Brain Research Bulletin 192 (2023) 156–167

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

Sex-specific role of hippocampal NMDA-Erk-mTOR signaling in fear extinction of adolescent mice

Emilija Glavonic [a,*], Milos Mitic [a], Ester Francija [a], Zorica Petrovic [a,b], Miroslav Adzic [a]

[a] Department of Molecular Biology and Endocrinology, "VINČA" Institute of Nuclear Sciences-National Institute of the Republic of Serbia, University of Belgrade, Belgrade, Serbia
[b] Dominick P. Purpura Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY 10461, USA

ARTICLE INFO

Keywords:
Fear-related learning
Development
Sex differences
Synaptic plasticity
Glutamate receptors
MTOR pathway

ABSTRACT

Adolescence is a key phase of development for perturbations in fear extinction, with inability to adequately manage fear a potent factor for developing psychiatric disorders in adulthood. However, while behavioral correlates of adolescent fear regulation are established to a degree, molecular mediators of extinction learning in adolescence remain largely unknown. In this study, we observed fear acquisition and fear extinction (across 4 and 7 days) of adolescent and adult mice of both sexes and investigated how hippocampal levels of different plasticity markers relate to extinction learning. While fear was acquired evenly in males and females of both ages, fear extinction was found to be impaired in adolescent males. We also observed lower levels of GluA1, GLUN2A and GLUN2B subunits in male adolescents following fear acquisition, with an increase in their expression, as well as the activity of Erk-mTOR pathway over subsequent extinction sessions, which was paralleled with improved extinction learning. On the other hand, we detected no changes in plasticity-related proteins after fear acquisition in females, with alterations in GluA1, GluA4 and GLUN2B levels across fear extinction sessions. Additionally, we did not discern any pattern regarding the Erk-mTOR activity in female mice associated with their extinction performance. Overall, our research identifies sex-specific synaptic properties in the hippocampus that underlie developmentally regulated differences in fear extinction learning. We also point out hippocampal NMDA-Erk-mTOR signaling as the driving force behind successful fear extinction in male adolescents, highlighting this pathway as a potential therapeutic target for fear-related disorders in the adolescent population.

1. Introduction

Fear-related disorders, such as post-traumatic stress disorder (PTSD), as well as anxiety disorders are highly prevalent, afflictive psychiatric ilnesses (Hoppen and Morina, 2019; Remes et al., 2016) characterized by aberrant processing of fear-inducing experiences resulting in a psychological state of persistent, generalized fear (Lissek, 2012; Norrholm and Jovanovic, 2018). It is believed that the key role in the onset and maintenance of this unremitting form of fear belongs to dysregulated acquisition of fear and its subsequent extinction (Pitman et al., 2012). Furthermore, the still most effective treatment for PTSD and anxiety – cognitive-behavioral exposure therapy – relies on the principles of fear extinction learning (de Quervain et al., 2017). This emphasizes the importance of fear extinction paradigms as vital methods for understanding behavioral and molecular mechanisms involved in the pathophysiology of PTSD and anxiety as well as for optimizing and improving upon existing treatment protocols.

While current treatments for fear-related disorders are administered with moderate efficiency in adults (Popiel et al., 2015; Rauch et al., 2019; Zoellner et al., 2019), less attention is paid to their application in the adolescent population (Mavranezouli et al., 2020; Williams et al., 2022). Adolescence is a developmental window of vulnerability for the onset of a number of psychiatric illnesses (Paus et al., 2008), most notably anxiety (Beesdo et al., 2009; Siegel and Dickstein, 2012), and it is estimated that around 75 % of adults with fear-related disorders met diagnostic criteria during late childhood and adolescence (Kim-Cohen et al., 2003). It is therefore not surprising that adolescence is marked by impaired fear extinction compared to adulthood (Baker and Richardson, 2015; Kim et al., 2011; Lu et al., 2019; McCallum et al., 2010; Pattwell et al., 2012), which highlights the importance of understanding

* Correspondence to: Department of Molecular Biology and Endocrinology, "VINČA" Institute of Nuclear Sciences-National Institute of the Republic of Serbia, University of Belgrade, P.O. Box 522-MBE090, 11001 Belgrade, Serbia.
E-mail address: emilija.glavonic@vin.bg.ac.rs (E. Glavonic).

https://doi.org/10.1016/j.brainresbull.2022.11.011
Received 5 August 2022; Received in revised form 26 September 2022; Accepted 17 November 2022
Available online 18 November 2022

molecular mechanisms of fear regulation in this developmental period.

Furthermore, even though anxiety and PTSD are twice as likely to occur in female subjects (Catuzzi and Beck, 2014; Haskell et al., 2010), a single behavioral study to date explored extinction in adolescent females (Perry et al., 2020), creating the need for deeper analyses of molecular mediators of extinction learning in female mice, particularly adolescents.

In recent years, relatively few rodent studies examined molecular mechanisms of fear extinction during adolescence. Within the existing literature, Pattwell and colleagues demonstrated electrophysiological alterations of glutamatergic transmission and changes in synaptic plasticity in brain regions of adolescent mice involved in extinction learning processes (Pattwell et al., 2012). However, it remains unclear how different N-methyl-D-aspartate (NMDA) and α-amino-3--hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits affect extinction performance in adolescent cohorts. Furthermore, we also explored the role of other plasticity-related proteins in fear extinction, such as neural cell adhesion molecules (NCAMs) and mammalian target of rapamycin (mTOR), along with its upstream stimulator-extracellular signal regulated kinase (Erk) and downstream effector, P70 S6 kinase (P70S6K). Given that hippocampus is heavily involved in different types of associative learning (Corbit and Balleine, 2000; Corcoran et al., 2005; Rajji et al., 2006; Zhang et al., 2017), including both contextual (Corcoran et al., 2005; Lacagnina et al., 2019) and cued extinction (Soler-Cedeno et al., 2019; Zhang et al., 2017), we aimed to investigate how hippocampal levels of these plasticity-related proteins relate to fear extinction across development in both male and female mice.

2. Experimental procedures

2.1. Animals

All experiments were carried out on adolescent and adult (aged 29 and 70 days) male and female C57BL/6 mice acquired from the Jackson Laboratory and bred in our facilities. Animals were housed 4 per standardized cage and had ad libitum access to food and water. They were held under a 12 h light/dark cycle in a room with controlled temperature of 20 ± 2 °C. To account for litter-driven effects, animals from different litters were randomly assigned to each experimental group. All experimental procedures were approved by the Ethical Committee of "VINČA" Institute of Nuclear Sciences-National Institute of the Republic of Serbia (Application No. 10/2019) and by the Ethical Committee of the Veterinary Directorate of the Republic of Serbia (Approval No. 323–07–00275/2020–05). All animal procedures were carried out in compliance with the U.K. Animals (Scientific procedures) Act, 1986 and the EU Directive 2010/63/EU for animal experiments.

2.2. Experimental design

Upon reaching postnatal days 29 and 70 (P29 and P70) – that is, adolescence and adulthood –, animals were subjected to cued fear conditioning and extinction protocols that lasted up to 8 days total. Namely, mice were randomly assigned to one of the three experimental protocols: fear conditioning (FC), fear extinction 4 days (E4) and fear extinction 7 days (E7). Each protocol subset was made up of 4 experimental groups: P29 males, P70 males, P29 females and P70 females, bringing the total to 12 distinct groups, consisting of 12–15 animals each (Fig. 1.).

FC groups were comprised of mice undergoing just the fear conditioning procedure. On the other hand, animals assigned to E4 and E7 groups were fear conditioned and subsequently subjected to 4 or 7 consecutive days of extinction respectively (Fig. 1.). One hour after their last behavioral test, all animals were euthanised by cervical dislocation and their hippocampi were isolated for further molecular analyses.

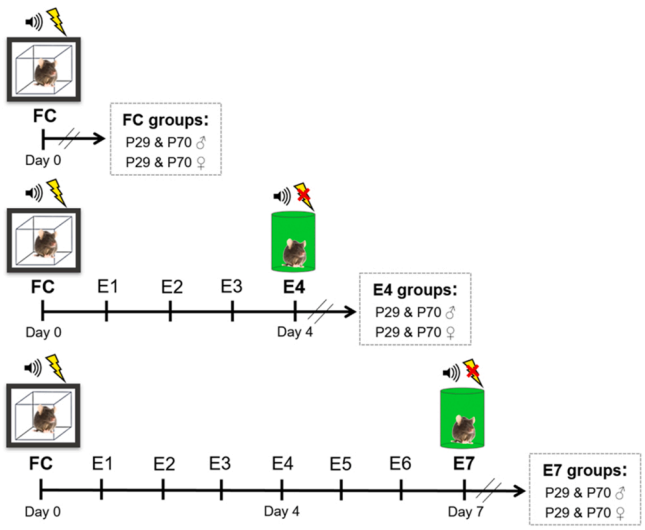

Fig. 1. *Schematic representation of experimental procedures*. Abbreviations – FC: fear conditioning; P29: postnatal day 29; P70: postnatal day 70; E1-E7: fear extinction days 1–7.

2.3. Behavioral analyses

Cued fear conditioning was performed in a specialized square chamber set in a sound-attenuated box (Ugo Basile S.R.L., Gemonio, Italy), with foot shocks delivered via metal rods located on the chamber floor. Within a single trial, mice were first habituated to the conditioning chamber (sprayed with 0.1 % (vol) peppermint extract dissolved in 70 % EtOH) for 2 min and then fear conditioned using three tone-shock presentations. These stimuli pairings included a 30 s (5 kHz, 70 dB) tone (conditioned stimulus, CS) which coterminated with a 0.7-mA foot shock (unconditioned stimulus, US) during the last second. Stimuli presentations were separated by intervals of 30 s (intertrial intervals, ITIs). After the final CS-US presentation, animals were allowed to recover in the conditioning arena for 1 min before being returned to their home cages. The arena was scrubbed clean in between each animal with peppermint-scented ethanol.

24 h following the conditioning procedure, mice were subjected to the first of 4 or 7 extinction trials depending on the group in question. Each subsequent trial was performed 24 h after the previous one. Extinction sessions were carried out in a new arena (green cylindrical chamber scented with 0.1 % (vol) lemon extract dissolved in 70 % EtOH) in order to eliminate the influence of conditioning context on FE. Animals were given 2 min for acclimation to chamber conditions, after which they were exposed to five 30 s tones (5 kHz, 70 dB) in the absence of US. ITIs between the tones lasted 30 s. After the final tone presentation, mice remained in the arena for 1 min before being returned to their home cages. The arena was cleaned after each animal with lemon-scented ethanol.

All behavioral tests were computer-controlled using the EthoVision XT behavioral software (Noldus, Wageningen, the Netherlands) and mice were videotaped for later analysis of freezing behavior by researchers blind to experimental groups. Freezing is defined as the lack of visible muscle movements except ones required for respiration (Pattwell et al., 2012) and was quantified as a species-specific measure of fear. Percentage of time spent freezing was calculated by dividing the number of seconds spent freezing during CS presentation by the duration of the stimulus (30 s). Fear conditioning and fear extinction procedures were performed following protocols established by Pattwell and colleagues.

