Contextual fear memory modulates PSD95 phosphorylation, AMPAr subunits, PKMζ and PI3K differentially between adult and juvenile rats

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ABSTRACT

It is well known that young organisms do not maintain memories as long as adults, but the mechanisms for this ontogenetic difference are undetermined. Previous work has revealed that the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAr) subunits are trafficked into the synaptic membrane following memory retrieval in adults. Additionally, phosphorylated PSD-95-pS295 promotes AMPAr stabilization at the synapse. We investigated these plasticity related proteins as potential mediators in the differential contextual stress memory retrieval capabilities observed between adult and juvenile rats. Rats were assigned to either pedestal stress (1 h) or no stress control (home cage). Each animal was placed alone in an open field for 5 min at the base of a 6×6 sq inch pedestal (4ft high). Stress subjects were then placed on this pedestal for 1hr and control subjects were placed in their home cage following initial exploration. Each animal was returned to the open field for 5 min either 1d or 7d following initial exposure. Freezing postures were quantified during the memory retrieval test. The 1d test shows adult (P90) and juvenile (P26) stressed rats increase their freezing time compared to controls. However, the 7d memory retrieval test shows P90 stress rats but not P26 stress rats freeze while in the fear context. Twenty minutes after the memory retrieval test, hippocampi and amygdala were micro-dissected and prepared for western blot analysis. Our results show that 1d fear memory retrieval induced an upregulation of PSD-95 and pS295 in the adult amygdala but not in the juvenile. However, the juvenile animals upregulated PKMζ, PI3K and GluA2/3, GluA1-S845 in the dorsal hippocampus (DH), but the adults did not. Following the 7d memory retrieval test, adults upregulated GluA2 in the amygdala but not the juveniles. In the DH, adults increased PSD-95 and pS295 but not the juveniles. The adults appear to preferentially increase amygdala-driven processing at 1d and increase DH-driven context specific processing at 7d. These data identify molecular processes that may underlie the reduced fear-memory retrieval capability of juveniles. Together these data provide a potential molecular target that could be beneficial in treatment of anxiety disorders and PTSD.

1. Introduction

Infants and young children can learn and remember but show a reduced capability to retain new memories (Bauer, 2006; Rovee-Collier, 1990; Rovee-Collier and Cuevas, 2009). This reduced memory capacity in children is not due to poor encoding of information, since younger children given equivalent learning or overlearning still forget (Bauer and Larkina, 2014; Guskjolen et al., 2017). Additionally, memory retention improves with maturation, but the mechanisms underlying this developmental shift are largely unknown (Carver and Bauer, 2001;

Klein and Meltzoff, 1999; Lukowski and Milojevich, 2016; Morgan and Hayne, 2011; Tustin and Hayne, 2010; Van Abbema and Bauer, 2005; Wolfe and Bell, 2007). Even in the absence of behavioral evidence of fear learning, memories of the event can appear later (S. S. Pattwell, K. G. Bath, B. J. Casey, I. Ninan, & F. S. Lee, 2011). Further, stress and traumatic experiences in early life may lead to physiological and molecular changes to accelerate the development of the adult memory system, which indicates that these experiences can cause long lasting effects on memory (Callaghan and Tottenham, 2016; Cowan et al., 2016; Richardson et al., 2016). In order to delineate molecular

mechanisms that are associated with an acute stress- or fear-memory retrieval, our study focused on two distinct development periods, juvenile and adult rats, and their ability to maintain a fear memory across either a recent or long interval.

Acute stress and/or fear affects many different molecular and cognitive processes including synaptic transmission, neural plasticity and memory (de Kloet et al., 2005). There has been a growing understanding for how these experiences exert specific changes on glutamatergic transmission in the brain (Popoli et al., 2011) in ways that can vary across time domains and brain regions (Dorey et al., 2012; M. Joels, Sarabdjitsingh and Karst, 2012; Karst et al., 2010). Stress/fear responses include corticosteroid-dependent changes to plasticity-related protein activity that can dramatically alter the neurophysiology underlying cognitive processes. Of particular importance, corticosterone can affect AMPAR-mediated transmission by increasing surface mobility (Groc et al., 2008; Whitehead et al., 2013) and that behavioral fear/stress increases synaptic AMPAR levels (Conboy and Sandi, 2010; Iniguez et al., 2016; Sebastian et al., 2013; Zanca et al., 2015). While these molecular adaptations are considered critical for modulating synaptic strength that can alter learning in-vivo, the signaling pathways mediating these corticosterone-driven effects have not been fully characterized. More importantly, the developmental shifts in synaptic signaling have not been investigated thoroughly, which may provide an understanding of how young animals show reduced fear memory capacity compared to adults (Oliver et al., 2016; Richardson et al., 2016). To delineate the molecular mechanisms associated with fear memory retrieval, we used an elevated platform to produce a context specific fear memory, which has been shown to increase corticosterone and alter GluA2 and PKMζ expression within 1 h (Sebastian et al., 2013). In our present study, we evaluated the retrieval of this platform stress as a contextual fear memory followed by assessment of synaptic markers in the dorsal hippocampus (DH) and amygdala.

The amygdala and DH are known to have discrete roles in contextual fear memory (Malin and McGaugh, 2006; Paz et al., 2006). The amygdala, and basolateral amygdala (BLA) in particular, modulate consolidation of various types of memories (McIntyre et al., 2012), including contextual fear memory (N. C. Huff and Rudy, 2004; LaLumiere et al., 2003). Furthermore, studies show that the BLA is more liberally involved in modulating the formation of emotionally arousing memories whereas the DH is specifically involved in potentiating the contextual aspects of fear memories (Malin and McGaugh, 2006). Moreover, the engagement of these two brain regions appear linked, in that BLA stimulation can enhance hippocampal LTP (Frey et al., 2001; Ikegaya et al., 1995) and enhance avoidance memory performance (McIntyre et al., 2005; McReynolds et al., 2014). While there are direct projections from BLA to ventral hippocampus (VH) (Felix-Ortiz and Tye, 2014; Pikkarainen et al., 1999), studies show that VH is involved in emotion and anxiety (Bannerman et al., 2003; Kjelstrup et al., 2002; Maren and Holt, 2004) and in the retention of foot shock learning with no role in the retention of the context learning (M. L. Huff, Emmons, Narayanan and LaLumiere, 2016). Thus, characterizing the activity in the DH following fear conditioning can help us understand the context-specific effects of stress on the brain.

Studies using shock and predator odor have shown that GluA1-2 containing AMPAr subunits increased during consolidation and retrieval of fear memory in the hippocampus (Bhattacharya et al., 2017) and amygdala (Opendak et al., 2018), while reconsolidation was shown to increase PKMζ levels in the amygdala (Oliver et al., 2016). Furthermore, surface GluA2 increased on mushroom spines in the DH, displaying the importance of AMPA-receptor subunits in the maintenance and retrieval of a contextual fear memory (Sase et al., 2015). Our study demonstrates that exposure to the elevated platform as a fear-inducing stimulus produces fear learning comparable to these studies using shock and predator odor. Both juveniles and adults showed equivalent contextual memory 1d after training, but only the adults expressed a contextual memory at 7d. We hypothesized that

these behavioral results are driven by developmentally-regulated synaptic plasticity molecules, acting as markers for enhanced or dampened neural activity. Our molecular analyses included markers PSD95, PKMζ, AMPA-receptor subunits GluA1, A2, and A3, as well as upstream signaling marker PI3K. Additionally, we also analyzed the developmental shifts in levels of phosphorylated synaptic plasticity markers, that could more readily indicate activation states of these proteins. Our data identify a developmentally-sensitive fear-memory capacity driven by developmentally-regulated synaptic plasticity marker expression.

