REGULAR RESEARCH ARTICLE

PI3K-Akt Signaling in the Basolateral Amygdala Facilitates Traumatic Stress Enhancements in Fear Memory

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Abstract

Background: A core symptom of posttraumatic stress disorder is persistent fear memory, which can be defined as fear memory that is resistant to updating, inhibition, or extinction. Posttraumatic stress disorder emerges after traumatic stress exposure, but neurobiological mechanisms via which traumatic stress leads to persistent fear memory are not well defined. Akt signaling within the amygdala (Amy) is enhanced with traumatic stress, and phosphatidylinositol kinase 3 (PI3K) activation of Akt within the basolateral Amy (BLA) has been implicated as critical to fear memory formation. These findings raise the possibility that traumatic stress enhances PI3K→Akt signaling in the BLA, which leads to persistent fear memory.

Methods: To test this hypothesis, rats were exposed to traumatic stress using the single prolonged stress model, and changes in Akt phosphorylation were assayed in the Amy at 0 and 30 minutes after fear conditioning (FC). In a separate experiment, we inhibited PI3K→Akt signaling in the BLA prior to FC and observed the effect this had on acquisition, expression, and extinction of FC in stressed and control rats.

Results: Enhanced Akt phosphorylation in the Amy at both time points was observed in stressed rats, but not in control rats. PI3K→Akt inhibition in the BLA had no effect on freezing in control rats but decreased freezing during extinction training and testing in stressed rats.

Conclusion: These findings suggest that PI3K→Akt signaling in the BLA could be a mechanism via which traumatic stress leads to fear memory that is resistant to extinction.

Key Words: Basolateral amygdala, extinction, fear memory, PTSD, single prolonged stress

Introduction

Posttraumatic stress disorder (PTSD) is a debilitating disorder that has a lifetime prevalence rate of 7%–9% of the US population (Kessler et al., 1995). A core PTSD symptom is the persistence of memories that accompany traumatic events that initiated PTSD (i.e., traumatic fear memory) (Kessler et al., 1995; Milad et al., 2009; American Psychiatric Association, 2013). Persistent fear memory can be defined as memory that is resistant to inhibition, updating, or extinction (Giustino and Maren, 2015; Bergstrom, 2016; Careaga et al., 2016). Persistent fear memory in PTSD can emerge because of enhanced fear memory (Morgan et al., 1995; Jovanovic et al., 2011; Inslicht et al., 2013) or deficits in inhibitory extinction memory...
Significance Statement

Previous studies have observed that Akt phosphorylation (i.e., activation of Akt) is enhanced with traumatic stress exposure, and PI3K→Akt signaling in the basolateral amygdala (BLA) is necessary for fear memory formation. This suggests that traumatic stress may enhance fear memory by enhancing PI3K→Akt signaling in the BLA. The results of this study suggests that PI3K→Akt signaling in the BLA is not important for fear memory per se but is a molecular signaling pathway that facilitates traumatic stress-induced changes in fear memory. In turn, these findings raise the possibility that PI3K→Akt signaling might contribute to enhancements in fear memory observed in traumatic stress-induced disorders such as posttraumatic stress disorder.

Materials and Methods

Animals

A total 114 male Sprague-Dawley rats (approximately 150–250 g on arrival) obtained from Charles River Inc. were used in this study. This strain and sex was used because the SPS model is well characterized in this strain and sex of animal (Yamamoto et al., 2009; Lisiieski et al., 2018). On arrival, rats were housed in pairs during a 5-day acclimation period with ad libitum access to food and water. Experiments commenced following the animals’ acclimation period. Following SPS and control procedures, rats were individually housed and restricted to 23 g/d of standard rat chow per the manufacturer’s recommendation (LabDiet, St. Louis, MO) with ad libitum access to water. The rats were on a 12-hour-light/12-dark cycle, and all experimental procedures were performed in the animals’ light cycle between the hours of 9:00 am and 4:00 pm. All experiments were approved by the University of Delaware Institutional Animal Care and Use committee following guidelines established by the NIH.