2.4. Preparation of the synaptosomal fraction

After cervical dislocation, brains were swiftly taken out and

hippocampi dissection was performed on ice. Crude synaptosomal fractions were prepared in line with the protocol outlined by Ronald Duman's lab (Dwyer et al., 2012; Li et al., 2010). In short, tissue was homogenized in a solution containing 0.32 M sucrose, 20 mM HEPES (pH 7.3), 1 mM EDTA, 1 protease inhibitor cocktail, 5 mM NaF and 1 mM NaVO3 and then centrifuged for 10 min at 2800 rpm. Pellets (nuclear fraction) were discarded, while supernatants were additionally centrifuged at 12000 rpm for 10 min to acquire pellets with crude synaptosomes. These pellets were then resuspended and sonicated in RIPA lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 2 mM EDTA, 1 mM NaVO3, 5 mM NaF and 1 protease inhibitor cocktail.

2.5. Western blot analysis

Synaptosomal protein concentration was determined through a spectrophotometric method adjusted by Markwell (Markwell et al., 1978). Samples were then dissolved in denaturing buffer according to Laemmli (Laemmli, 1970) and incubated at 100 °C for 5 min 40 μg of total proteins were loaded on 7.5 % and 10 % gels and subjected to SDS-PAGE electrophoresis at a constant voltage (100 V). Using a standard blot system (mini Transblot, Bio-Rad), proteins were transferred onto a PVDF membrane (Immobilon-P membrane, Millipore) for 2 h at 400 mA or overnight at constant voltage (30 V). The membrane was then blocked for 1 h in 5 % non-fat dry milk dissolved in phosphate-buffered saline and incubated in primary (overnight at 4 °C) and secondary HRP-conjugated antibodies (2 h at room temperature). Dilutions and catalogue numbers of primary antibodies used in this study are presented in Table 1., with β-actin applied as the loading control. Secondary antibodies used for detection were rabbit anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP (Abcam). Signals were detected by enhanced chemiluminescent reagent (Pierce) and SuperSignal Femto Maximum Sensitivity Substrate (Thermo Scientific) after exposure to X-ray film (Fuji Photofilm). Protein bands were analyzed by densitometry using Image J analysis PC software (NIH, Bethesda, MD) and quantities of all analyzed proteins were normalized to β-actin levels.

2.6. Statistical analyses

Data analysis was performed using IBM SPSS Statistics 20, STATISTICA 7 and GraphPad Prism 8 software and within-sex comparisons were carried out on both the behavioral and molecular level. Freezing behavior across fear conditioning and each day of fear extinction was analyzed with two-way repeated measures ANOVA, with Bonferroni correction for post hoc analysis. In addition, we also calculated several behavioral parameters which cannot be mutually compared between different experimental protocols (FC vs E4 vs E7 groups), and this data was consequently analyzed by independent samples t-test. Western blot results were processed by two-way ANOVA, with post hoc Tukey test used for determining between-group differences in order to assess the influence of age, experimental protocol or their interaction on the measured protein levels in male and female mice. All data are presented as a mean value ± S.E.M. and statistical significance was accepted at $p < 0.05$.

Table 1
Dilutions and catalogue numbers of primary antibodies used for Western blot analysis. Abbreviations – GluA1: AMPA receptor subunit 1; GluA4: AMPA receptor subunit 4; GLUN2A: NMDA receptor subunit 2A; GLUN2B: NMDA receptor subunit 2B; psaNCAM: polysialylated neural cell adhesion molecule; NCAM: neural cell adhesion molecule; pErk1/2: phosphorylated extracellular signal-regulated kinase 1/2; Erk1/2: extracellular signal-regulated kinase 1/2; pmTOR: phosphorylated mammalian target of rapamycin; mTOR: mammalian target of rapamycin; pP70S6K: phosphorylated P70 S6 kinase; P70S6K: P70 S6 kinase.

Protein	Dilution	Company	Cat. No.
GluA1	1:500	Santa Cruz Biotechnology	sc-55509
GluA4	1:500	Cell Signaling Technology	#8070
GLUN2A	1:500	Santa Cruz Biotechnology	sc-390094
GLUN2B	1:1000	Millipore	MAB5782
psaNCAM	1:1000	Millipore	MAB5324
NCAM	1:2000	Millipore	AB5032
pErk1/2 (Thr202/Tyr204)	1:1000	Cell Signaling Technology	#9101
Erk1/2	1:1000	Cell Signaling Technology	#9102
pmTOR (Ser 2448)	1:500	Santa Cruz Biotechnology	sc-101738
mTOR	1:600	Santa Cruz Biotechnology	sc-136269
pP70S6K (Thr389)	1:1000	Cell Signaling Technology	#9205
P70S6K	1:1000	Cell Signaling Technology	#9202
β-Actin	1:4000	Abcam	ab8227

3. Results

3.1. Fear acquisition and fear extinction across development

In our research, we performed a detailed examination of fear-related behavior of adolescent and adult mice of both sexes. We monitored animals' freezing behavior and calculated parameters indicative of their performance in different phases of fear-related learning.

First, we wanted to determine whether fear is equally acquired in male and female mice regardless of their age. To that end, we analyzed animals' freezing behavior across the fear conditioning paradigm. Even though male adolescents generally froze more than their adult counterparts, we found no statistically significant differences in fear acquisition across development in either of the sexes (Fig. 2. A and B). We should note that the same behavioral pattern was also observed in animals that went on to experience 4 and 7 days of extinction respectively (data shown in supplementary information, Fig. 1. SI and Fig. 2. SI).

Next, we explored animals' freezing behavior across 4 and 7 days of fear extinction, which allowed us to perform a comprehensive analysis of extinction learning in adolescent and adult mice of both sexes. We found that adolescent males exhibit higher freezing percentages during the first trial of Day 3 and Day 4 in both E4 (Day 3, age x trial: $F(4100)= 3.41$, $p < 0.05$; Day 4, age x trial: $F(4100)= 5.15$, $p < 0.05$; Fig. 3. A) and E7 groups (Day 3, age x trial: $F(4,92)= 4.05$, $p < 0.05$; Day 4, age x trial: $F(4,92)= 4.44$, $p < 0.05$; Fig. 3. C). This difference was abolished by Day 5, with comparable levels of freezing between adolescents and adults continuing until the end of the experiment. On the other hand, females failed to demonstrate any consistent differences in freezing behavior, with adult mice freezing more than adolescents during the second trial of Day 2 and Day 5, but only in the E7 group (Day 2, age: $F(1,22)= 14.27$, $p < 0.05$; Day 5, age: $F(1,22)= 4.37$, $p < 0.05$; Fig. 3. D).

In addition, based on the freezing percentages outlined in Fig. 3, we calculated two behavioral parameters indicative of long-term and short-term extinction learning. Spontaneous recovery of fear with time allows us to evaluate animals' ability to consolidate and retrieve extinction memory. Here, spontaneous recovery (SR) between different days was calculated by subtracting the percentage of freezing at the end of the previous day from the percentage of freezing at the start of the next day of FE. Our results show that adolescent males had greater fear recovery between first and second day in the E7 group (SR1: $t(23) = 3.32$, $p < 0.05$; Fig. 4. B), as well as between second and third (E4, SR2: $t(25) = 3.11$, $p < 0.05$; E7, SR2: $t(23) = 2.95$, $p < 0.05$; Fig. 4. A and 4. B), and third and fourth day of extinction in both E4 and E7 groups (E4, SR3: $t(25) = 3.52$, $p < 0.05$; E7, SR3: $t(23) = 2.97$, $p < 0.05$; Fig. 4. A and B) compared to adult mice. In females, there were some differences in fear recovery across the two ages, but they did not reach statistical significance for any day of FE.

Finally, whereas spontaneous recovery between extinction sessions is indicative of long-term learning processes, within-session reduction of fear can be taken as a measure of short-term extinction acquisition. Within-session extinction is calculated as a differential between the freezing percentage at the beginning and at the end of a single extinction session within any given day of FE. In males, adolescents exhibited greater within-session extinction (WSE) than adults for days 3 and 4 in

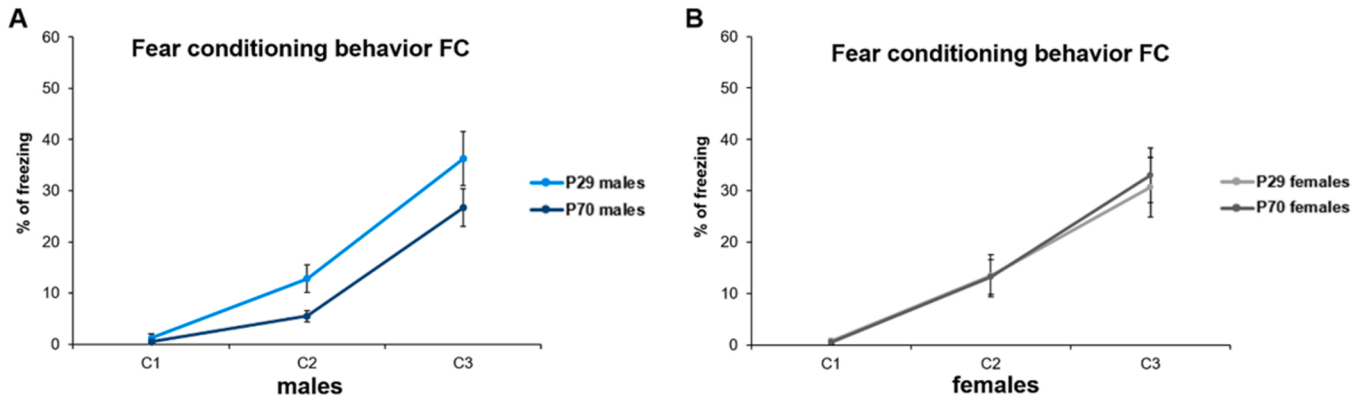

Fig. 2. *Cued fear conditioning across development in male and female mice.* (A) Males' freezing behavior across fear conditioning. (B) Females' freezing behavior across fear conditioning. Data are presented as mean ± SEM and were analyzed with two-way RM ANOVA within each sex, followed by post hoc Bonferroni correction, with conditioning trials as repeated variables and age as the independent variable.

E4 groups (WSE3: $t(25) = 2.21$, $p < 0.05$; WSE4: $t(25) = 3.84$, $p < 0.05$; Fig. 5. A), and for days 3, 4, 6 and 7 in E7 groups (WSE3: $t(23) = 2.53$, $p < 0.05$; WSE4 $t(23) = 3.31$, $p < 0.05$; WSE6: $t(23) = 2.15$, $p < 0.05$; WSE7: $t(23) = 2.25$, $p < 0.05$; Fig. 5. B). We again detected no differences regarding this parameter in female mice, although adolescent females showed a trend towards higher WSE than adult females for Days 1 and 4 in the E7 group ($p < 0.1$).