2. Methods

2.1. Subjects

Male Sprague-Dawley rats from Envigo (Indianapolis, ID) were purchased at 12 weeks of age (275–325 g). Adult rats were housed individually at the Hunter College animal facility for 1 week prior to beginning any behavioral assessments. Previous work has shown that environmental enrichment during peri-adolescent periods can have significant positive cognitive and neural effects (Bredy et al., 2004). Thus, social buffering arising from group-housed subjects may impair fear-learning (Gunnar, 2017) and obstruct our molecular investigations. Time pregnant Female Sprague-Dawley rats at 18 days gestation were purchased. Male juveniles were weaned at postnatal day (PN) 21 and housed in groups of three. Group housing was chosen for juveniles due to the greater level of social interactions shown by juveniles, e.g., play (Pellis, 2017; Thor and Holloway, 1984), as well as the fact that the adults used were group housed when juveniles. Animal quarters were maintained at constant temperature (22 ± 1 °C) and relative humidity (40–50%) with a 12 h light/dark cycle (lights on at 8:00 h). Food (Harlan Teklad; Frederick, MD) and water were available *ad libitum*. All housing conditions conform to the Hunter College guidelines outlined by the Institutional Animal Care and Use Committee (IACUC).

2.2. Fear memory training and behavioral assessments

Cohorts of juvenile (PN26) and adult rats (PN90) were assigned to either a fear (adult, $n = 16$; juvenile, n = 14) or a no fear control (adult, $n = 16$; juvenile, $n = 18$) condition, and then into groups that would be tested at 1d or 7d post-fear-/or no fear exposure. Juvenile rats were trained at PN26 and adult rats were trained at PN90. Testing took place either 24 h or 7 days after training, resulting in PN34 Juveniles rats and PN98 Adult rats. On the training day, fear-assigned animals were individually placed in an open field (3′ x 3′ square enclosure) at the base of a 6 × 6 sq. inch, 4′ high pedestal for 5 min, before being placed on top of the pedestal for 60 min. Animals in the control group were given the same 5 min exposure to the open field and then taken back to their home cage immediately after. The test day was either 1 day or 7 days post-training, where all animals regardless of condition, were place in the open field for 5 min before being placed back into their home cages. Behavior was videotaped and analyzed off-line. Time freezing was recorded by timer and stopwatch by experimenters blind to the rats' condition. Animals who froze during training were dropped from the experiment. *Novel Context*: In order to determine context-specific fear behavior, a subset of animals was either assigned fear (adult, $n = 8$; juvenile, n = 7) or control (adult, $n = 8$; juvenile, $n = 9$) and tested for fear-based behaviors in a novel context that did not have the pedestal platform. Data analyzed as above. (See Fig. 1A)

2.3. Tissue sample collection and fractionation

After fear memory retrieval testing at 1d or 7d post exposure, animals were sacrificed within 30 min after being brought back to their home cage. Whole brains were flash frozen in 2-methyl-butane. Brains were sectioned at 100 μm and kept on microscope slides at −80 °C. Under a dissecting microscope, amygdala and dorsal hippocampus were
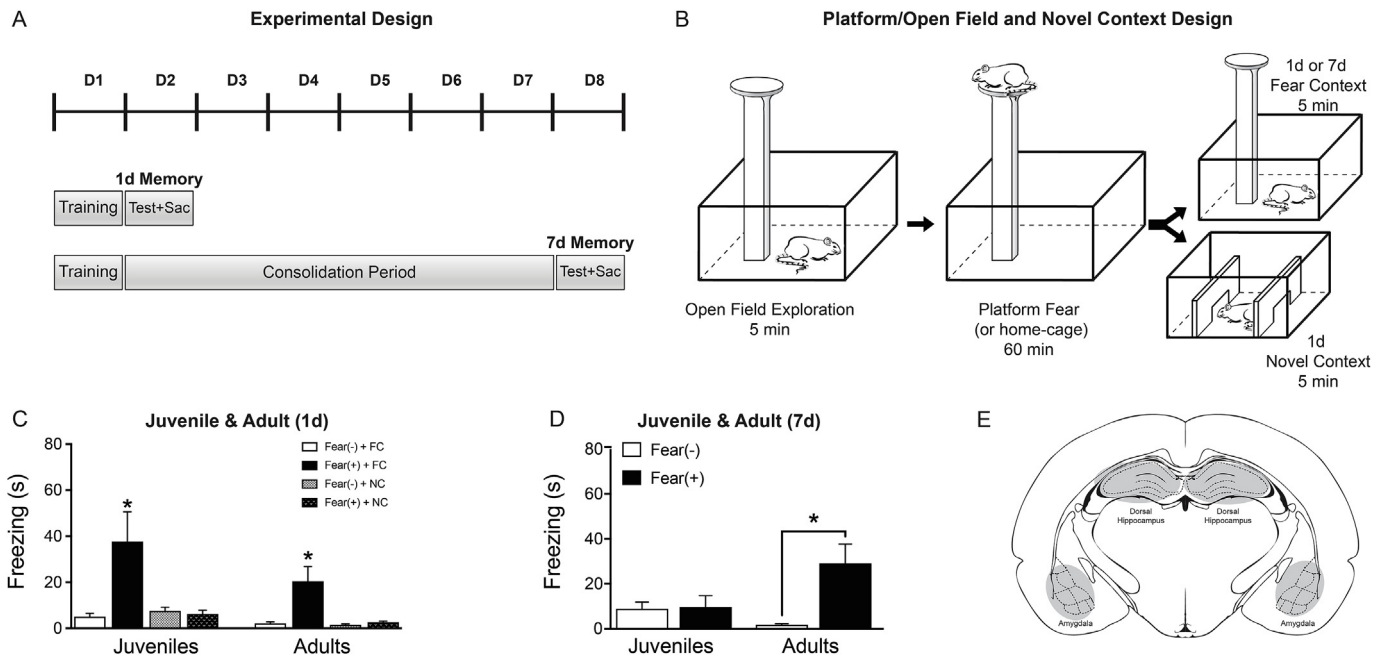
Fig. 1. *Memory retrieval test in Fear or Novel Context.* **A**) Rats were trained on day 1 and either tested the following day or on the 8th day. Tissue was collected 30 min after test. **B**) Rats were allowed to explore the open-field, were exposed to platform fear (or home-cage), and then re-exposed to fear context or a novel context. **C**) Fear (+) juveniles and adults increase freezing when tested in the fear context (FC) compared to all other conditions: juveniles (*$p < .05$); adult (**$p < .01$). **D**) 7d fear memory retrieval shows fear (+) adults increase freezing compared to fear (−) adults (*$p < .05$). Fear (+) juveniles vs. fear (−), juveniles are not significantly different. **E**) Anatomical representation of the dorsal hippocampus and amygdala tissue dissections in grey *(adapted from* Palombi et al., 2006.). Dorsal hippocampus and amygdala dissected between Interaural 7.12mm x Bregma −1.88mm and Interaural 5.20mm x Bregma −3.80mm.

dissected and prepared into cytosolic and synaptic fractions as previously reported (Braren et al., 2014) (See Fig. 1E). Briefly, tissues were thawed from frozen and immersed in a TEE (Tris 50 mM; EDTA 1 mM; EGTA 1 mM) buffer containing a SigmaFast, protease inhibitor cocktail (Sigma Aldrich) diluted to contain AEBSF (2 mM), Phosphoramidon (1 μM), Bestatin (130 μM), E-64 (14 μM), Leupeptin (1 μM), Aprotinin (0.2 μM), and Pepstatin A (10 μM). Tissues were homogenized in 200 μl of the TEE-homogenization buffer using 20 pumps with a motorized pestle. Homogenates were transferred to Eppendorf tubes and centrifuged at 3000 g (5 min at 4 °C), to remove pellet. The resulting supernatant was centrifuged at 100,000 g for 30 min. After ultracentrifugation, the supernatant was collected and stored as the cytosolic fraction. The remaining pellet was resuspended in 100 μl of homogenizing TEE buffer containing 0.001% Triton X-100, incubated on ice for 1 h and then centrifuged at 100,000 g for 1 h at 4 °C. The resulting pellet was resuspended in 50 μl of TEE buffer and stored as the synaptic fraction (Nogues et al., 1994). The Pierce bicinchoninic acid assay (BCA) (Thermo Scientific, Rockford, IL) was used to determine protein concentration for each sample. Samples were reduced with 4x Laemmli sample buffer equivalent to 25% of the total volume of the sample and then boiled and stored frozen at −80 °C.

