Research

Fear learning-induced changes in AMPAR and NMDAR expression in the fear circuit

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NMDA receptors (NMDARs) and AMPA receptors (AMPARs) in amygdala nuclei and the dorsal hippocampus (dHipp) are critical for fear conditioning. Enhancements in synaptic AMPAR expression in amygdala nuclei and the dHipp are critical for fear conditioning, with some studies observing changes in AMPAR expression across many neurons in these brain regions. Whether similar changes occur in other nodes of the fear circuit (e.g., ventral hippocampus [vHipp]) or changes in NMDAR expression in the fear circuit occur with fear conditioning have not been sufficiently examined. To address this we used near-infrared immunohistochemistry (IHC) to measure AMPAR and NMDAR subunit expression in several nodes of the fear circuit. Long-term changes in GluR1 and GluR2 expression in the ventral hippocampus (vHipp) and anterior cingulate cortex (ACC), enhanced NR2A expression in amygdala nuclei, and changes in the ratio of GluR1/NR2A and GluR2/NR2A in the dHipp was observed with fear conditioning. Most of these changes were dependent on protein synthesis during fear conditioning and were not observed immediately after fear conditioning. The results of the study suggest that global changes in AMPARs and NMDARs occur in multiple nodes within the fear circuit and raise the possibility that these changes contribute to fear memory. Further research examining how global changes in AMPAR, NMDAR, and AMPAR/NMDAR ratios within nodes of the fear circuit contribute to fear memory is needed.

[Supplemental material is available for this article.]

AMPA receptors (AMPARs) and NMDA receptors (NMDARs) have been consistently implicated in learning and memory (Malinow and Malenka 2002; Connor and Wang 2016; Diering and Huganir 2018). The role of these receptors in learning and memory have been extensively examined using fear conditioning. Fear conditioning involves presenting an innocuous conditioned stimulus (CS) such as a tone, light, or context with an aversive unconditioned stimulus (UCS). The most commonly used UCS is footshock. Prior to fear conditioning the CS does not elicit fear behavior, but after pairing the CS with an aversive event, robust fear behavior is observed (Davis 1992; Fanselow and LeDoux 1999; LeDoux 2000; Maren 2001; Pare et al. 2004; Orsini and Maren 2012).

Amygdala nuclei such as the basolateral amygdala (BLA) and central nucleus of the amygdala (CeA) are critical for fear memory (Miserendino et al. 1990; LeDoux 2000; Schafe and LeDoux 2000; Blair et al. 2001; Rodrigues et al. 2001; Bauer et al. 2002; Wilensky et al. 2006). Both AMPARs and NMDARs in amygdala nuclei have been implicated in fear conditioning. AMPAR and NMDAR antagonism in the BLA and CeA disrupt fear conditioning (Miserendino et al. 1990; Maren et al. 1996; Rodrigues et al. 2001; Bauer et al. 2002; Walker and Davis 2002; Goosens and Maren 2003; Walker et al. 2005; Zimmerman et al. 2007). Behavioral (Monfils et al. 2009), pharmacological (Joels and Lamprecht 2010), and genetic (Maren 2005) manipulations that disrupt GluR1 containing AMPAR expression or activation in the BLA disrupts fear memory. After fear conditioning, there is increased AMPAR insertion into sensory (cortical and thalamic) afferent and nonrelay thalamic synaptic input in different amygdala nuclei (McKernan and Shinnick-Gallagher 1997; Rogan et al. 1997; Rumpel et al. 2005; Humeau et al. 2007; Migues et al. 2010; Nedelescu et al. 2010; Thoeringer et al. 2012; Nabavi et al. 2014; Penzo et al. 2015). Blocking protein synthesis in the BLA blocks consolidation of fear memory (Schafe and LeDoux 2000; Wilensky et al. 2006). Together these studies suggest that AMPARs and NMDARs within amygdala nuclei are critical for fear memory, increases in AMPAR conductance within synapses in amygdala nuclei underlie fear memory, and increases in AMPARs are driven in part by novel protein synthesis during fear conditioning. Similar processes occurring in the dorsal hippocampus (dHipp) are believed to be critical for contextual representation (Maren et al. 1994; Goosens and Maren 2002; Zhou et al. 2009; Mitsushima et al. 2011; Penn et al. 2017).

Increases in expression of GluR1 subunits of AMPARs are observed after fear conditioning in the BLA and can be measured using immunohistochemistry (IHC) (Mei et al. 2005; Yeh et al. 2006). Such large increases in total AMPAR expression suggest up-regulation of AMPARs across many BLA neurons that is likely driven by enhanced transcription (Mei et al. 2005). Whether similar changes in AMPAR expression occur in other nodes of the fear circuit that are critical for associative or contextual memory has been insufficiently explored. The medial prefrontal cortex (mPFC) and ventral hippocampus (vHipp) are critical for representing contextual information during fear conditioning (Maren and Holt 2004; Rudy and Matus-Amat 2005; Maren et al. 2013; Rozeske et al. 2015; Heroux et al. 2017), the vHipp has been consistently implicated in CS–UCS associative fear memory (Bast et al. 2001; Maren and Holt 2004; Hunsaker and Kesner 2008; Staib

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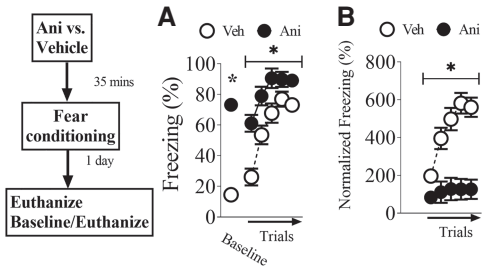

Figure 1. Effects of anisomycin treatment on freezing during fear conditioning. (*A*) Experimental design for experiment 1 and behavioral results. All rats (vehicle [Veh] = 9, anisomycin [Ani] = 9) showed increased freezing responses with repeated CS–UCS association (i.e., acquisition of fear memory), but subcutaneous administration of anisomycin prior to fear conditioning increased baseline freezing levels. (*B*) Freezing during CS-trials were then normalized to baseline freezing. These normalized freezing scores were lower in Ani-treated rats in comparison with Veh-treated rats. (*) *P*-value < 0.05, (* with bar) statistically significant comparisons across all trials.

et al. 2018), and the paraventricular nucleus of the thalamus (PVT) is also critical for fear memory (Do-Monte et al. 2015; Penzo et al. 2015). Furthermore, connectivity among these nodes within the fear circuit may also be critical for fear memory (Vetere et al. 2017). NMDAR activation in amygdala nuclei and the dHipp are critical for learning during fear conditioning (see above), but changes in expression of NMDARs in key nodes of the fear circuit after fear conditioning have not been extensively investigated.