3.2. Effects of fear extinction on glutamate receptors

In this paper, we investigated the effects of fear learning and extinction across development on the expression of different glutamate receptors, synaptic plasticity markers and kinases of the mTOR signaling pathway. Levels of all the detected proteins were examined in hippocampal synaptoneurosomes of both sexes. Graph values within each sex are presented as a % of their designated controls (FC P29 males and females respectively).

Regarding AMPA receptors, we observed an increase in GluA1 in fear conditioned (FC) male adults relative to adolescents (age x time point: $F(2,18) = 12.40$, $p < 0.05$; Fig. 6. A), whereas in E7 groups, adolescents exhibited higher expression of GluA1 than adults (age x time point: $F(2,18) = 12.40$, $p < 0.05$; Fig. 6. A). In addition, elevated levels of GluA1 as well as GluA4 subunit were detected in E4 and E7 groups of adolescents compared to FC mice (GluA1, time point: $F(2,18) = 12.62$, $p < 0.05$; GluA4, time point: $F(2,18) = 6.93$, $p < 0.05$; Fig. 6. A and B). We also found that E4 group of adolescent mice had greater levels of GluA4 than adults (age x time point: $F(2,18) = 5.01$, $p < 0.05$; Fig. 6. B). On the other hand, adolescent females had reduced levels of GluA1 and GluA4 subunit in E7 compared to E4 groups (GluA1, time point: $F(2,18) = 17.41$, $p < 0.05$; GluA4, time point: $F(2,17) = 15.37$, $p < 0.05$; Fig. 6. A and B), with the FC group also exhibiting lower levels of GluA4 than E4 adolescents (time point: $F(2,17) = 17.94$, $p < 0.05$; Fig. 6. B). In adult females, E4 group was associated with increased amount of GluA1 compared to E7 mice (time point: $F(2,18) = 17.41$, $p < 0.05$; Fig. 6. A), as well as with higher levels of GluA4 in comparison to FC mice (time point: $F(2,17) = 15.37$, $p < 0.05$; Fig. 6. B).

When it comes to NMDA receptors, male mice demonstrated the same pattern of expression for GLUN2A and GLUN2B subunits. Adult fear conditioned animals (FC group) exhibited elevated levels of both subunits compared to their adolescent counterparts (GLUN2A, age x time point: $F(2, 16) = 7.53$, $p < 0.05$; GLUN2B, age x time point: $F(2,17) = 7.81$, $p < 0.05$; Fig. 6. C and D), while GLUN2A and GLUN2B levels were also increased in E4 and E7 groups of adolescents compared to FC mice (GLUN2A, age x time point: $F(2, 16) = 7.53$, $p < 0.05$; GLUN2B, time: $F(2,17) = 4.93$, $p < 0.05$; Fig. 6. C and D). As for females, both adolescent and adult E7 mice had decreased expression of GLUN2B in relation to their respective FC and E4 groups (time point: $F(2,16) = 21.86$, $p < 0.05$; Fig. 6. D).

3.3. Effects of fear extinction on synaptic plasticity markers

In order to examine synaptic plasticity changes during fear learning and extinction in adolescence and adulthood more thoroughly, we detected levels of neural cell adhesion molecule, NCAM and its polysialylated form, psaNCAM. With the exception of E7 groups of female mice, adolescents demonstrated higher levels of psaNCAM than their adult counterparts regardless of their sex (FC, E4, E7 males, age: $F(1, 16) = 105.85$, $p < 0.05$; FC, E4 females, age: $F(1,17) = 101.88$, $p < 0.05$; Fig. 7. A). On the other hand, E7 group of adolescent females demonstrated a substantial drop in psaNCAM levels compared to both FC and E4 adolescents (time point: $F(2,17) = 38.33$, $p < 0.05$; Fig. 7. A).

In regard to NCAM, it is expressed in the form of 3 major isoforms: 120 kDa (NCAM 120), 140 kDa (NCAM 140) and 180 kDa (NCAM 180). We observed no significant changes in the expression of NCAM 120, while NCAM 140 was only altered in the E7 group of adolescent females, which demonstrated lower levels in relation to FC mice (time point: $F(2,17) = 5.37$, $p < 0.05$; Fig. 7. C). However, we observed several notable changes in NCAM 180. In adult males, E7 group exhibited significantly lower levels of NCAM 180 than both E7 adolescents (age: $F(1,17) = 6.56$, $p < 0.05$; Fig. 7. D) and the FC group of adult mice (time point: $F(2,17) = 5.19$, $p < 0.05$; Fig. 7. D). On the other hand, we observed reduced levels of NCAM 180 in the E7 group of adolescent females compared to both FC and E4 mice (time point: $F(2,16) = 8.61$, $p < 0.05$; Fig. 7. D).

3.4. Effects of fear extinction on mTOR signaling pathway

Finally, we took a closer look at how different kinases of the mTOR signaling cascade are altered with exposure to fear learning and its subsequent extinction in our two age cohorts. Since kinase activity is best represented as the ratio of phospho- over total- form, we have decided to showcase and discuss only the ratio of respective kinases of the mTOR pathway.

In males, adolescents of the E7 group had elevated ratios for both Erk1 and Erk2 compared to the adult E7 mice (Erk 1, age: $F(1,18) = 7.59$, $p < 0.05$; Erk 2, age: $F(1,18) = 42.80$, $p < 0.05$; Fig. 8. A and B), with E7 adolescents also exhibiting increased Erk2 ratio relative to their respective FC and E4 groups (age x time point: $F(2,18) = 19.65$, $p < 0.05$; Fig. 8. B). In addition, Erk1 ratio was significantly lower in the E7 adults in comparison to the E4 group (age x time point: $F(2,18) = 6.56$, $p < 0.05$; Fig. 8. A). Regarding females, we detected no changes in Erk 1 activity across our experiments, whereas the ratio of Erk2 was elevated in adolescents of the E7 group compared to the E7 adults (age x time point: $F(2,18) = 5.62$, $p < 0.05$; Fig. 8. B). Somewhat surprisingly,

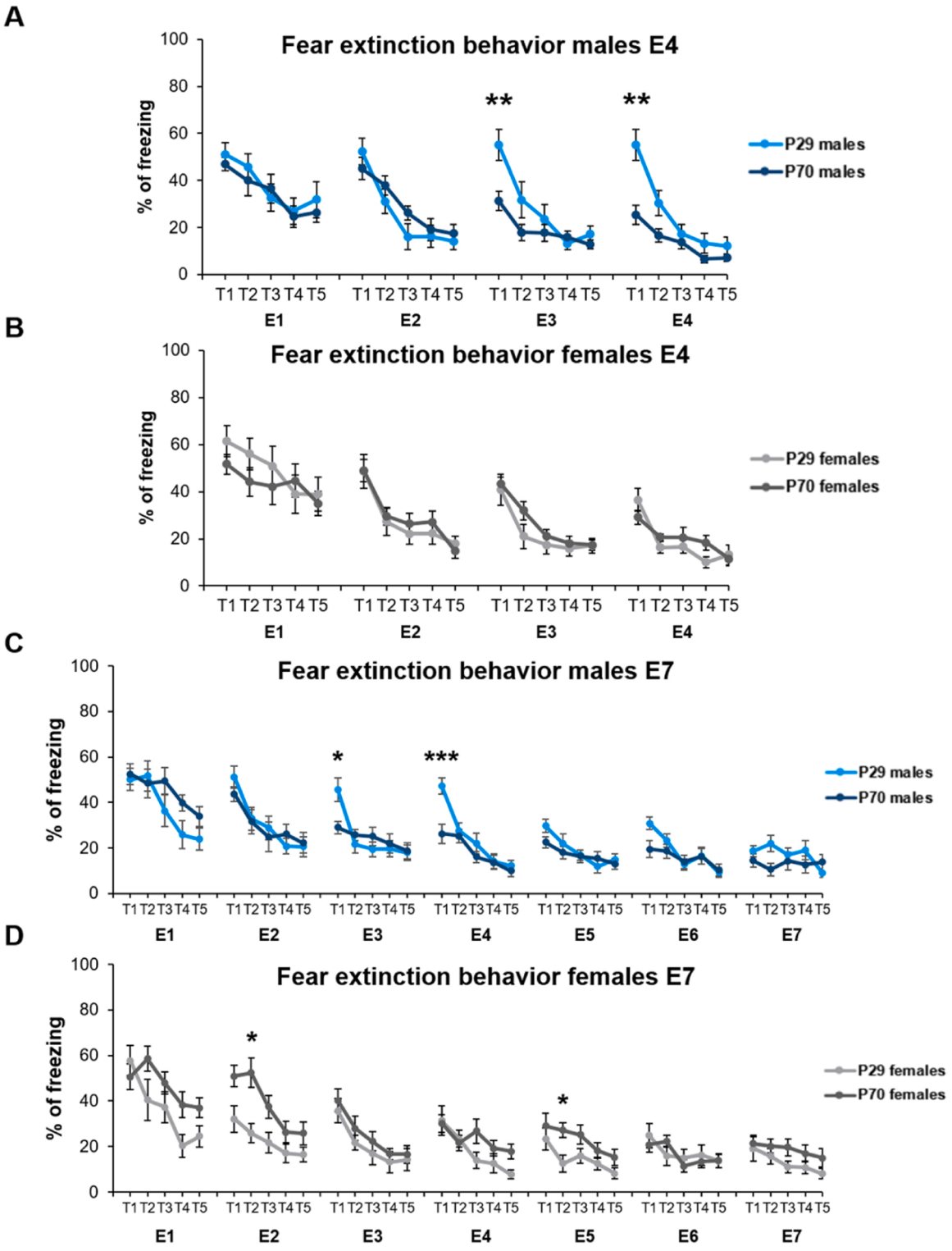

Fig. 3. *Cued fear extinction across development in male and female mice.* (A) Males' freezing behavior across 4 days of extinction. (B) Females' freezing behavior across 4 days of extinction. (C) Males' freezing behavior across 7 days of extinction. (D) Females' freezing behavior across 7 days of extinction. Data are presented as mean ± SEM and were analyzed with two-way RM ANOVA for each extinction day, followed by post hoc Bonferroni correction, with trial as repeated variable and age as an independent variable. Statistically significant differences are given as $p < 0.05$. (* P29 vs. P70).

there were no differences in the ratio of mTOR in either of the sexes (Fig. 8. C). However, we discovered a number of notable changes regarding the activity of the downstream kinase of mTOR, P70S6K. Namely, FC adolescent males had decreased ratio of P70S6K relative to their adult counterparts (age x time point: $F(2,16)= 31.48$, $p < 0.05$; Fig. 8. D), whereas P70S6K ratio was elevated in E4 and E7 adolescent males compared to E4 and E7 adult mice (age x time point: $F(2,16)= 31.48$, $p < 0.05$; Fig. 8. D). FC adolescents also exhibited lower ratio than E4 and E7 mice of the same age (age x time point: $F(2,16)= 31.48$, $p < 0.05$; Fig. 8. D), while FC adults had elevated ratio relative to their E4 and E7 groups (age x time point: $F(2,16)= 31.48$, $p < 0.05$; Fig. 8. D). Regarding females, differences between our two age cohorts could be