2.4. Protein quantification and western blot assessments

Samples (20 μg) were loaded onto a Tris/Gly 4–20% midi™ gel to resolve alpha-tubulin (55 kDa), GluA1 (102 kDa), GluA1 phosphorylated at serine 845 (102 kDa), GluA2 (102 kDa), GluA3 (102 kDa), PSD95 (95 kDa), PSD 95 phosphorylated at serine 295 (105 kDa), PKMζ (55 kDa), and PI3 Kinase (55 kDa). Every gel contained 3–4 lanes loaded with the same control sample, (all brain samples, ABS). ABS were used to standardize protein signals between gels. Gels were transferred to nitrocellulose membranes in IBlot® Dry Blotting System (Life Technologies; Carlsbad, CA) for 7 min. Nitrocellulose membranes were then incubated in blocking solution containing 5% sucrose in Tris Buffered Saline with Tween-20 (TBST; 0.1% Tween-20 in TBS) for

30 min at room temperature. Samples were incubated with the following primary antibodies overnight: alpha-tubulin (1:2000; Millipore, Burlington, MA), GluA2 (1:2000; Millipore, Burlington, MA), GluA1 (1:2000; Millipore, Burlington, MA), GluA1 phosphorylated serine 845 (1:2000, AbCam, Cambridge, UK), PKMζ (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA); and GluA3 (1:2000, Millipore, Burlington, MA), PSD-95 (1:2000; Millipore, Burlington, MA), PSD-95 phosphorylated serine 295 (1:2000, AbCam, Cambridge, UK), and PI3 Kinase (1:2000; Millipore, Burlington, MA). Membranes were washed in TBST for 20 min and probed with Horseradish Peroxidase (HRP) conjugated secondary antibody. Membranes were incubated with Enhanced Chemiluminescence (ECL) substrate and exposed on CL-X Posure Film (Thermo Scientific; Rockford, IL). α-tubulin was used as a control to standardize for protein concentration loaded on gels. Films were scanned for quantification with NIH Image J.

2.5. Statistical analyses

For behavioral analyses, a 3-way ANOVA was used in analyzing age x stress x context treatment interactions and a 2-way ANOVA was used in analyzing age x stress treatment interactions. Molecular analyses used 2-way ANOVA (age x stress treatment). Post-hoc analyses used a Tukey-corrected *t*-test. Prism GraphPad 6.0 Statistical Package (La Jolla, California) was used for all analyses.

3. Results

3.1. Contextual fear memory retrieval

Our results showed that both juveniles and adults demonstrated enhanced context specific freezing during a 1d fear memory retrieval test, ($F(3, 48) = 9.58$, **$p < .01$), with no effect of age ($F(3, 48) = 3.00$ $p = .09$), Fig. 1 C)]. Post-hoc, juvenile and adult fear (+) + fear context (FC) increased their freezing time compared to all other conditions: juveniles fear (−) + FC (*$p < .05$), fear (+) + novel
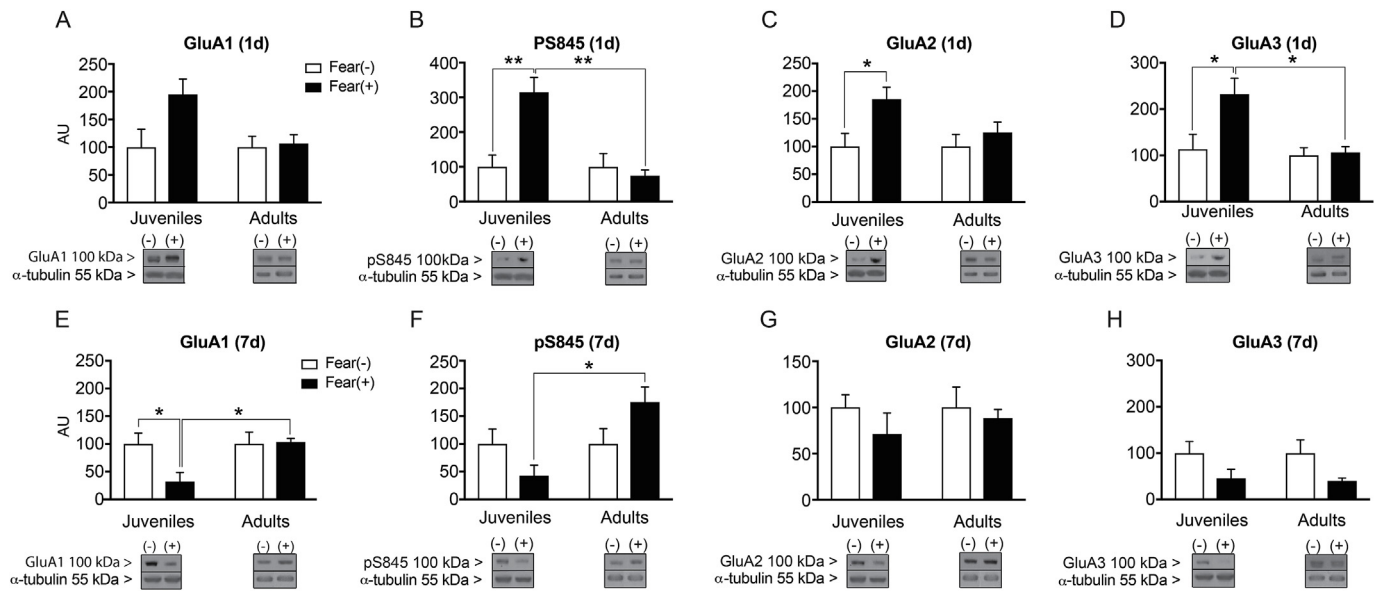
Fig. 2. *Dorsal hippocampus AMPAr synaptic expression following 1d or 7d memory retrieval.* 1d fear memory retrieval (**A-D**) and 7d fear memory retrieval (**E-H**). **A)** GluA1 expression showed an effect of fear. **B)** pS845 expression showed an effect of age and an interaction. Post hoc, fear (+) juveniles increased pS845 expression compared to fear (−) juveniles (**p < .01), and fear (+) adult (**p < .01). **C)** GluA2 expression showed an effect of fear. Post-hoc, fear (+) juveniles increased GluA2 expression compared to fear (−) juveniles (*p < .05). **D)** GluA3 expression showed an effect on age, stress and an interaction. Post-hoc, fear (+) juveniles increased GluA3 expression compared to fear (−) juveniles (*p < .05) and fear (+) adults (*p < .05). **E)** GluA1 expression showed an effect of age and an interaction. Post-hoc, fear (+) juveniles decreased GluA1 expression compared to fear (−) juveniles (*p < .05) and fear (+) adults (*p < .05). **F)** pS845 expression showed an effect of age and an interaction. Post-hoc, fear (+) juveniles decreased pS845 expression compared to fear (+) adults (*p < .05). **G)** GluA2 expression showed no significant effect of age, fear or an interaction. **H)** GluA3 expression showed an effect of fear. Western blot images correspond to conditions of the above bar graph.

context (NC) (*p < .05), fear(−) + NC (*p < .05); Fig. 1C, adult fear (−) + FC (**p < .01), fear (+) + novel context (NC) (**p < .01), fear(−) + NC (**p < .01). During the 7d fear memory retrieval test freezing behavior, two-way ANOVA reveals an effect of fear (F(1, 27) = 9.29, **p < .01), with no effect of age (F(1, 26) = 1.33 p = .26) (Fig. 1D). Post hoc, fear (+) adults show an increase in freezing compared to fear (−) adults, (*p < .05). Conversely, there was no significant difference in freezing behavior between fear (+) and fear (−) juveniles at 7d.