In this study, we measured changes in AMPARs and NMDARs in several nodes of the fear circuit after fear conditioning using near-infrared IHC. Near-infrared immunofluorescence (680- to 780-nm wavelength) is outside of the range of autofluorescence in the brain and can be used to quantify protein expression in brain tissue (Spitzer et al. 2011; Kimmelmann-Shultz et al. 2019). With sufficiently high-resolution (e.g., 21 µm) expression of proteins in relatively small brain regions can be characterized (Kimmelmann-Shultz et al. 2019). In experiment 1 we examined whether changes in AMPAR and NMDAR expression observed 1 d after fear conditioning (i.e., long-term changes) could be detected using near-infrared IHC and whether these changes were dependent on protein transcription. In experiment 2 we examined whether any changes observed in experiment 1 were observed immediately after fear conditioning.

Overall, the results suggest that long-term changes in AMPAR and NMDAR expression in several nodes of the fear circuit occur after fear conditioning and most of these changes are dependent on protein synthesis. Furthermore, changes in the ratio of AMPAR/NMDAR expression in brain regions after fear conditioning could not be explained by changes in individual AMPAR or NMDAR expression, which suggests that changes in the ratio of AMPAR/NMDAR levels after fear conditioning may represent a unique molecular signature that could be relevant to fear learning and memory.

Results

Experiment 1

The behavioral results for experiment 1 are illustrated in Figure 1. All rats acquired fear conditioning, but administration of anisomycin enhanced conditioned freezing during baseline and it is likely this effect carried over during fear conditioning trials (drug × treatment interaction: $F_{(5,80)} = 8.8$, $P < 0.001$) (Fig. 1A). To address this, we normalized freezing during CS presentation relative to baseline for all rats. Analysis of these normalized freezing scores revealed that normalized freezing scores across fear conditioning trials were lower in anisomycin-treated rats in comparison with vehicle-treated rats (main effect of treatment: $F_{(1,16)} = 30.829$, $P < 0.001$) (Fig. 1B; Supplemental Fig. S1).

Validation assays for GluR2/NR2A assays are in S1 and representative images of near-infrared IHC for GluR1, GluR2, and NR2A in all brain regions are presented in the Supplemental Figure S2, A–E, as well as baseline values for all protein and protein ratios (Tables 1, 2).

Validation of GluR1/NR2A IHC has been previously published (Kimmelmann-Shultz et al. 2019). Figure 2 shows results for changes in protein expression in brain regions with experimental treatments. In animals in the vehicle treatment, there were decreases in GluR1 expression in the ACC ($t_{(9)} = 2.799$, $P = 0.021$) and increases in the vCA3 ($t_{(7)} = 2.82$, $P = 0.026$) and vCA1 ($t_{(7)} = 2.748$, $P = 0.029$) vHipp regions. All effects were absent with anisomycin treatment (ACC: $t_{(10)} = 1.607$, $P = 0.139$; vCA3: $t_{(9)} = 0.236$, $P = 0.819$; vCA1: $t_{(9)} = 0.035$, $P = 0.973$), though it should be noted that the ACC statistic may have been driven by an increase in variability as opposed to an increase in GluR1 levels. These results are illustrated in Figure 2A. There was an increase in GluR2 expression in the ACC ($t_{(6)} = 2.634$, $P = 0.039$) and this effect was absent with anisomycin treatment ($t_{(7)} = 1.982$, $P = 0.088$). However, this effect of anisomycin may have been driven by increases in variability as opposed to an increase in GluR2 expression. These results are illustrated in Figure 2B. NR2A levels were increased in all amygdala regions (LA: $t_{(9)} = 3.255$, $P = 0.01$; BA: $t_{(9)} = 2.826$, $P = 0.02$; CeA: $t_{(9)} = 3.357$, $P = 0.008$), and this increase was absent with anisomycin treatment in all amygdala regions (LA: $t_{(10)} = 0.996$, $P = 0.343$; BA: $t_{(10)} = 0.56$, $P = 0.567$; CeA: $t_{(9)} = 1.953$, $P = 0.083$). However, decreases in NR2A levels with anisomycin treatment were observed in the dCA1 ($t_{(11)} = 2.438$, $P = 0.033$), dDG ($t_{(11)} = 3.437$, $P = 0.006$), and PVT ($t_{(10)} = 3.391$, $P = 0.007$), while no effects were observed in the vehicle-treated animals (*P*s > 0.05). However, the nonsignificant effect in vehicle-treated animals with dDG NR2A levels may have been driven by increased variance in this measure. These results are illustrated in Figure 2C.

Decreases in GluR1/NR2A ratios were observed in the ACC ($t_{(8)} = 3.786$, $P = 0.004$), LA ($t_{(8)} = 2.618$, $P = 0.031$), BA ($t_{(8)} = 2.38$, $P = 0.045$), CeA ($t_{(8)} = 2.434$, $P = 0.041$), and dCA3 ($t_{(9)} = 3.105$, $P = 0.013$). These effects were absent in rats treated with anisomycin in the ACC ($t_{(10)} = 1.511$, $P = 0.162$), CeA ($t_{(10)} = 0.391$, $P = 0.708$), and dCA3 ($t_{(11)} = 0.686$, $P = 0.507$), but not the LA ($t_{(10)} = 3.583$, $P = 0.005$) or BA ($t_{(10)} = 3.702$, $P = 0.004$). GluR1/NR2A

Table 1. Baseline values for proteins and protein ratios used in experiment 1

	ACC	*PL*	*IL*	*BA*	*LA*	*CeA*
GluR1	155.88 ± 23.66	124.6 ± 4.27	128.05 ± 5.47	182.28 ± 31.69	165 ± 22.91	196.75 ± 2.32
GluR2	121.5 ± 5.8	120.43 ± 5.57	124.08 ± 6.56	145.02 ± 21.3	114.91 ± 12.43	121.65 ± 15.37
NR2A	152.07 ± 12.68	128.66 ± 9.34	132.14 ± 11.22	138.02 ± 12.43	126.88 ± 12.12	130.34 ± 11.04
GluR1/NR2A	105.02 ± 5.26	104.41 ± 2.22	105.28 ± 2.77	130.96 ± 8.58	127.42 ± 6.8	155.53 ± 10.27
GluR2/NR2A	86.63 ± 9.04	92.67 ± 7.78	94.18 ± 8.47	85.98 ± 15.37	84.18 ± 11.08	93.8 ± 17.22

Baseline values (in signal value [percentage]) for protein and protein ratios in the mPFC and amygdala nuclei in experiment 1.

