.
- Hong, I., Song, B., Lee, S., Kim, J., Choi, S., 2009. Extinction of cued fear memory involves a distinct form of depotentiation at cortical input synapses onto the lateral amygdala. *Eur J Neurosci* 30, 2089-2099.
- Juge, N., Omote, H., Moriyama, Y., 2013. Vesicular GABA transporter (VGAT) transports beta-alanine. *J Neurochem*.
- Karpova, N.N., Lindholm, J., Pruunsild, P., Timmusk, T., Castren, E., 2009. Long-lasting behavioural and molecular alterations induced by early postnatal fluoxetine exposure are restored by chronic fluoxetine treatment in adult mice. *Eur Neuropsychopharmacol* 19, 97-108.
- Karpova, N.N., Pickenhagen, A., Lindholm, J., Tiraboschi, E., Kuleskaya, N., Agustsdottir, A., Antila, H., Popova, D., Akamine, Y., Bahi, A., Sullivan, R., Hen, R., Drew, L.J., Castren, E., 2011. Fear erasure in mice requires synergy between antidepressant drugs and extinction training. *Science* 334, 1731-1734.
- Kim, J., Lee, S., Park, K., Hong, I., Song, B., Son, G., Park, H., Kim, W.R., Park, E., Choe, H.K., Kim, H., Lee, C., Sun, W., Kim, K., Shin, K.S., Choi, S., 2007. Amygdala depotentiation and fear extinction. *Proc Natl Acad Sci U S A* 104, 20955-20960.
- Koester, C., Rudolph, U., Haenggi, T., Papilloud, A., Fritschy, J.M., Crestani, F., 2013. Dissecting the role of diazepam-sensitive gamma-aminobutyric acid type A receptors in defensive behavioral reactivity to mild threat. *Pharmacol Biochem Behav* 103, 541-549.
- Lange, M.D., Doengi, M., Lesting, J., Pape, H.C., Jungling, K., 2012. Heterosynaptic long-term potentiation at interneuron-principal neuron synapses in the amygdala requires nitric oxide signalling. *J Physiol* 590, 131-143.
- Lin, H.C., Mao, S.C., Gean, P.W., 2009. Block of gamma-aminobutyric acid-A receptor insertion in the amygdala impairs extinction of conditioned fear. *Biol Psychiatry* 66, 665-673.
- Lin, H.C., Mao, S.C., Su, C.L., Gean, P.W., 2010. Alterations of excitatory transmission in the lateral amygdala during expression and extinction of fear memory. *Int J Neuropsychopharmacol* 13, 335-345.
- Luscher, B., Shen, Q., Sahir, N., 2011. The GABAergic deficit hypothesis of major depressive disorder. *Mol Psychiatry* 16, 383-406.
- Mahan, A.L., Ressler, K.J., 2012. Fear conditioning, synaptic plasticity and the amygdala: implications for posttraumatic stress disorder. *Trends Neurosci* 35, 24-35.
- Mao, S.C., Hsiao, Y.H., Gean, P.W., 2006. Extinction training in conjunction with a partial agonist of the glycine site on the NMDA receptor erases memory trace. *J Neurosci* 26, 8892-8899.
- Melo, C.V., Mele, M., Curcio, M., Comprido, D., Silva, C.G., Duarte, C.B., 2013. BDNF regulates the expression and distribution of vesicular glutamate transporters in cultured hippocampal neurons. *PLoS One* 8, e53793.
- Myers, K.M., Davis, M., 2007. Mechanisms of fear extinction. *Mol Psychiatry* 12, 120-150.
- Otto, M.W., Bruce, S.E., Deckersbach, T., 2005. Benzodiazepine use, cognitive impairment, and cognitive-behavioral therapy for anxiety disorders: issues in the treatment of a patient in need. *J Clin Psychiatry* 66 Suppl 2, 34-38.
- Owens, M.J., Nemeroff, C.B., 1998. The serotonin transporter and depression. *Depress Anxiety* 8 Suppl 1, 5-12.
- Patterson, S.L., Abel, T., Deuel, T.A., Martin, K.C., Rose, J.C., Kandel, E.R., 1996. Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron* 16, 1137-1145.

- Pereira, M.E., Rosat, R., Huang, C.H., Godoy, M.G., Izquierdo, I., 1989. Inhibition by diazepam of the effect of additional training and of extinction on the retention of shuttle avoidance behavior in rats. *Behav Neurosci* 103, 202-205.
- Peters, J., Dieppa-Perea, L.M., Melendez, L.M., Quirk, G.J., 2010. Induction of fear extinction with hippocampal-infralimbic BDNF. *Science* 328, 1288-1290.
- Quirk, G.J., Mueller, D., 2008. Neural mechanisms of extinction learning and retrieval. *Neuropsychopharmacology* 33, 56-72.
- Quirk, G.J., Pare, D., Richardson, R., Herry, C., Monfils, M.H., Schiller, D., Vicentic, A., 2010. Erasing fear memories with extinction training. *J Neurosci* 30, 14993-14997.