SPS and Behavioral Procedures

All rats were randomly assigned to SPS or control stress. SPS was conducted as previously described (Liberonz et al., 1999; Knox et al., 2010) and consisted of 2 hours of restraint followed by 20 minutes of forced swim, then ether exposure until general anesthesia was induced. For control stress, rats were placed into a novel room in their home cages for the duration of SPS. A post-stress incubation period of 7–10 days was allowed to elapse prior to experimental testing, because this window of time is necessary to observe SPS effects (Liberonz et al., 1999; Knox et al., 2012b).

FC sessions were conducted as previously described (Knox et al., 2012a, 2012b) using 6 MedAssociates (Fairfax, VT) operant boxes. Briefly, FC consisted of 5 presentations of a 10-second auditory conditioned stimulus (CS, 2 kHz, 80 dB) that co-terminated with a 1-second, 1-mA footshock unconditioned stimulus. In some experiments, animals were also subjected to extinction training (30 CS-only presentations) and extinction testing (1 CS-only presentations). FC, extinction training, and testing sessions were conducted in unique contexts, and all sessions began with a 210-second baseline period and had inter-stimulus intervals of 60 seconds.
Western Blot

SPS and control rats were randomly assigned to 1 of 3 groups: baseline, FC0, or FC30. Rats in the baseline treatment were removed from the housing colony and immediately killed to determine basal Akt and pAkt levels in brain regions. All other rats were removed from the housing colony, subjected to FC, and removed from the operant boxes and killed either immediately (FC0) or 30 minutes (FC30) after FC. These timelines were selected because a previous report suggests maximal pAkt is observed 30 minutes after FC (Lin et al., 2001). All animals were killed via rapid decapitation, and their brains were immediately extracted and flash frozen in isopentane chilled on dry ice. Brains were then stored in a −80°C freezer until further processing. To dissect brain regions, brains were thawed to −13°C in a cryostat (Leica CM1350), and 300-μm coronal sections through the mPFC, Amy, dHipp, and vHipp were taken and these brain regions dissected out and placed into 1.5-mL microtubes. Dissected brain regions were then stored in a −80°C freezer until further processing.

At the start of sample preparation, brain regions were homogenized in 250 μL of buffer (50 mM Tris buffer, 10% sucrose, 1 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol, 1 mM benzamidine, 0.3 mM phenylmethylsulfonyl fluoride) by a motor-driven homogenizer (Fisher Scientific, PowerGen125). The homogenate was then centrifuged at 14,800 × g for 45 minutes at 4°C and the supernatant treated as the total cellular extract of brain tissue. The protein concentration of samples was then determined using Bradford assay per the manufacturer’s instructions (Pierce BCA Protein Assay Kit). Also 0.5× Laemmli sample buffer was mixed with approximately 15 μg of protein from each sample. These samples were stored in a −80°C until western blot.

At the start of western blot, protein samples were heated at 70°C for 7 minutes before being loaded into 10% Tris-HCl polyacrylamide gels and separated by SDS polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred from gels to nitrocellulose membranes. The membranes were subsequently left to dry for 30 minutes at 37°C followed by rehydration washes in 0.5 M Tris-buffered saline (TBS). Membranes were blocked for 1 hour at room temperature in TBS containing 5% nonfat milk. Nitrocellulose membranes were first probed for pAkt and β-actin (reference protein) by incubating overnight at 4°C with a polyclonal rabbit pAkt antibody (1:500, Cell Signaling, #4060, ser473 site) and a mouse β-actin antibody (1:2000, Cell Signaling Inc. #8H10D10) in TBS. After 18–20 hours, the membranes were subjected to several washes in 0.5 M TBS with 0.1% Tween-20 and then a 2-hour incubation at room temperature with polyclonal goat anti-rabbit (800CW, 1:2000, LI-COR Lincoln, NE) and anti-mouse IgG (680RD, 1:5000, LI-COR) secondary antibodies in 0.5M TBS containing 0.1% Tween and 5% nonfat milk. Nitrocellulose membranes were then washed in TBS and scanned in the Li-cor Odyssey Clx scanner under the following settings: resolution, 169 μm; quality, lowest; focus offset, 0.0 mm. This procedure was then repeated again in all membranes to image Akt bands using a polyclonal rabbit pan-Akt antibody (1:500, Cell Signaling, #4691) and the 800CW goat anti-rabbit antibody.