3.2. Hippocampal synaptic AMPAr subunit expression

Western blot analysis of AMPAr subunits (GluA1, pS845 GluA1, GluA2 and GluA3) expression following 1d or 7d fear memory retrieval was conducted. Synaptic GluA1 expression (Fig. 2A) showed a significant effect of fear ($F_{(1, 28)}$ = 4.2, p = .05) but no effect of age and no post-hoc differences. Synaptic pS845 expression (Fig. 2B) showed an effect of age ($F_{(1, 26)}$ = 11, p < .01) and an interaction ($F_{(1, 26)}$ = 11, p < .01). Post-hoc, fear (+) juveniles displayed an increase in pS845 expression compared to the fear (−) juveniles (**p < .01) and fear (+) adults (**p < .01). Synaptic GluA2 expression (Fig. 2C) showed an effect of fear ($F_{(1, 28)}$ = 6.688, p < .05), but no effect of age or interaction. Post-hoc, fear (+) juveniles showed an increase in GluA2 expression compared to fear (−) juveniles, (*p < .05). Synaptic GluA3 expression (Fig. 2D) showed an effect on age, fear and an interaction, respectively [($F_{(1, 26)}$ = 6.68, p = .016); $F_{(1, 26)}$ = 5.38, p = .03); $F_{(1, 26)}$ = 4.35, p = .04)]. Post-hoc, fear (+) juveniles show an increase in GluA3 compared to fear (−) juveniles (*p < .05) and fear (+) adults (*p < .05). Increases in AMPAr subunit activity in juveniles following 1d fear memory retrieval suggests the DH is specifically engaged via AMPAr subunit modulation. Following 7d memory retrieval synaptic GluA1 expression (Fig. 2E) showed an effect of age [$F_{(1, 27)}$ = 4.3, p < .05)], and an interaction [$F_{(1, 27)}$ = 4.3, p < .05)]. Post-hoc, fear (+) juveniles showed a decrease in synaptic GluA1expression compared to fear (−) juveniles (*p < .05) and fear (+) adults (*p < .05).

Synaptic pS845 expression (Fig. 2F) showed an effect of age [$F_{(1, 29)}$ = 6.33, p < .05)], and an interaction [$F_{(1, 29)}$ = 6.33, p < .05]. Post-hoc, pS845 expression decreased in fear (+) juveniles compared to fear (+) adults (*p < .05). Synaptic GluA2 expression (Fig. 2G) showed no effect of age, fear or an interaction (p > .05). Synaptic GluA3 expression (Fig. 2H) showed an effect of fear [$F_{(1, 29)}$ = 6.39, p < .05)] and no post-hoc differences.

3.3. Amygdala synaptic AMPAr subunit expression

AMPAr subunit expression following 1d fear memory retrieval shows no effect of fear, age or interaction for GluA1, pS845, GluA2 or GluA3 expression (p > .05) (Fig. 3A–D). 7d fear memory retrieval shows not effect of fear, age or interaction for GluA1 expression (Fig. 3E). Synaptic pS845 expression (Fig. 3F) showed effect of age ($F_{(1, 28)}$ = 8.64, p < .01) and an interaction ($F_{(1, 28)}$ = 8.6, p < .05). Post-hoc, fear (+) juveniles decreased pS845 expression compared to fear (+) adults (**p < .01). Synaptic GluA2 expression (Fig. 3G) showed an effect of age, fear, and an interaction, respectively [($F_{(1, 28)}$ = 16.6, p < .001), ($F_{(1, 28)}$ = 22, p < .001), ($F_{(1, 28)}$ = 16.6, p < .001)]. Post-hoc, GluA2 expression increased in fear (+) adults compared to fear (−) adults (**p < .01) and fear (+) juveniles (**p < .01). GluA3 expression (Fig. 3H) showed no effect of age, fear or an interaction.

3.4. Hippocampal cytosolic PKMζ and PI3K expression

Fig. 4A shows cytosolic PKMζ expression following 1d fear memory retrieval shows an effect of age, fear, and an interaction, respectively [($F_{(1,28)}$ = 11.6, p < .01), ($F_{(1,28)}$ = 12.3, p < .01), ($F_{(1,29)}$ = 11.6, p < .01)]. Post-hoc, PKMζ expression increased in fear (+) juveniles compared to fear (−) juveniles (**p < .01) and fear (+) adults (**p < .01). Cytosolic PI3K (Fig. 4b) expression following 1d fear memory retrieval shows an interaction, ($F_{(1,25)}$ = 10.5, p < .01). Post-hoc, PI3K expression increased in fear (+) juveniles compared to fear (−) juveniles (*p < .05). Conversely, fear (−) juveniles decreased
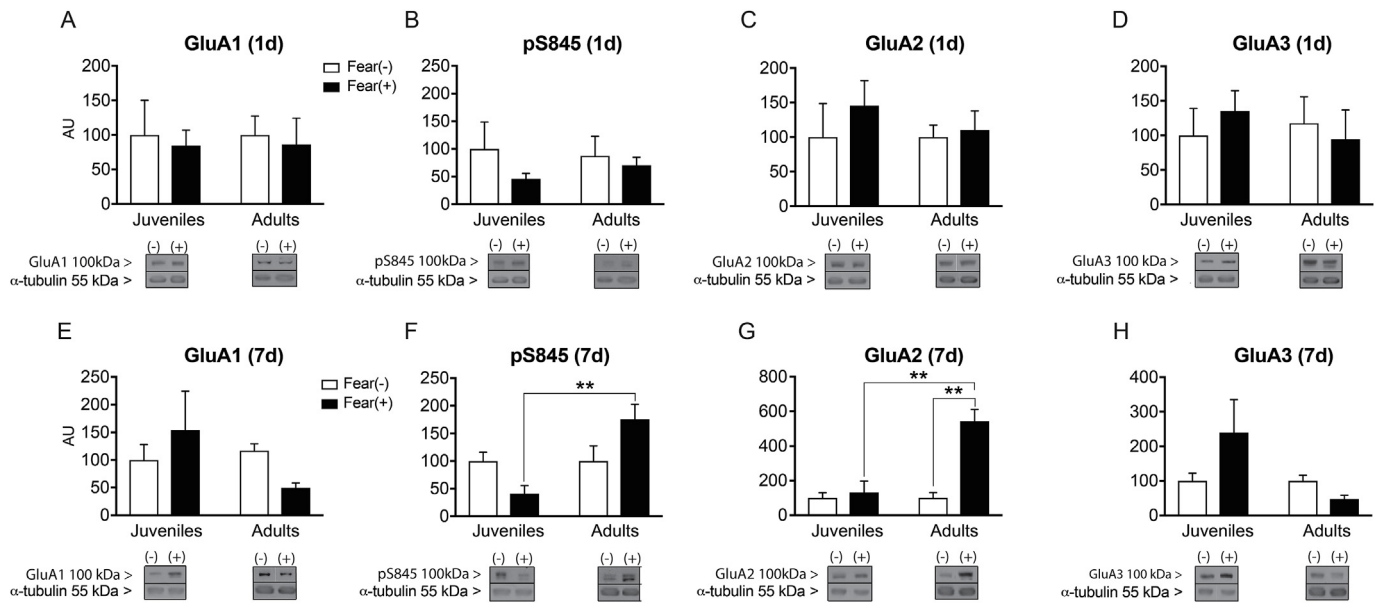
Fig. 3. *Amygdala AMPAr synaptic expression following 1d or 7d memory retrieval.* **A-D)** 1d fear memory retrieval shows no effect of age, fear or an interaction. **E-H)** 7d fear memory retrieval. **E)** GluA1 expression showed no effect of age, fear or an interaction. **F)** pS845 expression showed an effect of age and an interaction. Post-hoc, fear (+) juveniles decreased pS845 expression compared to fear (+) adults (**$p < .01$). **G)** GluA2 expression showed an effect of age, fear, and an interaction. Post-hoc, fear (+) adults increases GluA2 expression compared to fear (−) adults (**$p < .01$) and fear (+) juveniles (**$p < .01$). **H)** GluA3 expression showed no significant differences. Western blot images correspond to conditions of the above bar graph.