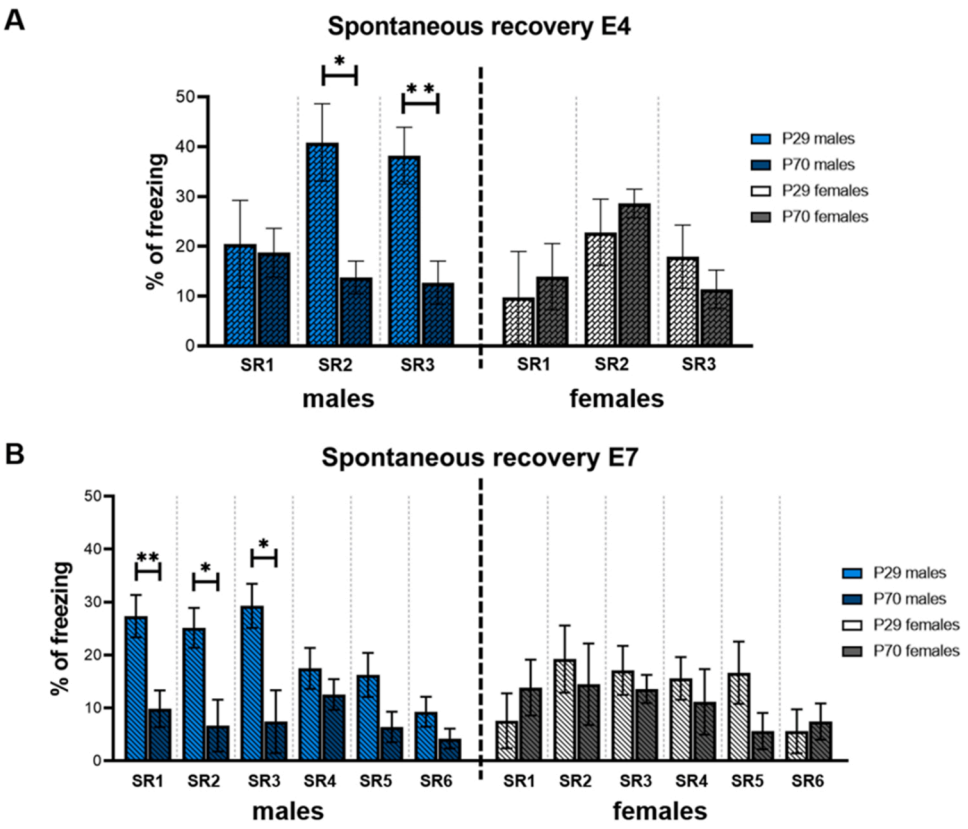

Fig. 4. *Spontaneous recovery of fear across development in male and female mice*. Freezing percentages for spontaneous recovery in E4 (A) and E7 (B) groups, calculated as follows: SR1 = E2T1-E1T5 ([Ext Day 2, Tone 1] - [Ext Day 1, Tone 5]); SR2 = E3T1-E2T5 ([Ext Day 3, Tone 1] - [Ext Day 2, Tone 5]); SR3 = E4T1-E3T5 ([Ext Day 4, Tone 1] - [Ext Day 3, Tone 5]); SR4 = E5T1-E4T5 ([Ext Day 5, Tone 1] - [Ext Day 4, Tone 5]); SR5 = E6T1-E5T5 ([Ext Day 6, Tone 1] - [Ext Day 5, Tone 5]); SR6 = E7T1-E6T5 ([Ext Day 7, Tone 1] - [Ext Day 6, Tone 5]). Data are presented as mean ± SEM and were analyzed within each sex with independent t-tests for each spontaneous recovery parameter. Statistically significant differences are given as $p < 0.05$. (* P29 vs. P70).

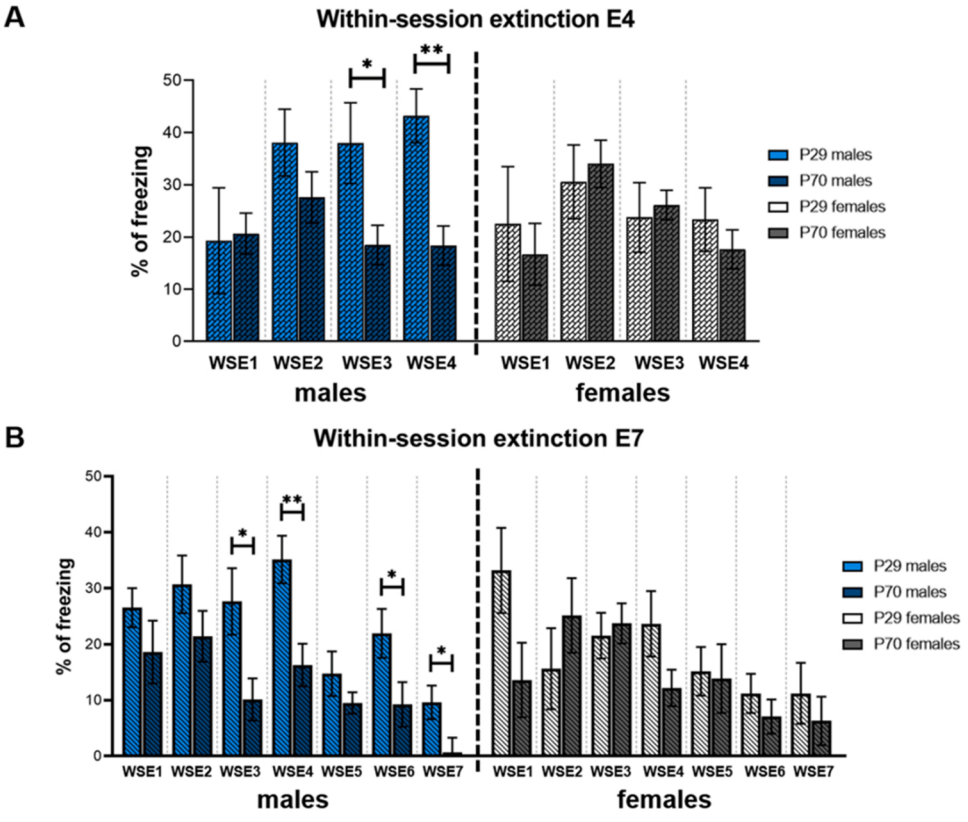

Fig. 5. *Within-session extinction of fear across development in male and female mice*. Freezing percentages for within-session extinction in E4 (A) and E7 (B) groups, calculated as follows: WSE1 = E1T1-E1T5 ([Ext Day 1, Tone 1] - [Ext Day 1, Tone 5]); WSE2 = E2T1-E2T5 ([Ext Day 2, Tone 1] - [Ext Day 2, Tone 5]); WSE3 = E3T1-E3T5 ([Ext Day 3, Tone 1] - [Ext Day 3, Tone 5]); WSE4 = E4T1-E4T5 ([Ext Day 4, Tone 1] - [Ext Day 4, Tone 5]); WSE5 = E5T1-E5T5 ([Ext Day 5, Tone 1] - [Ext Day 5, Tone 5]); WSE6 = E6T1-E6T5 ([Ext Day 6, Tone 1] - [Ext Day 6, Tone 5]); WSE7 = E7T1-E7T5 ([Ext Day 7, Tone 1] - [Ext Day 7, Tone 5]). Data are presented as mean ± SEM and were analyzed within each sex with independent t-tests for each day of extinction. Statistically significant differences are given as $p < 0.05$. (* P29 vs. P70).

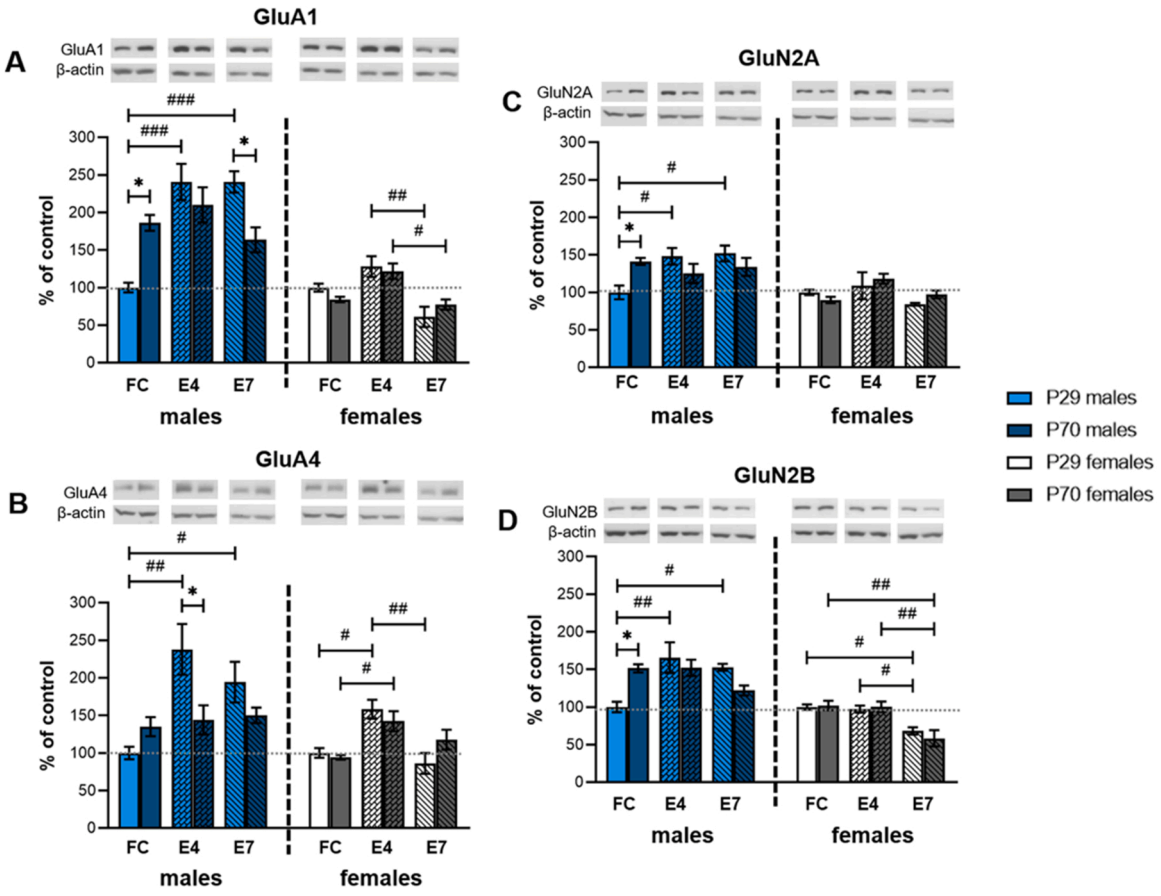

Fig. 6. *Protein expression of glutamate receptor subunits*. Levels of AMPA (A, B) and NMDA (C, D) subunits were detected in the hippocampal synaptosomal fraction of fear conditioned (FC) and fear extinguished (E4 and E7) adolescent and adult mice of both sexes. Graph values within each sex are presented as a % of their designated controls (FC P29 males and females respectively). Data are presented as mean ± SEM and were analyzed with two-way ANOVA within each sex, followed by Tukey's post hoc test with age and time point as independent variables. Statistically significant differences are given as $p < 0.05$. (* P29 vs. P70; # FC vs. E4 vs. E7).

seen in the E4 and E7 groups. While E4 adolescents had decreased activity of P70S6K relative to E4 adults (age x time point: $F(2,16) = 27.54$, $p < 0.05$; Fig. 8. D), E7 group of adolescents demonstrated greater kinase ratio than adult E7 mice (age x time point: $F(2,16) = 27.54$, $p < 0.05$; Fig. 8. D). Furthermore, while P70S6K activity in adolescents was lower in the E4 group versus both FC and E7 groups (time point: $F(2,16) = 13.99$, $p < 0.05$; Fig. 8. D), adult females exhibited decreased P70S6K ratio in E7 mice relative to their respective FC and E4 groups (time point: $F(2,16) = 13.99$, $p < 0.05$; Fig. 8. D).