Table 2. Baseline values for proteins and protein ratios used in experiment 1

	dCA1	*dCA3*	*dDG*	*vCA1*	*vCA3*	*vDG*	*PVT*
GluR1	252.17 ± 26.04	201.21 ± 27.47	198.77 ± 26.25	159.63 ± 21.16	152.77 ± 24.38	151.34 ± 20.75	133.05 ± 14.85
GluR2	156 ± 2.94	140.77 ± 2.18	140.42 ± 3.3	155.8 ± 14.4	161.73 ± 17.56	143.88 ± 10.88	115.07 ± 2.16
NR2A	146.95 ± 16.22	132.21 ± 12.88	141.99 ± 14.94	123.49 ± 7.9	120.62 ± 7.45	129.1 ± 7.73	133.93 ± 15.9
GluR1/NR2A	175.8 ± 6.97	133.56 ± 4.29	144.16 ± 5.11	131.33 ± 7.78	132.38 ± 10.12	123.99 ± 8.33	107.14 ± 3.87
GluR2/NR2A	105.89 ± 8.05	113.48 ± 5.56	99.2 ± 8.37	119.33 ± 2.39	108.9 ± 5.3	107.26 ± 1.55	86.75 ± 8.7

Baseline values (in signal value [percentage]) for protein and protein ratios in the hippocampus and PVT in experiment 1.

ratios were enhanced in the PVT of anisomycin-treated rats ($t_{(8)}$ = 2.841, P = 0.022), but not vehicle treated rats ($t_{(6)}$ = 1.143, P = 0.207). These results are illustrated in Figure 3A. GluR2/NR2A ratios were enhanced in the dDG ($t_{(7)}$ = 3.493, P = 0.01) and this effect was absent in rats treated with anisomycin ($t_{(7)}$ = 1.884, P = 0.102), though this noneffect may have been driven by enhanced variability in rats treated with anisomycin. These results are illustrated in Figure 3B.

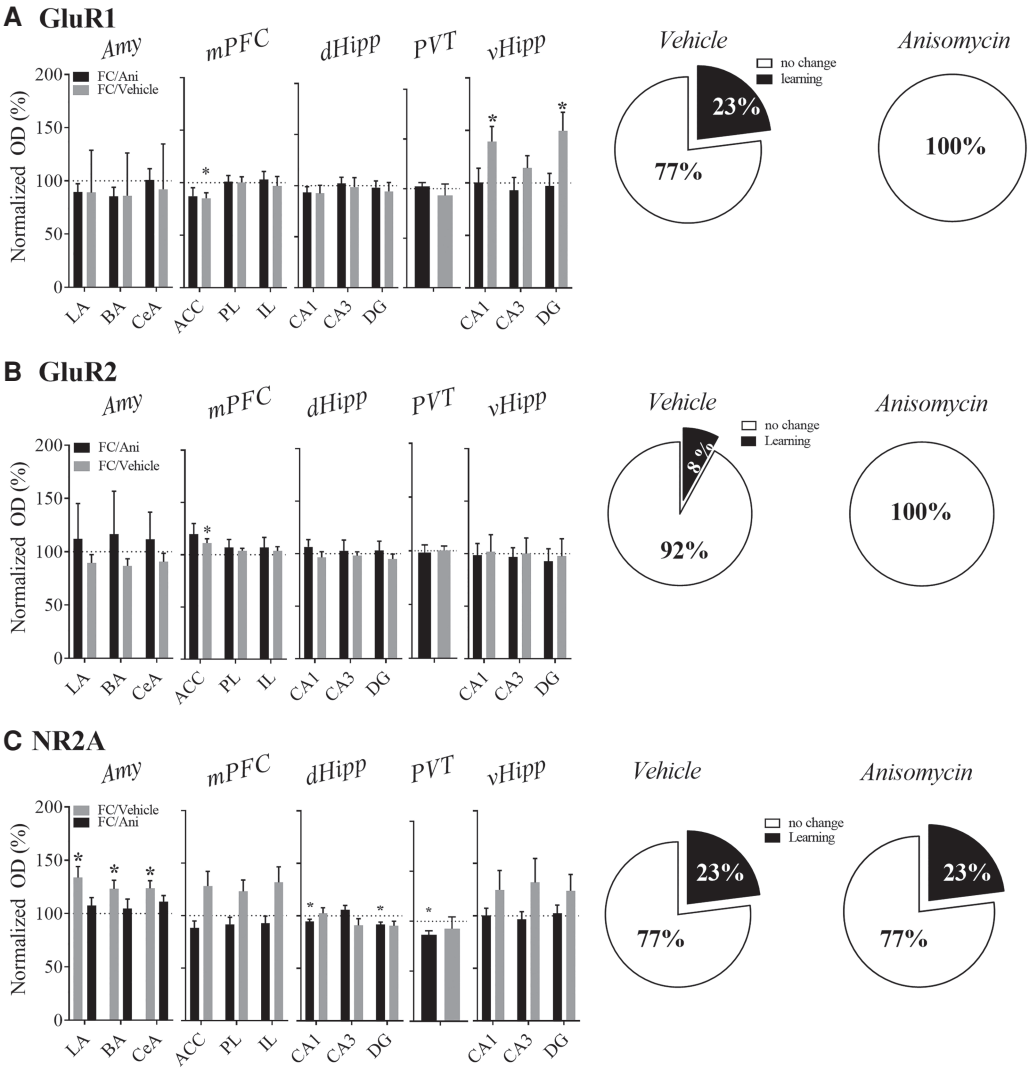

Figure 2. Long-term changes in AMPAR and NMDAR subunit expression with fear conditioning. Graphs show changes in receptor subunits and pie charts show the percentage of brain areas sampled that show changes in subunits with fear conditioning in anisomycin-treated and vehicle-treated animals. (*A*) GluR1 levels were decreased in the ACC and increased in the vCA1 and vDG. These changes were blocked with administration of anisomycin prior to fear conditioning, though effects on ACC GluR1 levels with anisomycin may have been driven by enhanced variance. (*B*) There was an increase in GluR2 expression in the ACC that was not observed in rats treated with anisomycin. This effect may have been due to increased variance in the anisomycin-treated animals. (*C*) In all amygdala nuclei, NR2A expression increased with fear conditioning and was blocked with anisomycin treatment. Anisomycin also decreased NR2A expression in dCA1, dDG, and the PVT. Effects with anisomycin on dDG NR2A levels may have been due to enhanced variability in these group of animals. (*) Significant one-sample *t*-test.