PI3K expression compared to fear (−) adults (*$p < .05$). Increases in PKMζ and PI3K activity in juveniles following 1d retrieval suggests that the DH is specifically engaged via PKMζ and PI3K upregulation and activity dependent mechanisms. Following 7d fear memory retrieval PKMζ and PI3k did not significantly alter their expression (Fig. 4C–D).

3.5. Amygdala cytosolic PKMζ and PI3K expression

1d fear memory retrieval does not show an effect of age, fear or an interaction for either cytosolic PKMζ or PI3k ($p > .05$, Fig. 5A–B. 7d fear memory retrieval shows an effect of age [$F_{(1,29)} = 4.78, p < .05$] and an interaction [$F_{(1,29)} = 4.78, p < .05$] for PKMζ, (Fig. 5C). Post-hoc, cytosolic PKMζ expression increased in fear (+) juveniles compared to fear (+) adults (*$p < .05$). PI3k expression following 7d fear memory retrieval is not significantly altered (Fig. 5D).

3.6. Hippocampal PSD-95 and S295 phosphorylation expression

Synaptic PSD-95 and pS295 expression following 1d fear memory retrieval show no effect of fear, age, or interaction ($p > .05$; Fig. 6AB). Following 7d fear memory retrieval synaptic PSD-95 expression showed

an effect of fear ($F_{(1, 28)} = 8.06, p < .01$; Fig. 6C). Post-hoc, PSD-95 expression increased in fear (+) adults compared to fear (−) adults (**$p < .01$) and fear (+) juveniles (**$p < .01$). Synaptic pS295 expression showed an effect of age, fear and an interaction, respectively [($F_{(1, 29)} = 14.3, p < .01$), ($F_{(1, 29)} = 4.6, p < .05$), ($F_{(1, 29)} = 14.32, p < .01$); Fig. 6D]. Post-hoc, pS295 expression increased in fear (+) adults compared to fear (−) adults (**$p < .01$) and fear (+) juveniles (**$p < .01$).

3.7. Amygdala PSD-95 and S295 phosphorylation expression

1d fear memory retrieval shows an effect of age ($F_{(1, 24)} = 8.22, p < .01$) and an interaction ($F_{(1, 24)} = 8.22, p < .01$) for synaptic PSD-95 expression (Fig. 7A). Post-hoc, PSD-95 expression increased in fear (+) adults compared to fear (−) adults (*$p < .05$) and fear (+) juveniles (**$p < .01$). Synaptic pS295 expression (Fig. 7B) showed an effect of age ($F_{(1, 23)} = 5.66, p < .05$), and an interaction ($F_{(1, 23)} = 8.45, p < .01$). Post-hoc, pS295 expression increased in fear (+) adults compared to fear (−) adults (*$p < .05$) and fear (+) juveniles (**$p < .01$). Following 7d fear memory retrieval, synaptic PSD-95 and pS295 showed no effect of fear, age, or interaction (Fig. 7C–D).
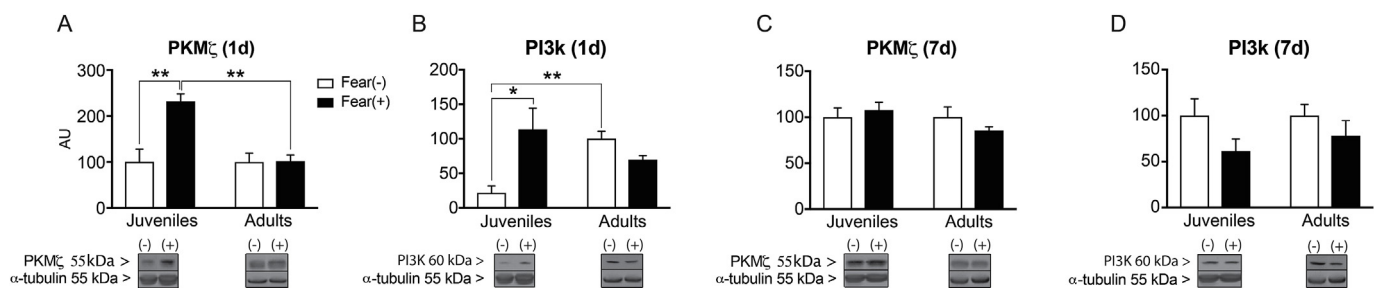

Fig. 4. *Dorsal hippocampus PKMζ and PI3k cytosolic expression following 1 or 7d memory retrieval.* 1d fear memory retrieval (**A-B**) and 7d fear memory retrieval (**C-D**). **A)** PKMζ expression showed an effect of age, fear, and an interaction. Post-hoc, PKMζ expression increased in fear (+) juveniles compared to fear (−) juveniles (**$p = < .01$) and fear (+) adults (**$p = < .01$). **B)** PI3K expression showed an interaction. Post-hoc, PI3K expression increased in fear (+) juveniles compared fear (−) juveniles (*$p = < .05$). Fear (−) juveniles decreased PI3K expression compared to fear (−) adults (*$p < .05$). **C-D)** No significant differences. Western blot images correspond to conditions of the above bar graph.

Fig. 5. *Amygdala PKMζ and PI3k cytosolic expression following 1 or 7d memory retrieval.* 1d fear memory retrieval for PKMζ and PI3k (**A-B**) shows no effect of age, fear or an interaction. **C-D)** 7d fear memory retrieval for PKMζ and PI3k. **C)** PKMζ expression showed an effect of age and an interaction. Post hoc, PKMζ expression increased in fear (+) juveniles compared to fear (+) adults (*$p < .05$). **D)** No significant differences. Western blot images correspond to conditions of the above bar graph.
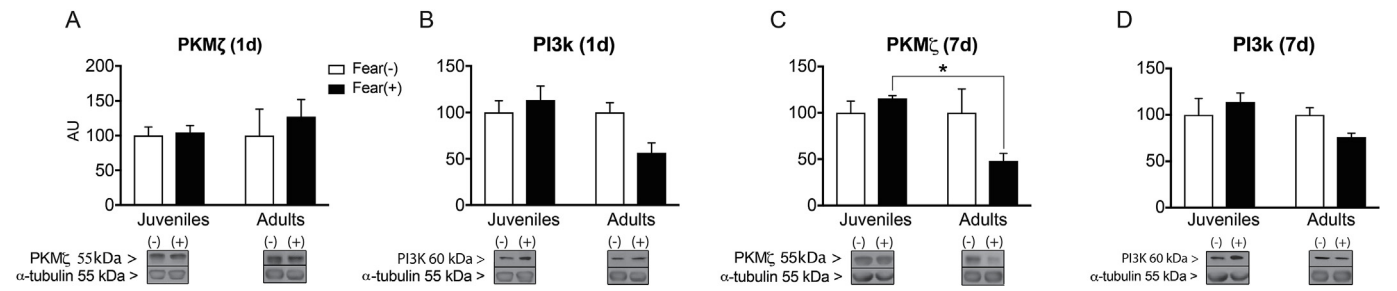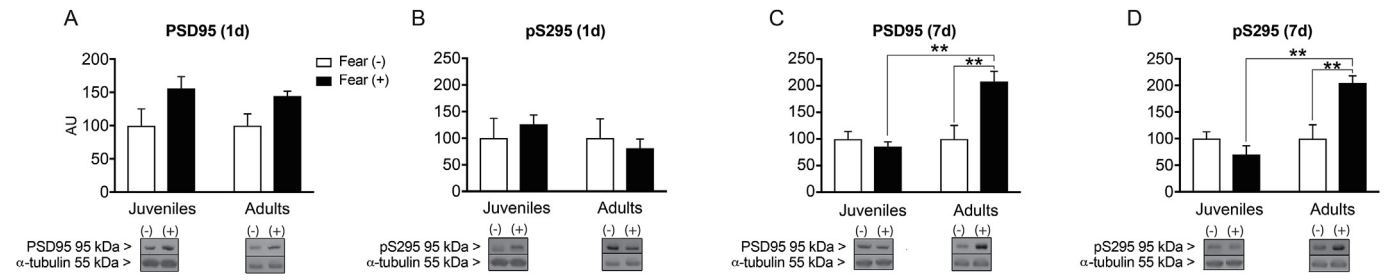

Fig. 6. *Dorsal hippocampus PSD-95 and pS295 synaptic expression following 1 or 7d memory retrieval.* 1d fear memory retrieval for PSD-95 and pS295 expression (**A-B**) showed no effect of age, fear or an interaction. **C-D)** 7d memory retrieval test. **C)** PSD-95 expression showed an effect of fear. Post-hoc, PSD-95 expression increased in fear (+) adults compared to fear (−) adults (**$p < .01$), and fear (+) juveniles (**$p < .01$). **D)** pS295 expression showed an effect of age, fear and an interaction. Post-hoc, pS295 expression increased in fear (+) adults compared to fear (−) adults (**$p < .01$), and fear (+) juveniles (**$p < .01$). Western blot images correspond to conditions of the above bar graph.