4. Discussion

In our study, we observed fear extinction behavior of adolescent and adult mice of both sexes across 4 and 7 days of extinction sessions. Furthermore, we explored the role of hippocampal glutamate receptors, cell adhesion molecules and Erk-mediated mTOR signaling in extinction learning across development in both males and females.

4.1. Adolescence is marked by impaired fear extinction consolidation in a sex-specific manner

Since alterations in extinction behavior can be a consequence of differences in fear acquisition, we first demonstrated that males and females of both age cohorts acquire fear pretty evenly. While Florido and colleagues reported higher freezing in adolescent mice across fear conditioning compared to adults (Florido et al., 2021), several other studies showed that adolescents and adults exhibit comparable freezing levels during conditioning (Colon et al., 2018; Kim et al., 2011; Lu et al., 2019; McCallum et al., 2010). Coupled with our results, this indicates that any changes in extinction across development depend solely on altered extinction processes.

Next, we showed that adolescent males have significantly higher freezing percentages at the beginning of Day 3 and Day 4 than adults, indicating less efficient fear extinction learning of this cohort. In addition, they also demonstrated higher recovery of fear responses across the first 4 days. As spontaneous recovery relates to long-term learning processes, this indicates that the observed impairment is related to diminished consolidation, retention, and/or recall of extinction memory (Quirk and Mueller, 2008). On the other hand, the efficacy of short-term learning and acquisition of extinction is reflected in the performance within individual extinction sessions (Quirk and Mueller, 2008). Here, adolescents had similar or even higher reduction of freezing than adults, which, contrary to the findings of Pattwell and colleagues (Pattwell et al., 2012), suggests they had no trouble acquiring fear extinction. However, as we previously argued, they struggled with extinction consolidation, retention and/or recall. Several studies, carried out in rats, reported elevated fear recovery and problems with extinction retention in male adolescents (Kim et al., 2011; McCallum et al., 2010). Interestingly, while there are some notable differences in fear processing between mice and rats (Bisby et al., 2021), our results closely matched those obtained in adolescent rats (Kim et al., 2011; McCallum et al., 2010). McCallum and peers also showed that doubling the number of extinction trials can significantly improve long-term extinction in adolescents (McCallum et al., 2010), which led us to conduct extinction

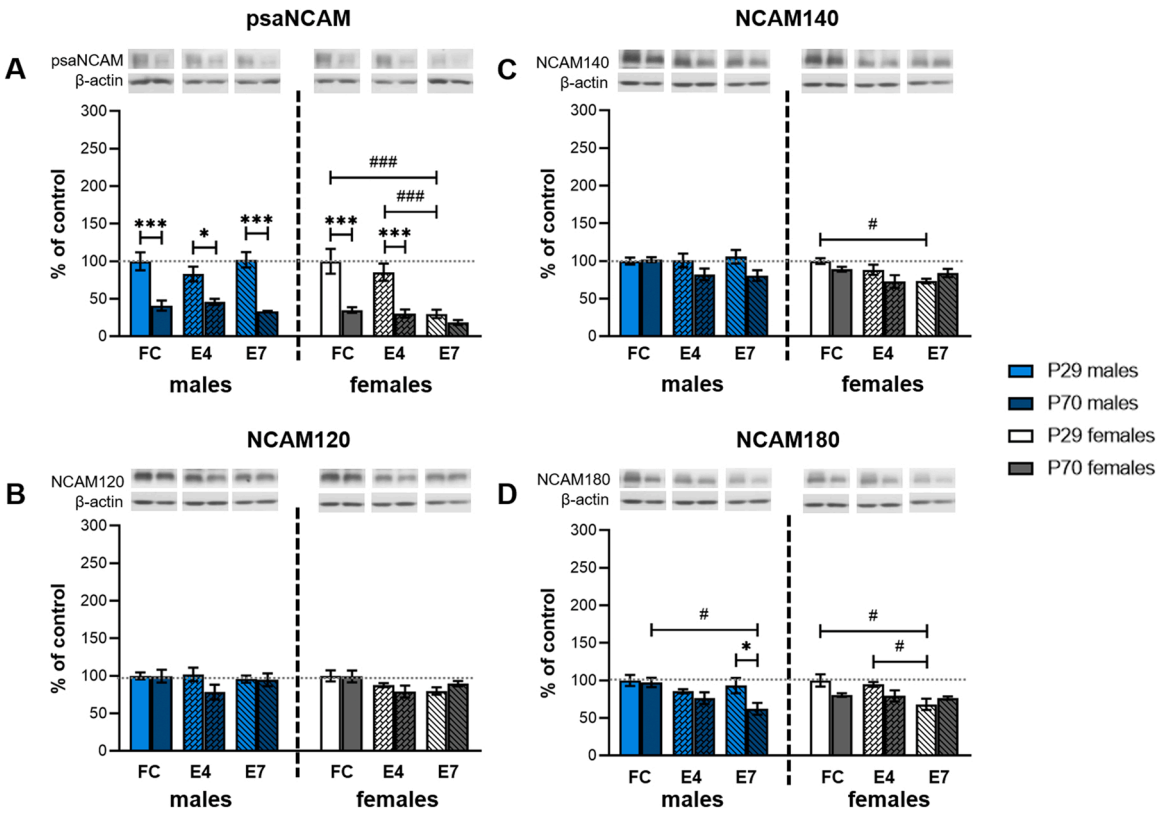

Fig. 7. *Protein expression of neural cell adhesion molecules.* Levels of psaNCAM (A) and NCAM (B, C, D) isoforms were detected in the hippocampal synaptosomal fraction of fear conditioned (FC) and fear extinguished (E4 and E7) adolescent and adult mice of both sexes. Graph values within each sex are presented as a % of their designated controls (FC P29 males and females respectively). Data are presented as mean ± SEM and were analyzed with two-way ANOVA within each sex, followed by Tukey's post hoc test with age and time point as independent variables. Statistically significant differences are given as $p < 0.05$. (* P29 vs. P70; # FC vs. E4 vs. E7).

sessions over the additional 3 days. Male adolescents exhibited marked improvement in extinction learning at Day 5 already, as they demonstrated comparable freezing levels to adult mice. This pattern then continued across the following 2 days, solidifying the link between the number of sessions and efficiency of extinction learning in adolescents.

To the best of our knowledge, our research also represents the first direct comparison of extinction between adolescent and adult females. Here, adolescent females failed to show any impairments in extinction learning compared to adults, with adult mice even freezing more during two separate trials. However, this was not consistenly replicated across our experiments. Coupled with a lack of differences in fear recovery and within-session extinction, we argue that, overall, females have equally successful extinction learning across development. This absence of developmental differences in extinction of female mice could be attributed to the beneficial effects of gonadal hormones, especially estradiol, on extinction learning (Lebron-Milad and Milad, 2012). However, females have only just begun producing significant concentrations of gonadal hormones during adolescence (Bell, 2018), with elevated estradiol even associated with less efficient extinction (Perry et al., 2020), suggesting that other mechanisms could be responsible for the absence of developmental impairment in females' extinction learning. We should also note here that Perry and colleagues examined fear extinction in rats, and this should be considered when discussing extinction of adolescent mice, due to species-related differences in fear processing of rats and mice (Bisby et al., 2021).

4.2. Glutamate receptors are involved in fear extinction across development in both male and female mice

The dysregulation of the glutamatergic system across the corticolimbic circuitry is heavily associated with fear-related psychopathology (Averill et al., 2017), highlighting the crucial role of glutamate receptors in processing fear-inducing experiences. In our study, fear conditioned adolescent males exhibited lower levels of GluA1, GLUN2A and GLUN2B subunits compared to adults. This was not associated with any differences in fear conditioning, possibly due to fear learning being a primarily amygdala-dependent process (Keifer et al., 2015; Maren and Quirk, 2004). However, it likely contributed to impaired extinction of adolescent males down the line, as AMPA and NMDA receptors are important for successful extinction learning (Ren et al., 2013; Xue et al., 2014; Zhang et al., 2017), owing to their role in activity-dependent synaptic plasticity (Sanderson et al., 2008; Shipton and Paulsen, 2014). As the expression of GluA1, GLUN2A and GLUN2B increased with extinction training sessions, adolescents normalized their freezing behavior. However, GluA1, GLUN2A and GLUN2B peaked at Day 4 already, whereas extinction improvement is not seen until Day 5, indicating they may be involved in extinction consolidation, which corresponds to previous studies linking GluA1 to consolidation of reward-based associative learning (Cai et al., 2013) and NMDA receptors to consolidation of fear extinction (Santini et al., 2001). In addition, GluA4 subunit is also up-regulated as adolescents undergo extinction sessions, exerting a similarly delayed effect as GluA1. Contrary to male mice, adolescent females did not experience any extinction deficits, with similar patterns of expression for GluA1, GluA4 and GLUN2B in both age cohorts. Our results suggest that GluA1, GluA4 and GLUN2B are involved in females' fear extinction during its initial days and regardless of their age, with subsequent decrease in GluA1 and GLUN2B possibly a result of self-regulatory synaptic scaling-down (Perez-Otano and Ehlers, 2005; Wang et al., 2012). Altogether, GluA1, GluA4 and GLUN2B are all implicated in fear extinction of female mice,