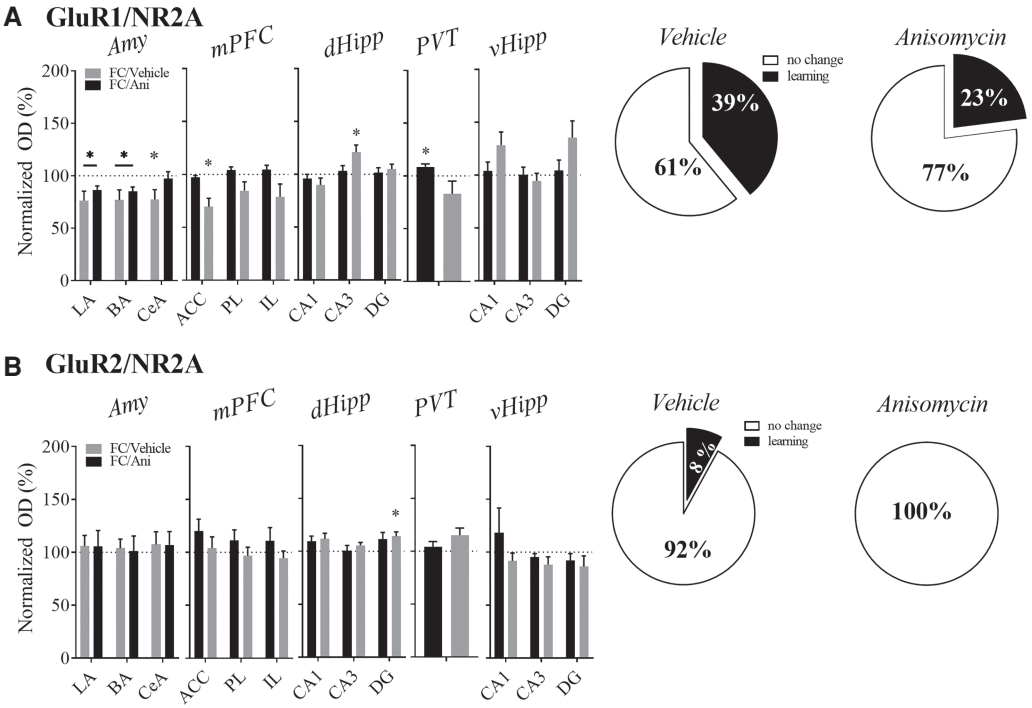

Figure 3. Long-term changes in AMPAR/NMDAR ratio expression with fear conditioning. Pie charts show the percentage of brain areas sampled that show changes in ratios with fear conditioning in anisomycin-treated and vehicle-treated animals. (*A*) GluR1/NR2A ratios were decreased in the ACC, amygdala nuclei, and dCA3. Only changes in the ACC, CeA, and dCA3 were blocked with anisomycin. Anisomycin treatment enhanced GluR1/NR2A ratios in the PVT. (*B*) There was an increase in GluR2/NR2A ratios in the dDG and this was blocked with anisomycin treatment, though this effect may have been driven by enhanced variance in the anisomycin-treated group. (*) Significant one-sample *t*-test.

Experiment 2

The behavioral results for experiment 2 are illustrated in Figure 4. Animals in the CS-Fear group showed significant increases in freezing behavior during CS presentation (trial × treatment interaction: $F_{(5,45)} = 105.825$, $P < 0.001$).

Representative images of near-infrared IHC for GluR1, GluR2, and NR2A in all brain regions for experiment 2 are presented in Supplemental Figure S3, A–E, as well as baseline values for all protein and protein ratios (Tables 3, 4).

Figure 5 shows results for changes in protein expression in brain regions with treatment. In animals in the CS-Fear treatment, there were decreases in GluR1 expression in all amygdala nuclei (LA: $t_{(12)} = 2.799$, $P = 0.021$; BA: $t_{(12)} = 3.21$, $P = 0.007$; CeA: $t_{(12)} = 3.09$, $P = 0.009$), dCA1 ($t_{(14)} = 2.959$, $P = 0.01$), and dDG ($t_{(14)} = 2.734$, $P = 0.016$). An increase in GluR1 expression was observed in the IL ($t_{(10)} = 2.904$, $P = 0.016$). For animals in the CS-Fear treatment there was an increase in GluR2 expression in the mPFC (ACC: $t_{(11)} = 2.554$, $P = 0.027$; PL: $t_{(11)} = 2.749$, $P = 0.019$; IL: $t_{(10)} = 2.793$, $P = 0.013$) and a decrease in the PVT ($t_{(13)} = 2.301$, $P = 0.039$). NR2A expression was decreased in the BA ($t_{(11)} = 2.697$, $P = 0.021$) and dCA3 ($t_{(13)} = 4.093$, $P = 0.001$) in animals in the CS-Fear treated animals. These results are illustrated in Figure 5.

There were increases in GluR2/NR2A ratios in the dCA3 ($t_{(13)} = 2.374$, $P = 0.034$) and PVT ($t_{(13)} = 4.632$, $P < 0.001$). For GluR2/NR2A ratios there were decreases in the amygdala (LA: $t_{(9)} = 3.338$, $P = 0.02$; BA: $t_{(9)} = 2.834$, $P = 0$, $P = 0.02$; CeA: $t_{(9)} = 4.699$, $P = 0.001$), dCA1 ($t_{(13)} = 3.544$, $P = 0.004$), and PVT ($t_{(13)} = 6.639$, $P < 0.001$). There were increases in GluR2/NR2A ratios in the dCA3 ($t_{(13)} = 2.984$, $P = 0.01$) and vCA1 ($t_{(10)} = 2.955$, $P = 0.01$) for the CS-Fear treatment. These results are illustrated in Figure 6.