See Table 1.

4. Discussion

We investigated contextual fear memory retrieval at recent (1d) and remote (7d) intervals in juveniles and adults to determine the role for AMPA receptor and PSD-95 expression in the amygdala and DH. We showed that both juveniles and adults were able to maintain a contextual fear memory for 1d, while only the adults demonstrated a remote 7d fear memory. Our molecular analyses revealed a dissociation between DH and amygdala following fear memory retrieval at 1d. Adults showed increased PSD-95 and pS295 expression in amygdala but no changes in DH, whereas juveniles showed no change in amygdala but an increase expression for AMPAr subunits, PKMζ and PI3K in the DH. At 7d, adults showed an increase in PSD-95 and pS295 expression in the DH and an increase in GluA2 expression in the amygdala, but the forgetful juveniles showed a decrease GluA1 expression in the hippocampus and no changes in the amygdala. These results highlight the role of AMPAr subunit distributions across the hippocampus-amygdala pathway in the context of fear/stress.

4.1. Developmental differences in fear memory retrieval: amygdala vs hippocampus

Our behavioral results showed a dissociation between juvenile and adult rats' memory capabilities and engagement of the amygdala and DH. Our use of the elevated fear-inducing platform paradigm identified that juveniles selectively engage the DH, associated with a 1d contextual fear memory, while the adults show selective activation of the amygdala associated with a 7d memory. In light of previous studies showing no expression of fear learning 1d post training in adolescent mice and its expression two weeks later (S. S. Pattwell, K. G. Bath, B. Casey, I. Ninan, & F. S. Lee, 2011; Pattwell et al., 2012), we conclude that the behavioral paradigm and species used to characterize the developmental shifts in fear-learning and synaptic plasticity should be an important consideration in this line of research. Our study uses a novel
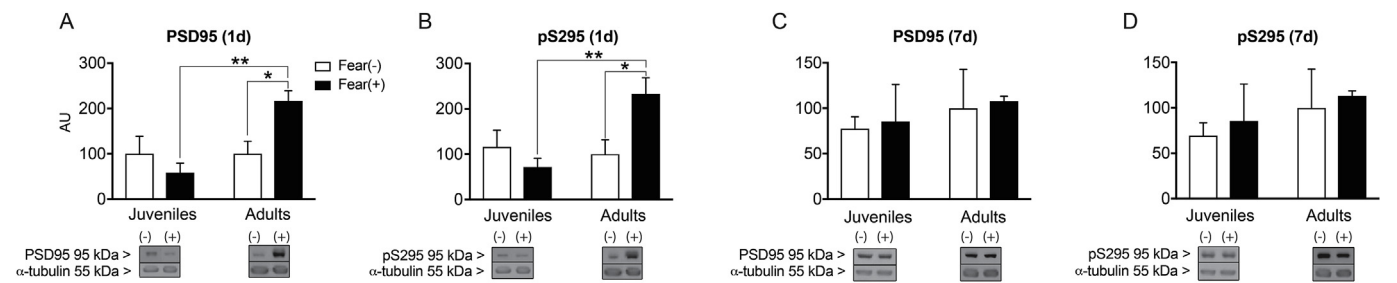

Fig. 7. *Amygdala PSD-95 and pS295 synaptic expression following 1 or 7d memory retrieval.* 1d fear memory retrieval for PSD-95 and pS295 expression (**A-B**) and 7d memory retrieval (**C-D**). **A)** PSD-95 expression showed an effect of age and an interaction. Post-hoc, PSD-95 expression increased in fear (+) adults compared to fear (−) adults (*$p < .05$) and fear (+) juveniles (**$p < .01$). **B)** pS295 expression showed an effect of age and an interaction. Post-hoc, pS295 expression increased in fear (+) adults compared to fear (−) adults (*$p < .05$), and fear (+) juveniles (**$p < .01$). **C-D)** PSD-95 and pS295 expression showed no effect of fear, age, or an interaction. Western blot images correspond to conditions of the above bar graph.

Table 1

Summary of marker findings between age groups and fear (+)/(−) conditions. Summary of markers used is separated by AMPA receptor subunits, synaptic proteins and cytosolic proteins and separated by brain region. Blue arrows represent significant post hoc effects and its direction between fear (+) and fear (−) conditions, within age groups. Orange arrows represent significant post hoc effects and its direction, between fear (+) juveniles and fear (+) adults. No arrow indicates no significant difference.

		Dorsal Hippocampus				Amygdala			
		1 Day		**7 Day**		**1 Day**		**7 Day**	
		Adult	Juvenile	Adult	Juvenile	Adult	Juvenile	Adult	Juvenile
AMPA receptor subunits	GluA1			↑	↓↓				
	GluA1- pS845	↓	↑↑	↑	↓			↓	↑
	GluA2		↑					↑↑	↓
	GluA3	↓	↑↑						
Synaptic Proteins	PSD-95			↑↑	↓	↑↑	↓		
	PSD-95-pS295			↑↑	↓	↑↑	↓		
Cytosolic Proteins	PI3K		↑						
	PKMζ	↓	↑↑					↓	↑

context-dependent platform design that produces significant immediate fear expression across young and aged rats. Our paradigm provides a method to investigate the neural effects of fear learning during this age period.

Our results are consistent with those reported using the contextual fear conditioning paradigm that dissociates behavioral roles between the DH and anterior cingulate cortex (Malin and McGaugh, 2006). Here, they found that the DH was selectively involved in the consolidation for the context learning but not the consolidation of the foot shock learning. Conversely, anterior cingulate cortex, but not the DH, was involved in the consolidation of the foot shock learning. The BLA, however, was found to enhance either context or footshock training (Malin and McGaugh, 2006). There has been an emphasis on the importance of the amygdala in the storage of contextual fear memory. Adult animals who are pre-exposed to the context before conditioning have greater retention levels than those who are immediately conditioned, a result that is abolished if the amygdala is inactivated before or after pre-exposure to the context (N. C. Huff and Rudy, 2004; N. C. Huff, Wright-Hardesty, Higgins, Matus-Amat and Rudy, 2005). This indicates that the amygdala plays an important role in modulating the storage of contextual fear memory that can occur through connections with the hippocampus, which may allow for long term memory consolidation. Recent work has shown that despite exhibiting equivalent projections between the BLA and the hippocampus, juveniles show decreased activation of the BLA projections to the VH-DH pathway during fear conditioning and extinction, but adults do not (Ganella and Kim, 2014; Ganella et al., 2018). It is possible that in the juveniles, the amygdala is not functioning as efficiently to modulate the storage of fear memory, only allowing for the expression of a fear-memory at 1 d with increased DH activity, followed by poor memory expression observed at 7d. We hypothesize that the increased activity observed in the adult amygdala (and presumably, the BLA) reflects the neural mechanism underlying consolidation and retrieval for the emotionality and the context aspects of the contextual fear memory. The juvenile rats appear to selectively encode contextual aspects over the emotional aspects of the fear memory. These results are consistent with the development of fear associations in the infant rat where PN8 rats fail to produce an avoidance response following odor/shock conditioning, unlike older rats > PN10 (Sullivan et al., 2000). This infantile learning system has the unique feature of being switched off if the odor-shock pairings are presented in the presence of the mother or when stress hormones are reduced (Moriceau and Sullivan, 2006; Opendak et al., 2018; Shionoya et al., 2007; Thompson et al., 2008; Upton and

Sullivan, 2010). We hypothesize that the juvenile rat amygdala continues to develop its support of the fear response memory system, identified here in the limited engagement of the amygdala compared to adults.