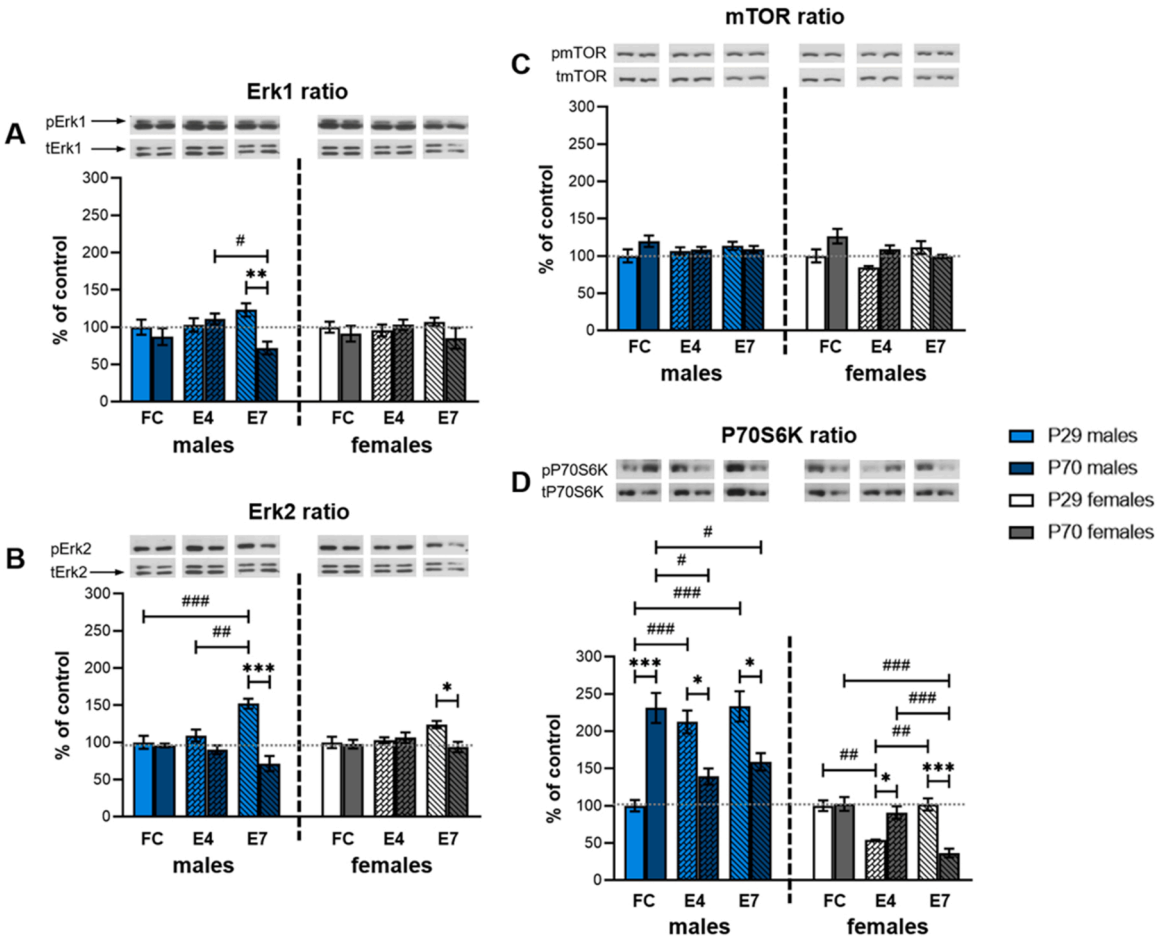

Fig. 8. *Protein expression of kinases of the mTOR signaling pathway.* Levels of Erk1, Erk2, mTOR, and P70S6K were detected in the hippocampal synaptosomal fraction of fear conditioned (FC) and fear extinguished (E4 and E7) adolescent and adult mice of both sexes. All proteins are represented as the ratio between their phospho- and total- forms. Graph values within each sex are presented as a % of their designated controls (FC P29 males and females respectively). Data are presented as mean ± SEM and were analyzed with two-way ANOVA within each sex, followed by Tukey's post hoc test with age and time point as independent variables. Statistically significant differences are given as $p < 0.05$. (* P29 vs. P70; # FC vs. E4 vs. E7).

with AMPA receptors seemingly playing a more prominent role. As AMPARs mediate faster synaptic transmission than NMDARs (Clements et al., 1998), this offers a potential explanation for females' overall more efficient extinction response. However, this result could also be attributed to the density of hippocampal AMPA receptors being significantly influenced by hormonal changes experienced during the estrous cycle (Palomero-Gallagher et al., 2003). On the whole, our results suggest that glutamate receptors play an important role in extinction learning of adolescent and adult mice of both sexes, with AMPA and NMDA up-regulation closely associated with extinction improvements in male adolescents.

4.3. *psaNCAM is developmentally regulated in both sexes*

Apart from glutamate receptors, cell adhesion molecules represent key regulators of synaptic plasticity, playing an essential role in the formation, maturation and maintenance of synapses (Sytnyk et al., 2017). While NCAM aids in the stabilization of potentiated synapses, the attachment of negatively charged PSA tail negatively influences cell adhesion, promoting axonal outgrowth and branching as well as synaptogenesis (Kleene and Schachner, 2004; Rutishauser and Landmesser, 1996). As expression of psaNCAM is most notable during development (Kleene and Schachner, 2004), it is not surprising that adolescents exhibited higher levels of psaNCAM than adults, regardless of their sex. However, psaNCAM levels of adolescent females at Day 7 fell to those of adult mice, whereas psaNCAM of adolescent males remained elevated relative to adults. Since polysialylated NCAM is required for the establishment of long-term memory (Lopez-Fernandez et al., 2007; Seymour et al., 2008), this indicates that adolescent males still actively consolidated extinction memory at this point, likely due to their initially impaired extinction learning. In addition, they maintained the levels of synapse-stabilizing NCAM 180 kDa isoform (Fux et al., 2003), further indicating their prolonged engagement in extinction learning. On the other hand, female adolescents' NCAM 140 and NCAM 180 levels were down-regulated by Day 7, as they demonstrated faster and more efficient extinction than their male counterparts. However, while NCAMs do contribute to stabilization of extinction memory, their levels, particularly that of psaNCAM, seem to be more developmentally regulated rather than associated with extinction performance.

4.4. *Erk-mediated mTOR pathway potentiates fear extinction of adolescent males*

Finally, mTOR is an integrator of cell signaling involved in dendritic translational control and the resulting long-term synaptic plasticity (Hoeffer and Klann, 2010), which forms the basis of long-term extinction memory. As mTOR helps shape neuronal response to activity-induced signals, its function is closely related to glutamate receptors and various protein kinases sensitive to extracellular signals (Hoeffer and Klann, 2010). Of them, Erk was consistently reported to be involved in extinction-related learning processes (Cestari et al., 2014; Matsuda et al., 2015), which prompted us to explore the role of Erk-mediated

mTOR pathway in extinction learning of adolescents and adults. In males, adolescent E7 group demonstrated greater ratios of Erk 1, Erk 2 and P70S6K than adults, with notable increase in kinase activity across 7 days of extinction for Erk 2 and P70S6K. As adolescents likely still consolidate extinction memory on Day 7, this suggests that Erk and P70S6K participate in their extinction consolidation, whereas the extinction-driven increase in Erk2 and P70S6K activity contributed to adolescents overcoming their initial extinction impairment. However, while we detected elevated kinase activity of adolescents up- and downstream of mTOR, its own activity was surprisingly unaltered. Additionally, the activity of P70S6K was partially independent of Erk, which could be explained by its complex regulation via numerous other kinases (Qiu et al., 2004; Romanelli et al., 2002). For the most part, our results match the existing literature in the field of fear extinction. Erk has a longstanding history of being involved in memory consolidation processes (Cestari et al., 2014), and its phosphorylation in the prefrontal cortex is necessary for fear extinction consolidation in adult and adolescent rats (Hugues et al., 2006; Kim et al., 2011). Meanwhile, the activity of P70S6K across the corticolimbic circuitry was previously implicated in extinction acquisition and recall (Girgenti et al., 2017; Huynh et al., 2018). Regarding mTOR, it is possible that we failed to observe changes in its phosphorylation status due to its quick dynamics, as associative learning can be followed by very rapid and transient mTOR activation in the hippocampus (Bekinschtein et al., 2007). This is further bolstered by a finding that fear conditioning had no effect on mTOR phosphorylation in the amygdala 40 mins after the behavioral session (Florido et al., 2021). Finally, Girgenti and colleagues demonstrated that facilitation of fear extinction via mTOR complex 1 signaling is not necessarily followed by mTOR phosphorylation, as long as its upstream (Erk) and downstream (P70S6K) kinases are active (Girgenti et al., 2017). As for female mice, we found no discernable pattern of expression for kinases across the Erk-mTOR pathway, suggesting some other molecular mechanism is responsible for their extinction learning.

Looking at the broader picture, Erk-mediated mTOR signaling is coupled to both NMDA and AMPA receptors. NMDAR activation evokes calcium spikes (Grienberger et al., 2014), with reports of both GLUN2A- and GLUN2B-dependent calcium influx contributing to Erk phosphorylation (El Gaamouch et al., 2012; Jin and Feig, 2010; Kim et al., 2005; Krapivinsky et al., 2003; Sun et al., 2018). Consequently, elevated Erk activity of male adolescents on Day 7 could be a result of increased and maintained GLUN2A and GLUN2B levels observed after extinction sessions. On the other hand, synaptic expression of GluA1 is one of the endpoints of mTOR signaling and P70S6K activation (Li et al., 2010). Here, P70S6K expression is mostly aligned with that of GluA1, with elevated GluA1 levels in male adolescents on Day 7, which highlights this subunit as one of the effectors of Erk-mTOR-P70S6K-dependent consolidation of extinction learning.

4.5. Limitations

Our research should be interpreted considering several limitations. Firstly, while we observed differences on behavioral and molecular level between male and female mice, we did not delve into the responsible mechanisms, as it was not a primary goal of this study. However, since gonadal hormones influence extinction learning (Lebron-Milad and Milad, 2012; Pace-Schott et al., 2013), it would be useful to analyze their levels and study the effects on plasticity-related proteins implicated in fear extinction. Additionally, owing to the complexity of experimental design and a large number of analyzed proteins, we pooled our samples, which precluded us from correlating protein levels to individual animals' behavior, an approach that would be better suited for characterizing individual differences in extinction learning.

4.6. Conclusion

Overall, our study shows that adolescent males have impaired fear extinction, more specifically impaired consolidation of extinction, with no significant differences found in female mice, indicating that adolescence is marked by aberrant fear extinction in a sex-specific manner. Furthermore, we identify sex-specific synaptic properties in the hippocampus that underlie developmentally regulated differences in extinction learning. Namely, our results point towards the hippocampal NMDA-Erk-mTOR signaling being the driving force behind successful extinction learning of adolescent males, highlighting this pathway as a potential target for augmenting fear extinction and ameliorating symptoms of fear-related disorders in the adolescent population.

CRediT authorship contribution statement

Emilija Glavonic: Investigation, Formal analysis, Writing – original draft. **Milos Mitic**: Investigation, Writing – review & editing. **Ester Francija**: Investigation. **Zorica Petrovic**: Investigation. **Miroslav Adzic**: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

Authors declare no conflict of interest. The funder of the study had no role in the study design, the collection, analysis and interpretation of the data, the writing of the report, and the decision to submit the article for publication.

Data Availability

Data will be made available on request.

Acknowledgement

This work was supported by the Ministry of Education, Science and Technological Development, Republic of Serbia, Grant Number 451-03-68/2022-14/200017. The funder had no role in the study design, the collection, analysis and interpretation of the data, the writing of the report, and the decision to submit the article for publication.

Author's statement

The authors state that this manuscript has not been published elsewhere and it is not under consideration by another journal. All authors have read the manuscript and approved its submission to Brain Research Bulletin.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.brainresbull.2022.11.011.