- Rantamaki, T., Hendolin, P., Kankaanpaa, A., Mijatovic, J., Piepponen, P., Domenici, E., Chao, M.V., Mannisto, P.T., Castren, E., 2007. Pharmacologically diverse antidepressants rapidly activate brain-derived neurotrophic factor receptor TrkB and induce phospholipase-Cgamma signaling pathways in mouse brain. *Neuropsychopharmacology* 32, 2152-2162.
- Santini, E., Muller, R.U., Quirk, G.J., 2001. Consolidation of extinction learning involves transfer from NMDA-independent to NMDA-dependent memory. *J Neurosci* 21, 9009-9017.
- Schoenfeld, T.J., Rada, P., Pieruzzini, P.R., Hsueh, B., Gould, E., 2013. Physical exercise prevents stress-induced activation of granule neurons and enhances local inhibitory mechanisms in the dentate gyrus. *J Neurosci* 33, 7770-7777.
- Smith, K.S., Engin, E., Meloni, E.G., Rudolph, U., 2012. Benzodiazepine-induced anxiolysis and reduction of conditioned fear are mediated by distinct GABAA receptor subtypes in mice. *Neuropharmacology* 63, 250-258.
- Soliman, F., Glatt, C.E., Bath, K.G., Levita, L., Jones, R.M., Pattwell, S.S., Jing, D., Tottenham, N., Amso, D., Somerville, L.H., Voss, H.U., Glover, G., Ballon, D.J., Liston, C., Teslovich, T., Van Kempen, T., Lee, F.S., Casey, B.J., 2010. A genetic variant BDNF polymorphism alters extinction learning in both mouse and human. *Science* 327, 863-866.
- Tordera, R.M., Totterdell, S., Wojcik, S.M., Brose, N., Elizalde, N., Lasheras, B., Del Rio, J., 2007. Enhanced anxiety, depressive-like behaviour and impaired recognition memory in mice with reduced expression of the vesicular glutamate transporter 1 (VGLUT1). *Eur J Neurosci* 25, 281-290.
- Walker, D.L., Ressler, K.J., Lu, K.T., Davis, M., 2002. Facilitation of conditioned fear extinction by systemic administration or intra-amygdala infusions of D-cycloserine as assessed with fear-potentiated startle in rats. *J Neurosci* 22, 2343-2351.
- Van Damme, P., Van Den Bosch, L., Van Houtte, E., Callewaert, G., Robberecht, W., 2002. GluR2-dependent properties of AMPA receptors determine the selective vulnerability of motor neurons to excitotoxicity. *J Neurophysiol* 88, 1279-1287.
- Vialou, V., Robison, A.J., Laplant, Q.C., Covington, H.E., 3rd, Dietz, D.M., Ohnishi, Y.N., Mouzon, E., Rush, A.J., 3rd, Watts, E.L., Wallace, D.L., Iniguez, S.D., Ohnishi, Y.H., Steiner, M.A., Warren, B.L., Krishnan, V., Bolanos, C.A., Neve, R.L., Ghose, S., Berton, O., Tamminga, C.A., Nestler, E.J., 2010. DeltaFosB in brain reward circuits mediates resilience to stress and antidepressant responses. *Nat Neurosci* 13, 745-752.
- Vlamos, I., Herry, C., Luthi, A., Aertsen, A., Kumar, A., 2011. Context-dependent encoding of fear and extinction memories in a large-scale network model of the basal amygdala. *PLoS Comput Biol* 7, e1001104.
- Wojcik, S.M., Katsurabayashi, S., Guillemin, I., Friauf, E., Rosenmund, C., Brose, N., Rhee, J.S., 2006. A shared vesicular carrier allows synaptic corelease of GABA and glycine. *Neuron* 50, 575-587.
- Wojcik, S.M., Rhee, J.S., Herzog, E., Sigler, A., Jahn, R., Takamori, S., Brose, N., Rosenmund, C., 2004. An essential role for vesicular glutamate transporter 1 (VGLUT1) in postnatal development and control of quantal size. *Proc Natl Acad Sci U S A* 101, 7158-7163.
- Wolf, B., Griffiths, R.R., 1991. Physical dependence on benzodiazepines: differences within the class. *Drug Alcohol Depend* 29, 153-156.
- Volk, D.W., Pierri, J.N., Fritschy, J.M., Auh, S., Sampson, A.R., Lewis, D.A., 2002. Reciprocal alterations in pre- and postsynaptic inhibitory markers at chandelier cell inputs to pyramidal neurons in schizophrenia. *Cereb Cortex* 12, 1063-1070.

Xue, L., Li, Z.D., Chen, Z.X., Wang, X.G., Shi, Y.W., Zhao, H., 2014. Fear response failed to return in AAB extinction paradigm accompanied with increased NR2B and GluR1 per845 in hippocampal CA1. *Neuroscience* 260, 1-11.