PI3K-akt Inhibition

For experiments involving PI3K-Akt inhibition prior to FC, the drug treatments were adapted from previous studies that show infusion of wortmannin into the BLA decreases phosphorylation of Akt at the ser273 site (Lin et al., 2001; Ou and Gean, 2006). Rats were first implanted with 26-gauge guide cannulas (Pl technologies, Roanoke, VA) 11 mm long. Cannulas were aimed at the BLA (AP: −2.3 mm, ML: ±5.1 mm, DV: −7.1 mm) using previously described techniques (Fitzpatrick et al., 2011). Guide cannulas were secured to the skulls of rats using dental acrylic. After 5 days of surgical recovery, rats were subjected to either SPS or control stress. Fifteen minutes prior to the start of FC, 0.2 μL/hemisphere of the selective PI3K inhibitor wortmannin (TOCRIS Inc.; 5 μg/μL) or vehicle was infused into the BLA at a rate of 0.2 μL/min. The dose of wortmannin was selected because it inhibits pAkt at the ser473 site (Lin et al., 2001; Ou and Gean, 2006). Wortmannin has a short half-life (10–15 minutes) and thus can only alter acquisition or consolidation of FC. Infusion was accomplished by using a micropump (Harvard Apparatus, Holliston, MA), infusion cannula (33 gauge projecting 1 mm beyond the guides), and PE-tubing connecting infusion cannula to 5-μL Hamilton syringes. Animals were then subjected to FC, extinction training, and extinction testing. After extinction testing, animals were infused with 0.2 μL of a 2% pontamine sky blue solution in physiological saline, under 2% isoflurane anesthesia, to locate infusion cannula tips. Animals were then killed by rapid decapitation and brains extracted and sliced in a cryostat at 30 μm. Sections were then Nissl stained as previously described (Knox and Keller, 2016).

Data and Statistical Analysis

Freezing behavior was analyzed using ANY-maze (Stoelting Inc., Wood Dale, IL) as previously described (Knox et al., 2012b). For FC involving western-blot experiments, baseline freezing was analyzed using a stress (SPS vs control) × condition (FC0 vs FC30) factor design. Conditioned freezing during FC trials was analyzed using a stress × condition × trial (1–5) factor design. For all PI3K-Akt inhibition experiments, conditioned freezing during behavioral sessions was analyzed at select critical points. For FC, this was baseline and trial 5 (to measure acquisition of fear memory), extinction training during baseline, the first 4 CS trials (to measure fear memory retrieval [FMR]), and the last 2 trials of extinction training (to measure acquisition of extinction), and extinction testing during baseline and the single CS trial. To maximize the power of statistical analyses for the PI3K-Akt inhibition experiments, the effects of infusing wortmannin into the BLA was analyzed separately in SPS and control rats using drug (wortmannin vs vehicle) × trial factor designs.

To reduce variability in western-blot data, representative rats from each independent factor (i.e., stress and condition) were always included in each protein assay and western blot. The integrated intensity of all bands were scored using ImageStudio. The profile curves of all bands were inspected to ensure that there was significant integrated intensity signal above the background within each lane in every western. For all statistical analyses, pAkt and Akt were expressed relative to β-actin to yield relative pAkt and Akt levels. Relative pAkt and Akt levels from FC experiments were normalized relative to baseline and subjected to a stress × condition (FC0 vs FC30) factor design.