References

Averill, L.A., Purohit, P., Averill, C.L., Boesl, M.A., Krystal, J.H., Abdallah, C.G., 2017. Glutamate dysregulation and glutamatergic therapeutics for PTSD: evidence from human studies. Neurosci. Lett. 649, 147–155. https://doi.org/10.1016/j.neulet.2016.11.064.

Baker, K.D., Richardson, R., 2015. Forming competing fear learning and extinction memories in adolescence makes fear difficult to inhibit. Learn. Mem. 22 (11), 537–543. https://doi.org/10.1101/lm.039487.114.

Beesdo, K., Knappe, S., Pine, D.S., 2009. Anxiety and anxiety disorders in children and adolescents: developmental issues and implications for DSM-V. Psychiatr. Clin. North Am. 32 (3), 483–524. https://doi.org/10.1016/j.psc.2009.06.002.

Bekinschtein, P., Katche, C., Slipczuk, L.N., Igaz, L.M., Cammarota, M., Izquierdo, I., Medina, J.H., 2007. mTOR signaling in the hippocampus is necessary for memory formation. Neurobiol. Learn. Mem. 87 (2), 303–307. https://doi.org/10.1016/j.nlm.2006.08.007.

Bell, M.R., 2018. Comparing postnatal development of gonadal hormones and associated social behaviors in rats, mice, and humans. Endocrinology 159 (7), 2596–2613. https://doi.org/10.1210/en.2018-00220.

Bisby, M.A., Stylianakis, A.A., Baker, K.D., Richardson, R., 2021. Fear extinction learning and retention during adolescence in rats and mice: a systematic review. Neurosci. Biobehav. Rev. 131, 1264–1274. https://doi.org/10.1016/j.neubiorev.2021.10.044.
Cai, Y.Q., Wang, W., Hou, Y.Y., Zhang, Z., Xie, J., Pan, Z.Z., 2013. Central amygdala GluA1 facilitates associative learning of opioid reward. J. Neurosci.: Off. J. Soc. Neurosci. 33 (4), 1577–1588. https://doi.org/10.1523/JNEUROSCI.1749-12.2013.
Catuzzi, J.E., Beck, K.D., 2014. Anxiety vulnerability in women: a two-hit hypothesis. Exp. Neurol. 259, 75–80. https://doi.org/10.1016/j.expneurol.2014.01.023.
Cestari, V., Rossi-Arnaud, C., Saraulli, D., Costanzi, M., 2014. The MAP(K) of fear: from memory consolidation to memory extinction. Brain Res. Bull. 105, 8–16. https://doi.org/10.1016/j.brainresbull.2013.09.007.
Clements, J.D., Feltz, A., Sahara, Y., Westbrook, G.L., 1998. Activation kinetics of AMPA receptor channels reveal the number of functional agonist binding sites. The. J. Neurosci.: Off. J. Soc. Neurosci. 18 (1), 119–127.
Colon, L., Odynocki, N., Santarelli, A., Poulos, A.M., 2018. Sexual differentiation of contextual fear responses. Learn. Mem. 25 (5), 230–240. https://doi.org/10.1101/lm.047159.117.
Corbit, L.H., Balleine, B.W., 2000. The role of the hippocampus in instrumental conditioning. J. Neurosci.: Off. J. Soc. Neurosci. 20 (11), 4233–4239.
Corcoran, K.A., Desmond, T.J., Frey, K.A., Maren, S., 2005. Hippocampal inactivation disrupts the acquisition and contextual encoding of fear extinction. J. Neurosci.: Off. J. Soc. Neurosci. 25 (39), 8978–8987. https://doi.org/10.1523/JNEUROSCI.2246-05.2005.
Dwyer, J.M., Lepack, A.E., Duman, R.S., 2012. mTOR activation is required for the antidepressant effects of mGluR(2)/(3) blockade. Int. J. Neuropsychopharmacol. 15 (4), 429–434. https://doi.org/10.1017/S1461145711001702.
El Gaamouch, F., Buisson, A., Moustie, O., Lemieux, M., Labrecque, S., Bontempi, B., De Koninck, P., Nicole, O., 2012. Interaction between alphaCaMKII and GluN2B controls ERK-dependent plasticity. J. Neurosci.: Off. J. Soc. Neurosci. 32 (31), 10767–10779. https://doi.org/10.1523/JNEUROSCI.5622-11.2012.
Florido, A., Velasco, E.R., Soto-Faguas, C.M., Gomez-Gomez, A., Perez-Caballero, L., Molina, P., Nadal, R., Pozo, O.J., Saura, C.A., Andero, R., 2021. Sex differences in fear memory consolidation via Tac2 signaling in mice. Nat. Commun. 12 (1), 2496. https://doi.org/10.1038/s41467-021-22911-9.
Fux, C.M., Krug, M., Dityatev, A., Schuster, T., Schachner, M., 2003. NCAM180 and glutamate receptor subtypes in potentiated spine synapses: an immunogold electron microscopic study. Mol. Cell. Neurosci. 24 (4), 939–950. https://doi.org/10.1016/j.mcn.2003.07.001.
Girgenti, M.J., Ghosal, S., LoPresto, D., Taylor, J.R., Duman, R.S., 2017. Ketamine accelerates fear extinction via mTORC1 signaling. Neurobiol. Dis. 100, 1–8. https://doi.org/10.1016/j.nbd.2016.12.026.
Grienberger, C., Chen, X., Konnerth, A., 2014. NMDA receptor-dependent multidendrite Ca(2+) spikes required for hippocampal burst firing in vivo. Neuron 81 (6), 1274–1281. https://doi.org/10.1016/j.neuron.2014.01.014.
Haskell, S.G., Gordon, K.S., Mattocks, K., Duggal, M., Erdos, J., Justice, A., Brandt, C.A., 2010. Gender differences in rates of depression, PTSD, pain, obesity, and military sexual trauma among Connecticut War Veterans of Iraq and Afghanistan. J. Women's Health 19 (2), 267–271. https://doi.org/10.1089/jwh.2008.1262.
Hoeffer, C.A., Klann, E., 2010. mTOR signaling: at the crossroads of plasticity, memory and disease. Trends Neurosci. 33 (2), 67–75. https://doi.org/10.1016/j.tins.2009.11.003.
Hoppen, T.H., Morina, N., 2019. The prevalence of PTSD and major depression in the global population of adult war survivors: a meta-analytically informed estimate in absolute numbers. Eur. J. Psychotraumatol. 10 (1), 1578637. https://doi.org/10.1080/20008198.2019.1578637.
Hugues, S., Chessel, A., Lena, I., Marsault, R., Garcia, R., 2006. Prefrontal infusion of PD098059 immediately after fear extinction training blocks extinction-associated prefrontal synaptic plasticity and decreases prefrontal ERK2 phosphorylation. Synapse 60 (4), 280–287. https://doi.org/10.1002/syn.20291.
Huynh, T.N., Santini, E., Mojica, E., Fink, A.E., Hall, B.S., Fetcho, R.N., Grosenick, L., Deisseroth, K., LeDoux, J.E., Liston, C., et al., 2018. Activation of a novel p70 S6 kinase 1-dependent intracellular cascade in the basolateral nucleus of the amygdala is required for the acquisition of extinction memory. Mol. Psychiatry 23 (6), 1394–1401. https://doi.org/10.1038/mp.2017.99.
Jin, S.X., Feig, L.A., 2010. Long-term potentiation in the CA1 hippocampus induced by GLUN2A subunit-containing NMDA glutamate receptors is mediated by Ras-GRF2/Erk map kinase signaling. PLoS One 5 (7), e11732. https://doi.org/10.1371/journal.pone.0011732.
Keifer Jr., O.P., Hurt, R.C., Ressler, K.J., Marvar, P.J., 2015. The physiology of fear: reconceptualizing the role of the central amygdala in fear learning. Physiology 30 (5), 389–401. https://doi.org/10.1152/physiol.00058.2014.
Kim, J.H., Li, S., Richardson, R., 2011. Immunohistochemical analyses of long-term extinction of conditioned fear in adolescent rats. Cereb. Cortex 21 (3), 530–538. https://doi.org/10.1093/cercor/bhq116.
Kim, M.J., Dunah, A.W., Wang, Y.T., Sheng, M., 2005. Differential roles of GLUN2A- and GLUN2B-containing NMDA receptors in Ras-ERK signaling and AMPA receptor trafficking. Neuron 46 (5), 745–760. https://doi.org/10.1016/j.neuron.2005.04.031.
Kim-Cohen, J., Caspi, A., Moffitt, T.E., Harrington, H., Milne, B.J., Poulton, R., 2003. Prior juvenile diagnoses in adults with mental disorder: developmental follow-back of a prospective-longitudinal cohort. Arch. Gen. Psychiatry 60 (7), 709–717. https://doi.org/10.1001/archpsyc.60.7.709.
Kleene, R., Schachner, M., 2004. Glycans and neural cell interactions. Nat. Rev. Neurosci. 5 (3), 195–208. https://doi.org/10.1038/nrn1349.
Krapivinsky, G., Krapivinsky, L., Manasian, Y., Ivanov, A., Tyzio, R., Pellegrino, C., Ben-Ari, Y., Clapham, D.E., Medina, I., 2003. The NMDA receptor is coupled to the ERK pathway by a direct interaction between GLUN2B and RasGRF1. Neuron 40 (4), 775–784. https://doi.org/10.1016/s0896-6273(03)00645-7.
Lacagnina, A.F., Brockway, E.T., Crovetti, C.R., Shue, F., McCarty, M.J., Sattler, K.P., Lim, S.C., Santos, S.L., Denny, C.A., Drew, M.R., 2019. Distinct hippocampal engrams control extinction and relapse of fear memory. Nat. Neurosci. 22 (5), 753–761. https://doi.org/10.1038/s41593-019-0361-z.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (5259), 680–685. https://doi.org/10.1038/227680a0.
Lebron-Milad, K., Milad, M.R., 2012. Sex differences, gonadal hormones and the fear extinction network: implications for anxiety disorders. Biol. Mood Anxiety Disord. 2, 3. https://doi.org/10.1186/2045-5380-2-3.
Li, N., Lee, B., Liu, R.J., Banasr, M., Dwyer, J.M., Iwata, M., Li, X.Y., Aghajanian, G., Duman, R.S., 2010. mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. Science 329 (5994), 959–964. https://doi.org/10.1126/science.1190287.
Lissek, S., 2012. Toward an account of clinical anxiety predicated on basic, neurally mapped mechanisms of Pavlovian fear-learning: the case for conditioned overgeneralization. Depress Anxiety 29 (4), 257–263. https://doi.org/10.1002/da.21922.
Lopez-Fernandez, M.A., Montaron, M.F., Varea, E., Rougon, G., Venero, C., Abrous, D.N., Sandi, C., 2007. Upregulation of polysialylated neural cell adhesion molecule in the dorsal hippocampus after contextual fear conditioning is involved in long-term memory formation. J. Neurosci.: Off. J. Soc. Neurosci. 27 (17), 4552–4561. https://doi.org/10.1523/JNEUROSCI.0396-07.2007.
Lu, K., Jing, X.B., Xue, Q., Song, X.J., Wei, M.D., Wang, A.G., 2019. Impaired fear memory extinction during adolescence is accompanied by the depressive-like behaviors. Neurosci. Lett. 699, 8–15. https://doi.org/10.1016/j.neulet.2019.01.039.
Maren, S., Quirk, G.J., 2004. Neuronal signalling of fear memory. Nat. Rev. Neurosci. 5 (11), 844–852. https://doi.org/10.1038/nrn1535.
Markwell, M.A., Haas, S.M., Bieber, L.L., Tolbert, N.E., 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87 (1), 206–210. https://doi.org/10.1016/0003-2697(78)90586-9.
Matsuda, S., Matsuzawa, D., Ishii, D., Tomizawa, H., Sutoh, C., Shimizu, E., 2015. Sex differences in fear extinction and involvements of extracellular signal-regulated kinase (ERK. Neurobiol. Learn. Mem. 123, 117–124. https://doi.org/10.1016/j.nlm.2015.05.009.
Mavranezouli, I., Megnin-Viggars, O., Daly, C., Dias, S., Stockton, S., Meiser-Stedman, R., Trickey, D., Pilling, S., 2020. Research Review: Psychological and psychosocial treatments for children and young people with post-traumatic stress disorder: a network meta-analysis. J. Child Psychol. Psychiatry, Allied Discip. 61 (1), 18–29. https://doi.org/10.1111/jcpp.13094.
McCallum, J., Kim, J.H., Richardson, R., 2010. Impaired extinction retention in adolescent rats: effects of D-cycloserine. Neuropsychopharmacol.: Off. Publ. Am. Coll. Neuropsychopharmacol. 35 (10), 2134–2142. https://doi.org/10.1038/npp.2010.92.
Norrholm, S.D., Jovanovic, T., 2018. Fear processing, psychophysiology, and PTSD. Harv. Rev. Psychiatry 26 (3), 129–141. https://doi.org/10.1097/HRP.0000000000000189.
Pace-Schott, E.F., Spencer, R.M., Vijayakumar, S., Ahmed, N.A., Verga, P.W., Orr, S.P., Pitman, R.K., Milad, M.R., 2013. Extinction of conditioned fear is better learned and recalled in the morning than in the evening. J. Psychiatr. Res. 47 (11), 1776–1784. https://doi.org/10.1016/j.jpsychires.2013.07.027.
Palomero-Gallagher, N., Bidmon, H.J., Zilles, K., 2003. AMPA, kainate, and NMDA receptor densities in the hippocampus of untreated male rats and females in estrus and diestrus. J. Comp. Neurol. 459 (4), 468–474. https://doi.org/10.1002/cne.10638.
Pattwell, S.S., Duhoux, S., Hartley, C.A., Johnson, D.C., Jing, D., Elliott, M.D., Ruberry, E. J., Powers, A., Mehta, N., Yang, R.R., et al., 2012. Altered fear learning across development in both mouse and human. Proc. Natl. Acad. Sci. USA 109 (40), 16318–16323. https://doi.org/10.1073/pnas.1206834109.
Paus, T., Keshavan, M., Giedd, J.N., 2008. Why do many psychiatric disorders emerge during adolescence. Nat. Rev. Neurosci. 9 (12), 947–957. https://doi.org/10.1038/nrn2513.
Perez-Otano, I., Ehlers, M.D., 2005. Homeostatic plasticity and NMDA receptor trafficking. Trends Neurosci. 28 (5), 229–238. https://doi.org/10.1016/j.tins.2005.03.004.
Perry, C.J., Ganella, D.E., Nguyen, L.D., Du, X., Drummond, K.D., Whittle, S., Pang, T.Y., Kim, J.H., 2020. Assessment of conditioned fear extinction in male and female adolescent rats. Psychoneuroendocrinology 116, 104670. https://doi.org/10.1016/j.psyneuen.2020.104670.
Pitman, R.K., Rasmusson, A.M., Koenen, K.C., Shin, L.M., Orr, S.P., Gilbertson, M.W., Milad, M.R., Liberzon, I., 2012. Biological studies of post-traumatic stress disorder. Nat. Rev. Neurosci. 13 (11), 769–787. https://doi.org/10.1038/nrn3339.
Popiel, A., Zawadzki, B., Praglowska, E., Teichman, Y., 2015. Prolonged exposure, paroxetine and the combination in the treatment of PTSD following a motor vehicle accident. A randomized clinical trial - The "TRAKT" study. J. Behav. Ther. Exp. Psychiatry 48, 17–26. https://doi.org/10.1016/j.jbtep.2015.01.002.
Qiu, Q., Yang, M., Tsang, B.K., Gruslin, A., 2004. Both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signalling are required in epidermal growth factor-induced human trophoblast migration. Mol. Hum. Reprod. 10 (9), 677–684. https://doi.org/10.1093/molehr/gah088.
de Quervain, D., Schwabe, L., Roozendaal, B., 2017. Stress, glucocorticoids and memory: implications for treating fear-related disorders. Nat. Rev. Neurosci. 18 (1), 7–19. https://doi.org/10.1038/nrn.2016.155.