4.2. Recent contextual fear memory retrieval: PSD-95 expression in amygdala

As predicted both juvenile and adults maintained a 24 h fear memory. However, only the adults engaged the amygdala; showing increases in PSD-95 and in the specific pS295 phosphorylation site. These data are consistent with several reports showing increased PSD-95 expression in the amygdala, which is sensitive to extinction training resulting in the inhibition of fear (Mao et al., 2013; Mao et al., 2008). Additionally, pharmacological treatments known to upregulate PSD-95 including estrogen and insulin substrate-2 deletion improved spatial and fear memory (Avila et al., 2017; Irvine et al., 2011; Liu et al., 2008). A recent report has identified a unique role for PSD-95 in the maintenance of remote but not recent fear memories (Fitzgerald et al., 2015). Similarly, mutant mice with a ligand-binding-deficient knockin mutation of PSD-95 show deficient contextual fear memory expression one week, but not one day, after conditioning (Nagura et al., 2012). No behavioral studies have examined the role of phosphorylation pS295 on memory. However, reports show that pS295 phosphorylation by JNK1 promotes the synaptic accumulation of PSD-95 (Kim et al., 2007) and NMDA treatment induces rapid dephosphorylation of pS295 mediated by PP1/PP2A phosphatase(s) (Lin et al., 2007; Welsh and Proud, 1993). Additionally, the phosphomimetic mutant PSD-95-S295D blocks AMPA receptor internalization and subsequent LTD (Kim et al., 2007). Conversely, activation of PP2A can dephosphorylate pS295 promoting the phosphorylation of another PSD phosphorylation site (pT19, via GSK-3β) that enhances LTD (Morishita et al., 2001). This literature suggests that increased expression of pS295 in the adult amygdala reflects the stabilization of plasticity related proteins associated with LTP and in maintaining aspects of contextual fear memory.

4.3. Recent contextual fear memory retrieval: AMPAr/PKMζ/PI3K expression in dorsal hippocampus

We focused on the various AMPA receptor subunits expressed within the synaptic membrane. The AMPA receptor is made up of four distinct subunits (GluA1-4) which can affect synaptic plasticity and cognition (Chater and Goda, 2014). The calcium permeable (CP) AMPA

subunits include GluA1,3,4 and can be expressed as either homomers or heteromers (Passafaro et al., 2001; Shi et al., 2001). GluA2, the calcium non-permeable subunit, is [1] the rate-limiting factor for calcium influx after activation (Isaac et al., 2007), [2] highly expressed in the hippocampus in the form of two heterodimers, GluA1/GluA2 and GluA2/GluA3 (Wenthold et al., 1996), and [3] important for learning (G. Joels and Lamprecht, 2010) and [4] long-term memory (Henley and Wilkinson, 2013; Migues et al., 2010; Sebastian et al., 2013).

It is generally accepted that GluA1/2 subunits are trafficked by activity-driven mechanism, while GluA2/3 subunits are continuously cycled to maintain basal activity (Passafaro et al., 2001; Shi et al., 2001). Our results show, juvenile rats increase GluA2/3 and phosphorylation of GluA1-pS845 in the DH, an effect not shown in the adults. It is well known that AMPA receptor mediated-signaling and plasticity is critical to various forms of fear learning and memory and that PSD-95 plays a functional role in stabilizing synaptic AMPARs to promote synaptic function and spine growth (Avila et al., 2017; Iniguez et al., 2016). Decreasing PSD-95 leads to a loss of synaptic AMPAR content, synaptic weakening, deficient LTD, and spine elimination (Bhattacharyya et al., 2009; Nelson et al., 2013; Sturgill et al., 2009; Xu et al., 2008). Based on this literature, its intriguing to find that the juveniles are altering their AMPA receptor subunit expression without detectable changes in their PSD-95. Perhaps this is the molecular signature that highlights the short-term nature of the juvenile fear response that engages the DH, but not the amygdala. The results suggest that the juveniles are showing aspects of synaptic strengthening consistent with what is observed following LTP in the DH. For example, AMPAR subunits display distinct exocytosis properties where GluA2/3 heterodimers cycle continuously in and out of the membrane maintaining the surface pool of synaptic receptors (Passafaro et al., 2001; Shi et al., 2001). Conversely, GluA1/2 and GluA2/4 heterodimers are inserted into synapses in an activity-dependent manner (Shi et al., 2001). Together these studies suggest that the juvenile rats are both increasing AMPA subunits due to synaptic activity and are continuously maintaining these subunit expression levels. Alternatively, the increase in GluA2 without an increase in GluA1 suggests that a switch between these two subunits maybe occurring. This is consistent with results showing that LTP induction triggers a rapid but transient synaptic insertion of calcium permeable, (CP) AMPARs (GluA1,3,4) that are replaced by GluA2-containing AMPARs within 30 min, and blocking CP-AMPARs reduces the magnitude of potentiation and overall levels of CP-AMPARs (Plant et al., 2006). While our juvenile rats do not show an overall increase in GluA1, they do show increased expression of S845-GluA1 phosphorylation. Studies show activity-dependent synaptic trafficking of GluA1 is pS845 dependent (Esteban et al., 2003) which is essential for targeting GluA1 to the plasma membrane (Derkach et al., 2007; Oh et al., 2006). Additionally, phosphorylation of GluA1 at pS845 has been reported to play a role in stabilizing GluA1 homomers and retaining CP-AMPARs at peri-synaptic sites (He et al., 2009). Conversely, dephosphorylation of pS845 is correlated with LTD (S. H. Lee, Simonetta and Sheng, 2004) and down-regulation of cell surface GluA1 (Holman et al., 2007) and impaired spatial memory (H. K. Lee et al., 2003). Together these studies illustrate that enhancing pS845 phosphorylation promotes AMPA receptor plasticity and synaptic stabilization associated with fear memory retrieval.

Finally, we also focus on upstream plasticity related proteins that are known to participate in the trafficking of AMPA subunits, including protein kinase Mzeta (PKMζ). PKMζ is a specific autonomously active form of the atypical isozyme PKCζ (Hernandez et al., 2003). Recently PKCζ has been found to replace PKMζ function in the PKMζ-null mutant (Tsokas et al., 2016), revealing that wild-type animals and PKMζ-KO mice undergo LTP maintenance through different mechanisms. This finding has strengthened the hypothesis that PKMζ is vital to the mechanisms underlying synaptic plasticity in non-mutant animals. PKMζ has been shown to function in concert with GluA2 during synaptic plasticity (Ling et al., 2002; Yao et al., 2008). As the trafficking of the

GluA2 receptor subunit increases in the synapse during plasticity, clusters of PKMζ/GluA2/PSD-95 proteins develop (Shao, Sondhi, van de Nes and Sacktor, 2012), preventing AMPA receptors from undergoing endocytosis. Stabilization of AMPA receptors within the synaptic membrane is important for increasing mushroom spine heads (Sebastian et al., 2013), which in turn facilitates synaptic plasticity. Given that AMPA stabilization occurs within the post-synaptic membrane, we evaluated the role of PSD-95 and its specific phosphorylation site, S295, which is bidirectionally modulated by synaptic activity in neurons, promotes the synaptic accumulation of PSD-95 and enhances synaptic potentiation (Kim et al., 2007).