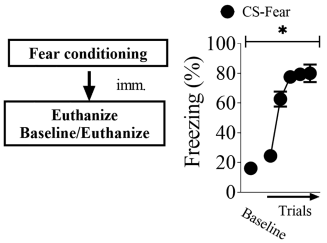

Figure 4. Experimental design and behavioral results for experiment 2. All animals ($n = 10$) acquired fear memory. (* with bar) *P*-value < 0.05 for statistical tests across trials.

Discussion

The results of experiments 1 and 2 are summarized in Tables 5 and 6. Using near-infrared IHC, we were able to detect a number of changes in AMPAR and NMDAR expression (relative to baseline) throughout critical nodes in the fear circuit. Within amygdala nuclei and dHipp, we did not observe changes in AMPAR expression with fear conditioning. While some studies have observed an increase in GluR1 expression in amygdala nuclei with fear conditioning (Mei et al. 2005), other studies have not (Yeh et al. 2006). However, there were increases in GluR1 expression in the vCA3 and vCA1 and an increase in GluR2 in the ACC with fear conditioning. Furthermore, AMPAR changes in the vHipp were eliminated with protein synthesis inhibition.

The vHipp has been consistently implicated in CS–UCS associative fear memory (Bast et al. 2001; Maren and Holt 2004; Hunsaker and Kesner 2008; Staib et al. 2018) and the ACC is critical for facilitating weak fear memory (Bissiere et al. 2008), remote

Table 3. Baseline values for proteins and protein ratios used in experiment 2

	ACC	*PL*	*IL*	*BA*	*LA*	*CeA*
GluR1	161.9 ± 12.82	121.58 ± 5.85	126.73 ± 8.19	224 ± 35.41	188.61 ± 19.78	223.28 ± 20.6
GluR2	154.47 ± 12.91	145.12 ± 10.71	161.28 ± 3.72	191.13 ± 20.1	170.62 ± 70.39	163 ± 31
NR2A	193.13 ± 14.27	158.02 ± 6.76	172.31 ± 8.53	176.19 ± 15.26	158.72 ± 13.73	155.07 ± 13.35
GluR1/NR2A	78.08 ± 6.05	76.92 ± 5.29	72.22 ± 6.33	121.79 ± 6.11	116.22 ± 5.02	142.53 ± 5.23
GluR2/NR2A	96.26 ± 2.05	106.53 ± 3.24	104.99 ± 3.7	118.53 ± 7.78	111.9 ± 8.6	111.56 ± 26.58

Baseline values (in signal value [percentage]) for protein and protein ratios in the mPFC and amygdala nuclei in experiment 2.

contextual fear memory (Einarsson and Nader 2012), and conditioned avoidance (Gabriel et al. 1991; Kang and Gabriel 1998). Changes in synaptic AMPAR conductance that facilitate neural plasticity critical for fear memory are believed to occur via increased synaptic AMPAR insertion via surface diffusion (Penn et al. 2017), changes in AMPAR trafficking (Rumpel et al. 2005; Migues et al. 2010; Mitsushima et al. 2011), and modification of the intracellular tail of AMPARs (Malinow and Malenka 2002). Thus, up-regulation of total AMPAR expression within a brain region (e.g., amygdala and dHipp) is not needed for enhancing synaptic AMPAR conductance. However, up-regulation of AMPAR subunits in the ACC and vHipp were observed and the up-regulation in the vHipp was dependent on protein synthesis. The meaning of this up-regulated response in these brain regions requires further research.

We observed increases in NR2A expression in all amygdala nuclei with fear conditioning and these increases were blocked with protein synthesis. NMDARs in BLA and CeA are critical for consolidation of fear memory, but synaptic plasticity that facilitates fear memory does not involve increases in synaptic NMDARs (Malinow and Malenka 2002; Nabavi et al. 2014; Connor and Wang 2016; Diering and Huganir 2018). What facilitating effect might enhanced NR2A expression in amygdala nuclei have after fear conditioning? There could be an overall increase in NMDARs that contain NR2A subunits without any overall change in NMDARs or fear conditioning could lead to an enhancement in extrasynaptic expression of NMDARs. Further research is needed to explore what role up-regulation in NR2A subunits in amygdala nuclei play in fear memory.

With the exception of increases in GluR2 expression in the ACC all changes in AMPARs and NMDARs observed 1 d after fear conditioning (i.e., long-term memory) were not observed immediately after fear conditioning. In the amygdala there were decreases in GluR1 (all amygdala nuclei) and NR2A (BA). Because these changes were not observed 1 d after fear conditioning it is likely that these changes represent a change in the configuration of receptors that occur immediately after fear conditioning and could be driven by acquisition of fear memory (Jarome et al. 2011) or an adapted response to enhanced excitation after fear conditioning (Zinebi et al. 2003). In the mPFC there was an up-regulation of GluR2 in all subregions examined and an increase in GluR1 in the IL. The mPFC has been implicated in contextual representation during fear learning (Maren et al. 2013; Rozeske et al. 2015; Heroux et al. 2017) and expression of fear behavior (Corcoran and Quirk 2007), but is also critical for stress reactivity (McKlveen et al. 2016; McKlveen et al. 2019; Page and Coutellier 2019). Further research is needed to examine how changes in mPFC AMPAR and NMDAR levels immediately after fear conditioning are linked to changes in memory or stress during fear conditioning.

Even though systemic administration of anisomycin blocked increases in protein expression in several brain regions and decreased CS-induced freezing relative to baseline, this drug treatment also significantly enhanced baseline freezing prior to fear conditioning and induced effects on protein expression (see Results). In agreement with previous research (Davis and Squire 1984), these findings suggest that systemic anisomycin administration may produce unwanted side effects and may be less preferable to localized protein synthesis inhibition when examining the role of protein synthesis within specific nodes of the fear circuit in learning and memory.