For all factor designs, main and simple effects were analyzed using ANOVA, while main and simple comparisons were analyzed using independent, paired sample, or 1 sample t test with a Bonferroni correction applied where appropriate. The reference value for all 1 sample t tests was set to 100. Statistical significance was assumed with P < .05 for all statistical tests. Outliers were excluded from data analysis and were defined as numbers that were 2 SDs away from a group mean.
RESULTS

Western Blot

Figure 1 shows the experimental design and freezing behavior during FC. There were no significant effects of stress or condition for baseline freezing (Ps > .05). There was a main effect of trial \(F_{(4,128)} = 95.825, P < .001\) for conditioned freezing during FC but no significant effects of stress or condition (Ps > .05). This suggested rats in all groups acquired fear memory in an equivalent manner.

The use of our western-blot protocol to separately measure Akt and pAkt bands is illustrated in supplementary Figure 1. Figure 2A shows representative westerns for Amy homogenates. SPS enhanced pAkt levels during FC in Amy homogenates without having any effect on Akt levels (Figure 2B–C). This was revealed by a significant main effect of stress for pAkt/actin ratios \(F_{(1,34)} = 5.493, P = .026\) not Akt/actin ratios (Ps > .05). This effect was most pronounced at the FC0 time point. pAkt/Akt ratios from Amy homogenates were not affected by any treatment (Ps > .05; Figure 2D). Figure 2E shows representative westerns for mPFC homogenates. In the mPFC, Akt levels were unaffected by any treatments (Ps > .05). pAkt levels were enhanced at FC30 in control rats but decreased in SPS rats. This resulted in a stress × condition effect for pAkt/actin and pAkt/Akt ratios [pAkt/actin: \(F_{(1,36)} = 5.493, P = .026\), pAkt/Akt: \(F_{(1,30)} = 5.264, P = .029\); Figure 2F–H]. Figure 3A show representative westerns for dHipp homogenates. Neither stress nor condition had any effect on pAkt or Akt ratios in the dHipp (Ps > .05; Figures 3B–D). Figure 3E shows representative westerns for vHipp homogenates. Akt/actin and pAkt/actin ratios of vHipp homogenates were unaffected by any treatments (Ps > .05; Figures 3F and 3G). SPS enhanced pAkt/Akt ratios at baseline in vHipp homogenates and this effect approached significance \(t_{(33)} = 1.945, P = .06\); Figure 3H). However, there was no significant effects of stress on pAkt/Akt ratios in the vHipp during FC (Ps > .05; Figure 3H).

PI3K-akt Inhibition

Figure 4A shows the design for this experiment. Figure 4B shows placements of cannulas for the drug condition for SPS (red/brown) and control (blue/purple) animals. Qualitative analysis revealed that irrespective of cannula placement, vehicle-treated animals within a stress group did not differ significantly regarding behavior. As a result, all SPS and control animals in the vehicle treatment were used for statistical analysis.

There was a main effect of trial (SPS: \(F_{(1,13)} = 73.028, P < .001\); control: \(F_{(1,13)} = 91.521, P < .001\), but no drug effects for SPS or control rats (Ps > .05) for conditioned freezing during FC (Figure 4C).