Our results show that juvenile rats, but not adults, also increased cytosolic PKMζ and PI3k in the DH. The increased expression in PKMζ is consistent with the elevated expression observed in GluA2 in the juveniles. GluA2 is known to be selectively trafficked to the synapse through the interaction with NSF and PKMζ (Ling et al., 2002; Sacktor, 2011; Tsokas et al., 2016). Moreover, pharmacologically disrupting the GluA2–NSF interaction inhibits PKMζ-induced synaptic potentiation (Yao et al., 2008) and the formation of fear memory (G. Joels and Lamprecht, 2010). These results show that PKMζ expression is critical for the maintenance of synaptic plasticity and fear memory similar to its role in the adult during spatial memory maintenance (Hsieh et al., 2017; Sacktor, 2011; Sebastian et al., 2013). Consistent with the increase in cytosolic PKMζ is the increased expression of PI3K. PI3K produces the lipid second messenger, phosphatidylinositol-3,4,5-trisphosphate (Ptdins-3,4,5-P3) that binds PDK1 to PKMζ. Positioned together, PDK1 can phosphorylate PKMζ converting it from inactive to an active conformation (Kelly et al., 2007). Thus, these results suggest that the juvenile rats are possibly maintaining contextual aspects of the fear memory involving PKMζ/GluA trafficking. Further pharmacological studies are needed to delineate this proposed mechanism.

An earlier study from our laboratory suggests that activation of the amygdala is involved in maintaining juvenile fear memory (Oliver et al., 2016). Similar to our present study, we showed that juveniles were only able to show contextual fear memory 1d after exposure to odor of a predator, but failed to maintain long-term memory when tested for fear response at 4d post-conditioning. When reconsolidation sessions were given 3d and 6d after initial training, however, fear memory was preserved 7d after conditioning. Elevated PKMζ expression was seen only in the amygdala, not DH, in juveniles that received reconsolidation sessions in comparison to juveniles that were not reexposed. These data suggest that repeated reminders and increased PKMζ maintain fear responses in juvenile animals that otherwise would not exhibit this behavior.

In the current study, 1d post-exposure to the platform fear paradigm, no change in PI3K expression was observed in either the adult or juvenile amygdala. This is somewhat consistent with a report showing differential effects of PI3K inhibition on contextual fear conditioning in juveniles and adults. PI3K is known to have differential effects in terms of acquisition and retrieval. Inhibition of PI3K after contextual fear conditioning with shock enhances contextual fear memory in adults but not in juveniles, while inhibition prior to memory retrieval caused juveniles to show enhanced freezing levels, with adults showing a reduction in freezing (Slouzkey and Maroun, 2016). This suggests that juveniles may be using different mechanisms than adults, who need PI3K in order to express fear memory. Despite seeing no changes in expression of PI3K in the adults at 1 d, it is possible that adults have sufficient PI3K to facilitate the retrieval of the fear memory. Further studies need to be done to elucidate this mechanism.

4.4. Remote contextual fear memory retrieval: Juvenile vs adult

Remote fear memory was maintained in the adults but not the juveniles. As predicted there were no changes in the juvenile amygdala while the adults show only a significant increase in GluA2. This non-CP AMPA subunit has a powerful influence on the ionic properties at the

synapse. AMPAR diffusion along the plasma membrane is receptor subtype specific which is dependent upon their synaptic location, as well as neuronal activity. In particular, GluA2 subunits diffuse more slowly in general as neurons mature, and exhibit trapping at synapses. The level of neuronal activity also affects the speed of diffusion, with increased activity slowing the movement of the subunits (Borgdorff and Choquet, 2002; Groc et al., 2004). That slowing of receptor diffusion within synapses may be mediated in part by the interaction with the synaptic scaffold proteins (Bats et al., 2007). These studies suggest that long-term increased expression of GluA2 reflects enhanced synaptic activity essential for stabilizing AMPA receptors associated with remote fear memory maintenance in the amygdala and perhaps allow rats with higher levels of GluA2 to more readily maintain other fear memories. Following remote fear memory retrieval, the adults show an increase in PSD-95 and pS295 expression in the DH, but not the juveniles. The adult pattern reflects what was observed at 1d in the amygdala. These results suggest that there may be a shift in the storage of the contextual fear memory and/or a shift in the reduction of the emotionality of the fear memory. The recent retrieval of the fear memory engaged the amygdala, while retrieval of the remote more directly engaged the contextual aspects of the fear memory and not the emotional aspects.

4.5. Long lasting consequences of fear memory consolidation in juvenile rats

Following remote fear memory testing in the juveniles, we did not expect changes in either the amygdala or DH since they failed to maintain a remote fear memory. The juvenile DH, however, shows a decrease in GluA1 expression. We interpret this result as a long-lasting consequence of fear-memory consolidation. Decreases in GluA1 would lower activity-dependent synaptic trafficking of GluA1 (Esteban et al., 2003) which is shown to enter spines upon stimulation and precede structural enlargement of the spine head (Kopec et al., 2006). These effects have also been demonstrated in fear conditioning in the lateral amygdala (Rumpel et al., 2005). Additionally, the rapid changes associated with synaptic plasticity are known to be driven by the insertion of GluA1 occurring within minutes of stimulation in neuronal cultures, followed by GluA2 and then GluA3 (Tanaka and Hirano, 2012). Together these studies suggest that long-lasting decreases in GluA1 expression may undermine many of the rapid GluA1-driven plasticity changes associated with increasing synaptic plasticity and memory.

4.6. Caveats

Two areas need to be addressed to confirm and extend our results. For reasons described in Methods, juvenile subjects were group housed, but adults were caged alone. Further, juveniles were stressed with the same platform as adults. Due to their smaller body, the stress may not have been severe. We do remind the reader, however, that the difference in memory between adults and juveniles matches many studies using shock or predator odor as the stressor (Bhattacharya et al., 2017; Oliver et al., 2016; Opendak et al., 2018).

5. Conclusions and clinical implications

Many psychopathologies including anxiety disorders, substance abuse, and PTSD have strong learning and memory components. Considerable basic and clinical research and even some clinical treatments are aimed at disrupting memory. This strategy is based on findings that, under certain circumstances, memories are labile when recalled. That is, the original memory can be altered by the presentation of new material or by pharmacological agents. To date, however, the results of clinical treatments have been inconsistent. Understanding the basic mechanisms of memory can only aid our search for efficacious treatments. Our research uses the 'natural' experiment provided by ontogenetic changes to uncover how memory is regulated biochemically. Young animals forget more quickly than adults. Investigation of

the differential neural responses in juveniles and adults in a contextual fear task is one step in elucidating the basic processes of memory. Our results identify differential expression involving PSD-95-pS295 and AMPA subunit expression across DH and amygdala following retrieval of a 1-day contextual fear memory in juvenile and adult rats. While both ages retain the fear memory for 1d, juveniles fail to engage amygdala driven PSD-95 expression unlike the adults. Conversely, the adults fail to show increased AMPA subunit and PKMζ expression, unlike the juveniles. These data highlight potential molecular signature for reduced contextual fear memory in juveniles involving the enhanced hippocampal and reduced amygdala activation. To focus on the developmental shift in expression of synaptic plasticity markers correlated to our behavioral paradigm, the scope of our current study is limited to only male subjects. Clinical reports show women are twice as more likely than men to develop anxiety and fear based disorders like post traumatic disorder (Tolin and Foa, 2006). Furthermore, studies on rodents show female rats in proestrus display an increase in spine morphology compared to males in the medial prefrontal cortex, in response to chronic stress (Garrett and Wellman, 2009). Future studies will explore the sex differences within our behavioral paradigm and the resulting molecular differences in the amygdala and hippocampus.

Significance statement

Young organisms do not maintain memories as long as adults, but the mechanisms for this ontogenetic difference are unknown. Our results identify differential expression involving PSD-95-pS295 and AMPA subunit expression across DH and amygdala following retrieval of a 1-day contextual fear memory in juvenile and adult rats. While both ages retain the fear memory for 1d, juveniles fail to engage amygdala driven PSD-95 expression unlike the adults. Conversely, the adults fail to show increased AMPA subunit and PKMζ expression, unlike the juveniles. These data highlight a potential molecular signature for reduced contextual fear memory in juveniles involving the enhanced hippocampal and reduced amygdala activation.

Author contributions

All authors contributed to designing the experiments and drafting the manuscript. RZ, SS, ER conducted all the behavioral and biochemical assays. RZ, SS, JA conducted all the statistical analyses. All authors approved the final manuscript for submission.

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