Using the Li-Cor scanner we were able to detect GluR1 and NR2A or GluR2 and NR2A subunits in the same brain region, which allowed us to construct GluR1/NR2A and GluR2/NR2A ratios in different nodes of the fear circuit. In some cases these ratios simply reflected changes in individual proteins. For example, in experiment 1, in the CeA there was an increase in NR2A levels, a corresponding decrease in GluR2/NR2A ratios, and both effects were sensitive to protein synthesis inhibition. However, in other instances, these ratios did not reflect changes in the individual proteins. In the ACC GluR2 levels were increased with fear conditioning, but there was no corresponding decrease in GluR2/NR2A levels. In the dCA3 and dDG there were increases in GluR1/NR2A and GluR2/NR2A ratios, respectively. However, fear conditioning did not change individual subunits of AMPARs or NMDARs. Instances where changes in GluR1/NR2A and GluR2/NR2A ratios did not correspond to changes in individual AMPAR or NMDAR subunit expression are italicized in Tables 5 and 6. An increase in the conductance of synaptic AMPARs relative to NMDARs drive the expression of neural plasticity (e.g., LTP) critical to formation and expression of fear memory and is an electrophysiological signature of memory (Malinow and Malenka 2002; Diering and Huganir 2018). Changes in the ratio of AMPAR/NMDAR expression could represent a unique molecular signature that is relevant to learning and memory, but further research exploring this possibility is needed.

Table 4. Baseline values for proteins and protein ratios used in experiment 2

	dCA1	*dCA3*	*dDG*	*vCA1*	*vCA3*	*vDG*	*PVT*
GluR1	259.29 ± 11.76	199.44 ± 13.77	198.02 ± 11.51	272.19 ± 50.28	227.41 ± 31.19	253.13 ± 51.36	122.77 ± 9.15
GluR2	179.73 ± 10.01	164.72 ± 7.67	162.85 ± 8.71	104.24 ± 5	104.37 ± 9.63	96.65 ± 4.52	139.08 ± 7.23
NR2A	142.94 ± 6.04	134.42 ± 8.52	132.57 ± 6.09	110.1 ± 3.45	107.49 ± 4.55	116.32 ± 3.6	128.38 ± 7.8
GluR1/NR2A	176.45 ± 4.59	146.68 ± 5.54	147.6 ± 5.09	169.68 ± 11.05	161.79 ± 9.89	147.4 ± 7.89	96.61 ± 6.21
GluR2/NR2A	132.89 ± 7.62	124.43 ± 10.78	124.69 ± 7.37	118.79 ± 3.33	106.95 ± 5.02	104.89 ± 2.02	120.78 ± 3.29

Baseline values (in signal value [percentage]) for protein and protein ratios in the hippocampus and PVT in experiment 2.

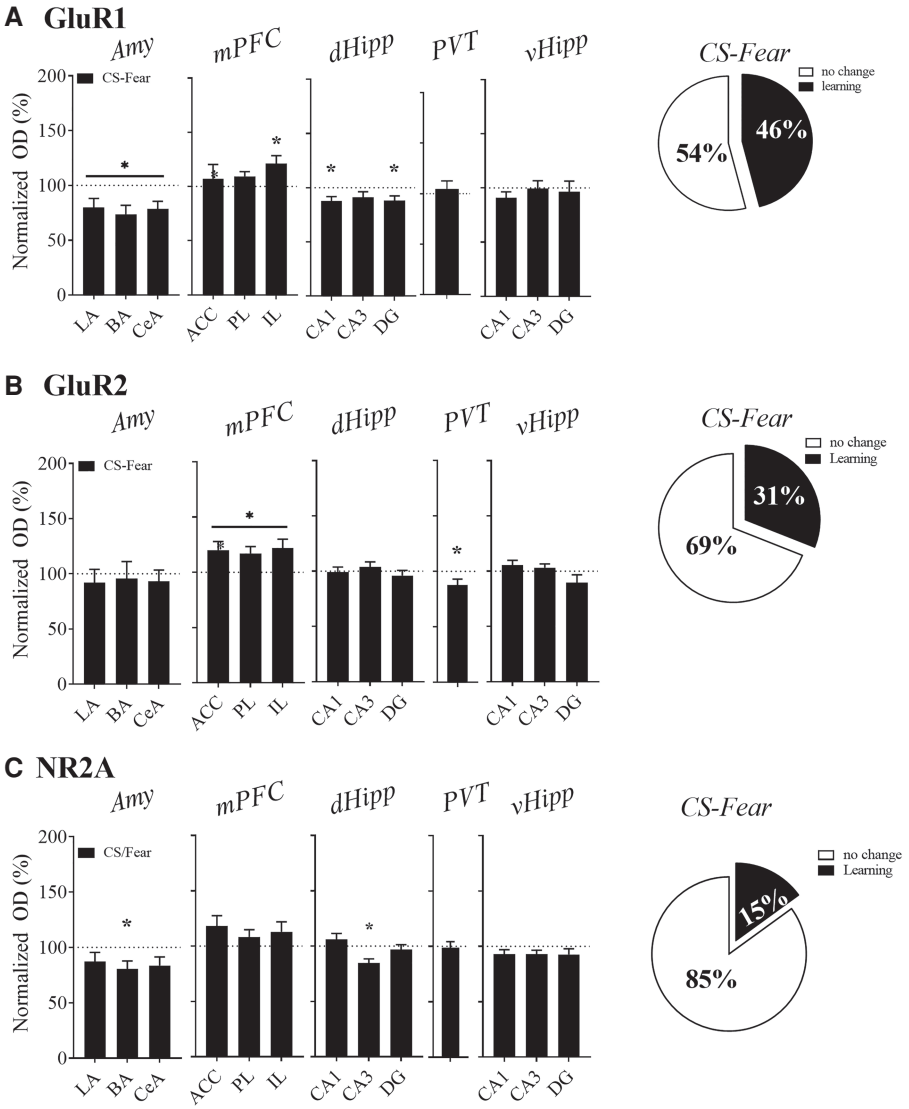

Figure 5. Short-term changes in AMPAR and NMDAR subunit expression with fear conditioning. Graphs show changes in receptor subunits and pie charts show the percentage of brain areas sampled that show changes in subunits with fear conditioning in anisomycin-treated and vehicle treated animals. (*A*) GluR1 levels were increased in the IL and decreased in amygdala nuclei, dCA1, and dDG. (*B*) There was an increase in GluR2 expression in the mPFC and a decrease in the PVT. (*C*) In BA and dCA3 there was a decrease in NR2A. (*) Significant one-sample t-test, (* with bar) significant one-sample *t*-test for all groups.