Quirk, G.J., Mueller, D., 2008. Neural mechanisms of extinction learning and retrieval. Neuropsychopharmacol.: Off. Publ. Am. Coll. Neuropsychopharmacol. 33 (1), 56–72. https://doi.org/10.1038/sj.npp.1301555.

Rajji, T., Chapman, D., Eichenbaum, H., Greene, R., 2006. The role of CA3 hippocampal NMDA receptors in paired associate learning. J. Neurosci.: Off. J. Soc. Neurosci. 26 (3), 908–915. https://doi.org/10.1523/JNEUROSCI.4194-05.2006.

Rauch, S.A.M., Kim, H.M., Powell, C., Tuerk, P.W., Simon, N.M., Acierno, R., Allard, C.B., Norman, S.B., Venners, M.R., Rothbaum, B.O., et al., 2019. Efficacy of prolonged exposure therapy, sertraline hydrochloride, and their combination among combat veterans with posttraumatic stress disorder: a randomized clinical trial. JAMA Psychiatry 76 (2), 117–126. https://doi.org/10.1001/jamapsychiatry.2018.3412.

Remes, O., Brayne, C., van der Linde, R., Lafortune, L., 2016. A systematic review of reviews on the prevalence of anxiety disorders in adult populations. Brain Behav. 6 (7), e00497 https://doi.org/10.1002/brb3.497.

Ren, J., Li, X., Zhang, X., Li, M., Wang, Y., Ma, Y., 2013. The effects of intra-hippocampal microinfusion of D-cycloserine on fear extinction, and the expression of NMDA receptor subunit GLUN2B and neurogenesis in the hippocampus in rats. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 44, 257–264. https://doi.org/10.1016/j.pnpbp.2013.02.017.

Romanelli, A., Dreisbach, V.C., Blenis, J., 2002. Characterization of phosphatidylinositol 3-kinase-dependent phosphorylation of the hydrophobic motif site Thr(389) in p70 S6 kinase 1. J. Biol. Chem. 277 (43), 40281–40289. https://doi.org/10.1074/jbc.M205168200.

Rutishauser, U., Landmesser, L., 1996. Polysialic acid in the vertebrate nervous system: a promoter of plasticity in cell-cell interactions. Trends Neurosci. 19 (10), 422–427. https://doi.org/10.1016/0166-2236(96)10041-2.

Sanderson, D.J., Good, M.A., Seeburg, P.H., Sprengel, R., Rawlins, J.N., Bannerman, D. M., 2008. The role of the GluR-A (GluA1) AMPA receptor subunit in learning and memory. Prog. Brain Res. 169, 159–178. https://doi.org/10.1016/S0079-6123(07)00009-X.

Santini, E., Muller, R.U., Quirk, G.J., 2001. Consolidation of extinction learning involves transfer from NMDA-independent to NMDA-dependent memory. J. Neurosci.: Off. J. Soc. Neurosci. 21 (22), 9009–9017.

Seymour, C.M., Foley, A.G., Murphy, K.J., Regan, C.M., 2008. Intraventricular infusions of anti-NCAM PSA impair the process of consolidation of both avoidance conditioning and spatial learning paradigms in Wistar rats. Neuroscience 157 (4), 813–820. https://doi.org/10.1016/j.neuroscience.2008.09.041.

Shipton, O.A., Paulsen, O., 2014. GluN2A and GluN2B subunit-containing NMDA receptors in hippocampal plasticity. Philos. Trans. R. Soc. Lond. Ser. B, Biol. Sci. 369 (1633), 20130163. https://doi.org/10.1098/rstb.2013.0163.

Siegel, R.S., Dickstein, D.P., 2012. Anxiety in adolescents: update on its diagnosis and treatment for primary care providers. Adolesc. Health Med. Ther. 3, 1–16. https://doi.org/10.2147/AHMT.S7597.

Soler-Cedeno, O., Torres-Rodriguez, O., Bernard, F., Maldonado, L., Hernandez, A., Porter, J.T., 2019. Plasticity of NMDA receptors at ventral hippocampal synapses in the infralimbic cortex regulates cued fear. eNeuro 6 (2). https://doi.org/10.1523/ENEURO.0354-18.2019.

Sun, Y., Xu, Y., Cheng, X., Chen, X., Xie, Y., Zhang, L., Wang, L., Hu, J., Gao, Z., 2018. The differences between GluN2A and GluN2B signaling in the brain. J. Neurosci. Res. 96 (8), 1430–1443. https://doi.org/10.1002/jnr.24251.

Sytnyk, V., Leshchyns'ka, I., Schachner, M., 2017. Neural cell adhesion molecules of the immunoglobulin superfamily regulate synapse formation, maintenance, and function. Trends Neurosci. 40 (5), 295–308. https://doi.org/10.1016/j.tins.2017.03.003.

Wang, G., Gilbert, J., Man, H.Y., 2012. AMPA receptor trafficking in homeostatic synaptic plasticity: functional molecules and signaling cascades. Neural Plast. 2012, 825364 https://doi.org/10.1155/2012/825364.

Williams, T., Phillips, N.J., Stein, D.J., Ipser, J.C., 2022. Pharmacotherapy for post traumatic stress disorder (PTSD. Cochrane Database Syst. Rev. 3, CD002795. https://doi.org/10.1002/14651858.CD002795.pub3.

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 GLUN2B and GluA1 per845 in hippocampal. Neuroscience 260 (1–11), CA1. https://doi.org/10.1016/j.neuroscience.2013.12.014.

Zhang, B., Li, C.Y., Wang, X.S., 2017. The effect of hippocampal NMDA receptor blockade by MK-801 on cued fear extinction. Behav. Brain Res. 332, 200–203. https://doi.org/10.1016/j.bbr.2017.05.067.

Zoellner, L.A., Roy-Byrne, P.P., Mavissakalian, M., Feeny, N.C., 2019. Doubly randomized preference trial of prolonged exposure versus sertraline for treatment of PTSD. Am. J. Psychiatry 176 (4), 287–296. https://doi.org/10.1176/appi.ajp.2018.17090995.