Discussion

SPS enhanced pAkt/actin ratios in the Amy of rats undergoing FC for up to 30 minutes after FC, but there was no enhancement in pAkt/actin ratios at baseline. This suggests SPS did not enhance pAkt levels on its own but enhanced pAkt signaling in SPS rats during fear memory formation. There was no change in the ratios of pAkt/Akt in Amy homogenates (see Results). The reason for this is not clear. One possibility could be a combined enhancement in Akt (too small to attain statistical significance in this study) with an enhancement in phosphorylation of Akt. Irrespective, the enhancement in pAkt/actin ratios in Amy homogenates suggests an enhancement in pAkt levels. Inhibiting PI3K→Akt signaling in the BLA reduced conditioned freezing in SPS rats during extinction training and testing. Importantly, inhibition of PI3K→Akt signaling had no effect on conditioned freezing at baseline or during CS presentation in FC. These findings suggest that wortmannin had no effect on locomotor activity or acquisition of fear memory. Overall, these findings suggest that PI3K→Akt signaling in the BLA during FC enhances fear memory in SPS rats and renders fear memory resistant to extinction. There was also an enhancement in conditioned freezing during extinction training in SPS/vehicle rats compared with SPS/drug rats that approached significance. This observation too is consistent with SPS enhancing fear memory. In control rats, there was no change in pAkt in the Amy with FC, and inhibiting PI3K→Akt signaling in the BLA had no effect on conditioned freezing in control rats. Thus, it would appear that PI3K→Akt signaling in the BLA during FC selectively modulates fear memory in stressed rats.

SPS decreased pAkt in the mPFC 30 minutes after FC. In control rats, FC increased pAkt at the same time point. The mPFC is not necessary for cued fear memory formation (Maren, 2001; Paré et al., 2004; Quirk et al., 2006; Corcoran and Quirk, 2007; Orsini and Maren, 2012). The mPFC has been implicated in contextual fear memory (Beeman et al., 2013; Rozeske et al., 2015), but previous studies have observed that inhibiting PI3K→Akt signaling in the mPFC during FC has no effect on contextual fear memory (Sui et al., 2008). We used cued FC to examine fear memory, which makes it unlikely that changes in pAkt observed in the mPFC 30 minutes after FC contributed to changes in fear memory in SPS or control animals. SPS may have enhanced pAkt in the vHipp at baseline, which is consistent with a previous finding (Eagle et al., 2013), but SPS had no effect on pAkt levels in the dHipp or vHipp during FC.
Figure 2. Changes in phosphorylated Akt (pAkt) and Akt in the amygdala (Amy) and medial prefrontal cortex (mPFC). (A) Representative Amy westerns. (B) Single prolonged stress (SPS) had no effect on Akt levels, but (C) enhanced pAkt/actin ratios in the Amy. (D) There was no effect of SPS on pAkt/Akt ratios. (E) Representative mPFC westerns. (F) In control animals, pAkt/actin ratios were enhanced 30' after fear conditioning (FC), but in SPS rats these ratios were decreased 30' after FC. (G) SPS had no effect on Akt/actin ratios. (H) In control animals, pAkt/Akt ratios were enhanced 30' after FC, but in SPS rats these ratios were decreased 30' after FC. Baseline levels (SPS = 18, control = 18) of Akt and pAkt in the Amy and mPFC were not affected by SPS. One SPS animal’s datum point was dropped from the Amy analysis, because it was more than 2 SDs away from the group mean. Error bars represent SEM, black * indicates main effect of stress, red * indicates stress × time interaction.
Figure 3. Changes in pAkt and Akt in the dorsal hippocampus (dHipp) and ventral hippocampus (vHipp). (A) Representative dHipp westerns. (B–D) Single prolonged stress (SPS) had no effect on Akt or phosphorylated Akt (pAkt) levels. (E) Representative vHipp westerns. (F and G) There were no effects observed for pAkt/actin or Akt/actin levels. (E) SPS rats had enhanced pAkt/Akt ratios at baseline (SPS = 18, control = 18), but this effect only approached significance (+ represents $P = .06$). SPS had no effect on pAkt/Akt ratios during fear conditioning.
The results of this study are consistent with previous findings that have observed enhancing pAkt is accompanied by enhanced conditioned fear responding (Dahlhoff et al., 2010; Yen et al., 2012). Previous findings have observed PI3K → Akt signaling is critical for fear memory in nonstressed rats (Lin et al., 2001; Ou and Gean, 2006), which at first seems inconsistent with the results of this study. Studies that have implicated PI3K → Akt signaling in fear memory have used fear potentiated startle paradigms, which involve presentation of startle probes prior to FC (Lin et al., 2001; Ou and Gean, 2006). Startle probes have an anxiogenic property (Smith et al., 2011; Furlong et al., 2016) and may lead to higher levels of acute stress in animals prior to FC compared with behavioral paradigms that use changes in freezing to measure emotional memory. This higher level of stress could have engaged PI3K → Akt signaling in the BLA and rendered this signaling pathway important for fear memory in the fear-potentiated startle paradigm.