Conclusions

The results of this study shows that near-infrared IHC can be used to detect long-term and short-term changes in protein levels that occur after fear conditioning. The use of IHC likely resulted in the detection of AMPARs and NMDARs in the membrane and cytoplasm (i.e., total) of many neurons within a specific brain region. What these changes represent can only be determined with more research. However, the finding that AMPARs are up-regulated in the vHipp and ACC after fear conditioning, and up-regulation of AMPARs in the vHipp were disrupted with protein synthesis prior to fear conditioning, do point to changes in AMPAR dynamics in these brain regions contributing to fear learning and memory. Also, the increase in NMDARs in amygdala nuclei with fear conditioning suggests a dynamic role (either up-regulation or a change in the makeup of NMDARs) for amygdala NMDARs in fear memory. Verification of these possibilities will require further research.

Materials and Methods

Animals

Adult male Sprague Dawley rats ($n = 52$) were purchased from Charles River, Inc., for use in this study. Upon arrival, rats were pair housed for 3–5 d with ad libitum access to food during this time period, but were then kept on a diet of 23 g of rat chow per day, which is the manufacturer's recommended diet (LabDiet). Throughout the study, rats had ad libitum access to water. Experimental manipulations commenced at a minimum of 5 d after rats had been in the housing colony. Rats were on a 12-h light–dark cycle. All experimental procedures were performed in the animals' light cycle between 9:00 a.m. and 4:00 p.m. All experiments were approved by the University of Delaware Institutional Animal Care and Use Committee following guidelines established by the National Institutes of Health.

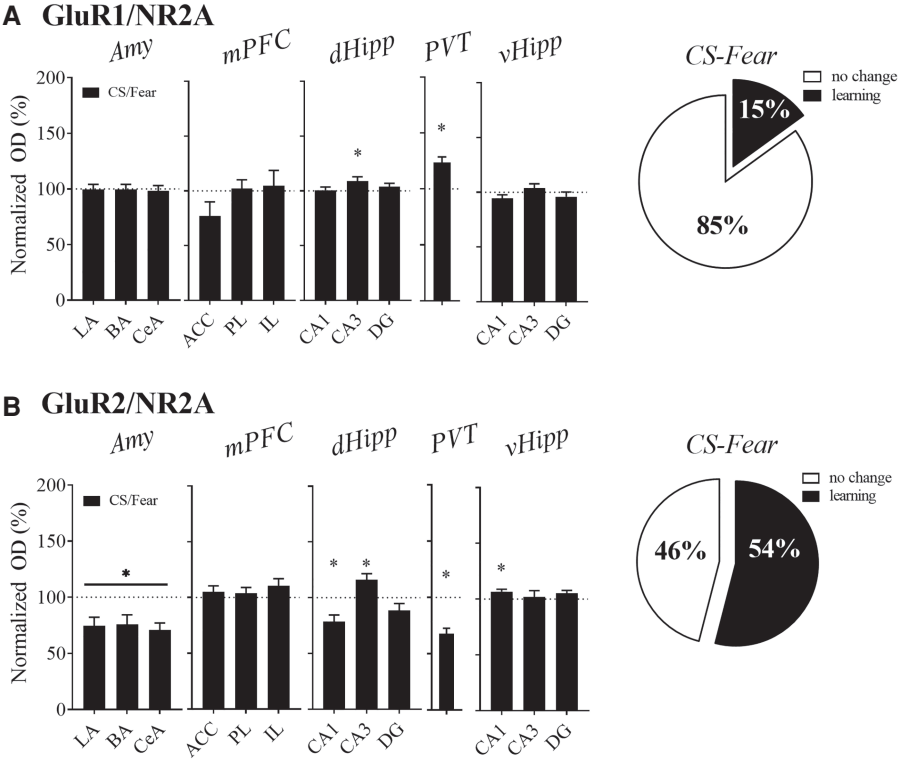

Figure 6. Short-term changes in AMPAR to NMDAR ratio expression with fear conditioning. Pie charts show the percentage of brain areas sampled that show changes in ratios with fear conditioning in animals. (*A*) GluR1/NR2A ratios were increased in the dCA3 and the PVT. (*B*) There were decreases in GluR2/NR2A ratios in amygdala nuclei, dCA1, and PVT; and increases in the dCA3 and vCA1. (*) Significant one-sample *t*-test, (* with bar) significant one-sample *t*-test for all groups.

Experimental procedures

Behavior

Fear conditioning was performed in MedAssociates, Inc., operant boxes as previously described (Knox et al. 2012a). Fear conditioning was conducted by presenting an auditory CS (10 sec, 80 dB, 2 kHz) that coterminated with a 1-sec, 1-mA footshock five times. The interstimulus interval was 60 sec. We conducted two experiments in this study. In experiment 1 rats were administered anisomycin (30 mg/kg in 1 mL/kg) in vehicle (1 N HCl adjusted to a final pH of 7.2 using NaOH) subcutaneously 35 min prior to fear conditioning. A previous report has shown this dose of anisomycin is sufficient to block protein synthesis in several brain regions (Davis et al. 1980). Another group of animals was administered vehicle prior to fear conditioning and another group of animals was administered vehicle, but left in the housing colony to establish baseline levels of proteins in different brain regions. The design for experiment 2 was similar to experiment 1, except all rats were euthanized immediately after fear conditioning and there was no drug treatment.

Near-infrared IHC

Rats were euthanized via rapid decapitation at the relative times listed above. Brains were then extracted and flash frozen in chilled isopentane and stored in a −80°C freezer until further processing. Brains were then thawed to −13°C in a cryostat (Leica CM1350) and 30 µm sections through the vmPFC (AP: 3.72–2.52 mm), dHipp, PVT, and BLA (AP: −2.4 mm to −4.2 mm), and vHipp (AP: −4.92 mm to −5.88 mm) were taken and mounted onto superfrost slides. All coordinates were based on the atlas of Paxinos and Watson (1998). Brain sections were then stored in a −80°C freezer until time of assay.