Figure 4. Effect of inhibiting PI3K → Akt signaling in the basolateral amygdala (BLA) during FC on fear memory. (A) Experimental design. (B) Representative placement of infusion tip in the BLA along with schematic of all BLA placements. Red/brown circles are single prolonged stress (SPS) rats and blue/purple circles are control rats. (C) Infusing wortmannin into the BLA prior to fear conditioning reduced conditioned freezing during extinction training (approached significance with P < .05, indicated with a + sign) and testing (P < .05, indicated with an asterisk). These effects were not observed in control rats. SPS/vehicle = 10, SPS/drug = 7, control/vehicle = 11, control/drug = 8. (D) Infusing wortmannin outside of the BLA had no effect on conditioned fear in SPS rats (n = 6). The SPS/vehicle group from C is plotted in this graph for comparison. One SPS/vehicle animal was excluded from all behavioral analyses because its mean for extinction testing was greater than 2 SDs away from the group mean. Error bars represent SEM.

How might SPS lead to enhanced PI3K → Akt signaling? PI3K can be activated by growth factors (e.g., BDNF), hormones (e.g., CRH), and G-protein coupled receptor activation (Brunet et al., 2001; Hauger et al., 2012; Borrie et al., 2017; Roux and Topisirovic, 2018). Enhanced BDNF and CRH expression and/or signaling in the BLA is observed with FC (Rattiner et al., 2004; Ou and Gean, 2007; Ou et al., 2010; Bijlsma et al., 2011; Mountney et al., 2011; Chou et al., 2014). SPS enhances BDNF signaling in the Hipp during contextual FC (Takei et al., 2011) and enhances CRH expression in the central nucleus of the Amy (Sabban et al., 2015), but the effect of SPS on BDNF, CRH, or other hormone and growth factor signaling in the BLA during FC is not clear. Previous research has observed that SPS leads to a tonic decrease in locus coeruleus-norepinephrine (LC-NE) system activity but enhances phasic activity of the LC-NE system (George et al., 2012). Postsynaptic adrenergic receptor (β-ARs and α-ARs) expression increases with a decrease in NE.
CONCLUSION

The results of this study suggest that enhancements in PI3K→Akt signaling within the BLA could be a mechanism via which traumatic stress exposure renders fear memory resistant to extinction. However, changes in PI3K→Akt signaling with traumatic stress have been observed in other brain regions (see Introduction and Results), and PI3K→Akt signaling is also critical for extinction memory (Kritman and Maroun, 2013), a process that is sensitive to traumatic stress (Yamamoto et al., 2008; Milad et al., 2009; Knox et al., 2012b, 2016; Keller et al., 2015a, 2015b; RaiseAbdullahi et al., 2019). These findings raise the possibility that SPS may have a selective effect on a neurobiological function that is somewhat independent of fear memory expression/retrieval. The magnitude of fear memory (often inferred by the magnitude of fear conditioned responses) can be independent of the resistance of fear memory to change or inhibition. Perineuronal nets within the Amy are critical for the resistance of fear memory to extinction but not FMR (Gogolla et al., 2009). The formation of perineuronal nets is a neurobiological mechanism that can account for why fear memory persistence is different in animals at different developmental stages (Gogolla et al., 2009; Baker et al., 2017). If traumatic stress could alter properties of perineuronal nets within the BLA, this could be a mechanism through which traumatic stress leads to persistent fear memory. While speculative, this hypothesis would be interesting to explore as it would provide a relatively novel mechanism through which traumatic stress alters emotional memory.

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Statement of Interest

None.

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