Double labeling IHC procedures were performed as previously described (Kimmelmann-Shultz et al. 2019). Either GluR1/NR2A or GluR2/NR2A double labeling procedures were performed in all brain regions. Sections were fixed in 4% paraformaldehyde in. 0.1 M phosphate-buffered saline (PBS). Sections were then incubated in Triton X-100, rinsed in 1 M tris buffered saline (TBS) and incubated in 3% goat serum. Sections were rinsed again in TBS and incubated with a rabbit polyclonal GluR1 (Millipore ABN241) and mouse monoclonal NR2A (Millipore MAB5216) primary antibodies at a concentration of 1:500 in PBS overnight at 4°C. Sections were then rinsed in TBS containing 0.01% Tween-20 (TBS-T). After this, sections were incubated in a solution consisting of TBS, 1.5% goat serum, 0.1% Triton X-100, and a 1:2000 dilution of goat antirabbit 800CW secondary antibody (Li-Cor Biotechnology, Inc.) and goat antimouse 680RD secondary antibody (Li-Cor Biotechnology, Inc.) for 2 h at room temperature. Sections were rinsed in TBS-T and TBS, and then deionized water. Sections were then left to air-dry overnight. A similar procedure was used to visualize GluR2 (Millipore MABN71) and NR2A in the fear circuit.

Data analysis and statistical analysis

Freezing behavior was scored using Any-maze (Stotelting Inc.) as previously described (Knox et al. 2012b). For experiment 1, freezing was subjected to a drug

Table 5. Summary of effects for experiment 1

	GluR1	GluR2	NR2A	GluR1/NR2A	GluR2/NR2A
ACC	↓Learning	↑Learning	X	↓*Learning* √Ani	*X*
PL	X	X	X	X	X
IL	X	X	X	X	X
LA	X	X	↑Learning √Ani	↓*Learning*	*X*
BA	X	X	↑Learning √Ani	↓*Learning*	*X*
CeA	X	X	↑Learning √Ani	↓Learning √Ani	*X*
dCA1	X	X	↓Ani	*X*	*X*
dCA3	X	X	X	↓*Learning* √*Ani*	X
dDG	X	X	X	*X*	↑*Learning*
vCA1	↑Learning √Ani	X	X	*X*	X
vCA3	↑Learning √Ani	X	X	*X*	X
vDG	X	X	X	X	X
PVT	X	X	↓Ani	↑Ani	*X*

Changes in AMPAR and NMDAR subunit expression with fear conditioning and sensitivity to protein synthesis inhibition with anisomycin (Ani) from experiment 1. (X) No effect, (↑) an increase, (↓) a decrease. Check mark (√) followed by drug means anisomycin blocked the observed effect. Terms in italics in the last two columns mean that changes in protein ratios did not reflect changes in individual protein levels. Effects that may have been driven by enhanced variability have been left out of the table.

Table 6. Summary of effects for experiment 2

	GluR1	GluR2	NR2A	GluR1/NR2A	GluR2/NR2A
ACC	X	↑CS-Fear	X	*X*	*X*
PL	X	↑CS-Fear	X	*X*	*X*
IL	↑CS-Fear	↑CS-Fear	X	*X*	*X*
LA	↓CS-Fear	X	X	*X*	↓*CS-Fear*
BA	↓CS-Fear	X	↓CS-Fear	*X*	↓*CS-Fear*
CeA	↓CS-Fear	X	X	*X*	↓*CS-Fear*
dCA1	↓CS-Fear	X	X	*X*	↓*CS-Fear*
dCA3	X	X	↓CS-Fear	↑CS-Fear	↑CS-Fear
dDG	↓CS-Fear	X	X	*X*	X
vCA1	X	X	X	X	↑*CS-Fear*
vCA3	X	X	X	X	X
vDG	X	X	X	X	X
PVT	X	↓CS-Fear	X	↑*CS-Fear*	↓*CS-Fear*

Changes in AMPAR and NMDAR subunit expression with fear conditioning from experiment 2. (X) No effect, (↑) an increase, (↓) a decrease. Terms in italics in the last two columns mean that changes in protein ratios did not reflect changes in individual protein levels.

(vehicle vs. anisomycin) x trial (baseline, 1–5) factor design. For experiment 2, freezing was subjected to a single factor (i.e., trial) repeated measures factor design. For all factor designs main and simple effects were analyzed using analysis of variance (ANOVA) while main and simple comparisons were analyzed using *t*-test with Bonferroni corrections applied where necessary.

Dried brain sections, treated for GluR1, GluR2, and NR2A IHC, were scanned at 21 µm, highest resolution, and an offset of 0 in the Odyssey scanner (Li-Cor Biotechnology, Inc.). Fluorescent activity measured in the Li-Cor scanner from acquired images remain constant irrespective of how an image is manipulated for viewing/presentation purposes in this system. Fluorescent activity in a particular brain region was then expressed as a percent change from activity in the corpus callosum. We referred to this as signal activity. As we have done in previous studies (Eagle et al. 2013; Knox et al. 2016, 2018; Della Valle et al. 2019), signal activity from protein in all brain regions was normalized with respect to animals in the baseline treatment (i.e., normalized activity). For experiments 1 and 2 the baseline condition consisted of 12 rats. Normalized activity was constructed so that if signal activity was equal to baseline signal activity a normalized score of 100% [i.e., (signal activity/averaged baseline activity) × 100] would be obtained.

For experiments 1 and 2, normalized activity in the fear circuit (i.e., IL, PL, dHipp [dCA1, dCA3, and dDG], BLA [lateral {LA} and basal {BA} regions separately], CeA, PVT, and vHipp [vCA1, vDG, and vCA3]) was subjected to separate one-sample *t*-tests with the test value being set to 100. For experiment 1 this procedure was adopted separately for the vehicle and drug treated animals. All statistical tests were performed in IBM SPSS statistic 23. Any additional post-hoc *t*-tests were subject to Bonferroni corrections. P-value < 0.05 was taken as statistical significance for all tests.

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Author contributions: B.S., A.F., and B.C. helped slice brains, developed a consistent method for scoring and analyzing images, performed near-infrared IHC assays, wrote parts of the manuscript, and reviewed the manuscript. N.M. helped develop the double labeling IHC assay for AMPARs and NMDARs, sliced brains, helped with near-infrared IHC assays, and reviewed the manuscript. D.K. designed experiment, helped develop the double labeling IHC assay for AMPARs and NMDARs, performed all statistical analyses, and wrote and edited the manuscript.

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