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Single prolonged stress enhances hippocampal glucocorticoid receptor and phosphorylated protein kinase B levels

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Abstract

Animal models of posttraumatic stress disorder (PTSD) can explore neurobiological mechanisms by which trauma enhances fear and anxiety reactivity. Single prolonged stress (SPS) shows good validity in producing PTSD-like behavior. While SPS-induced behaviors have been linked to enhanced glucocorticoid receptor (GR) expression, the molecular ramifications of enhanced GR expression have yet to be identified. Phosphorylated protein kinase B (pAkt) is critical for stress-mediated enhancement in general anxiety and memory, and may be regulated by GRs. However, it is currently unknown if pAkt levels are modulated by SPS, as well as if the specificity of GR and pAkt related changes contribute to anxiety-like behavior after SPS. The current study set out to examine the effects of SPS on GR and pAkt protein levels in the amygdala and hippocampus and to examine the specificity of these changes to unconditioned anxiety-like behavior. Levels of GR and pAkt were increased in the hippocampus, but not amygdala. Furthermore, SPS had no effect on unconditioned anxiety-like behavior suggesting that generalized anxiety is not consistently observed following SPS. The results suggest that SPS-enhanced GR expression is associated with phosphorylation of Akt, and also suggest that these changes are not related to an anxiogenic phenotype.

Keywords

single prolonged stress; Akt; glucocorticoid receptor; hippocampus; posttraumatic stress disorder; anxiety

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1. Introduction

Posttraumatic stress disorder (PTSD) presents symptoms including chronic re-experiencing of the traumatic event, hyperarousal, and avoidant behavior (American Psychiatric Association, 1994), and has been associated with enhanced negative feedback of the hypothalamo-pituitary-adrenal (HPA) axis (Weiss, 2007; Yehuda, 2001) and deficits in extinction retention of aversive memories (Jovanovic et al., 2009; Milad et al., 2008). Exploring neurobiological mechanisms by which traumatic stress exposure results in PTSD is difficult to accomplish in humans but can be explored in animal models. The SPS paradigm, originally developed by Liberzon and colleagues (1997), models certain PTSD symptoms, such as hyperarousal (Khan and Liberzon, 2004; Kohda et al., 2007), and certain PTSD characteristics, such as enhanced HPA-axis negative feedback (Liberzon et al., 1997; Liberzon et al., 1999), and enhanced contextual fear conditioning (Armario et al., 2008; Yamamoto et al., 2009). In addition, SPS also impairs the retention of a previously extinguished aversive memory (Knox et al., 2012a) and in some studies is reported to produce increased anxiety-like behaviors (Peng et al., 2010; Wang et al., 2010b).

One emerging neurobiological mechanisms by which traumatic stress exposure results in PTSD symptoms is glucocorticoid alterations in individuals with PTSD (Yehuda, 2009). Interestingly, SPS consistently enhances glucocorticoid receptor (GR) protein levels and mRNA expression in the hippocampus (Liberzon et al., 1999; Wang et al., 2009). This effect has been linked to and may directly contribute to SPS-induced phenotypes (Knox et al., 2012b, in press). To investigate the mechanism of GR overexpression and to validate our model with respect to those previous studies, GR protein levels were examined in the hippocampus and amygdala. These brain regions were selected because they are sensitive to traumatic stress (McEwen, 2007), critical for proper memory management as well as the behavioral expression of anxiety (Bannerman et al., 2004), and the pathophysiology of PTSD (Bremner et al., 2008).

While SPS-enhanced GR expression is known to contribute to SPS-induced phenotypes, the molecular mechanisms by which GRs mediate SPS-induced phenotypes are unknown. One of the non-genomic mechanisms by which GRs alter neural function is by modulating phosphorylation of kinases and phosphatases (Stahn and Buttgereit, 2008). Akt is a serine/threonine protein kinase that is widely expressed in emotional circuits such as the hippocampus and amygdala, and is phosphorylated (pAkt) at serine residue 473 and threonine residue 308 to produces downstream signaling events (LoPiccolo et al., 2008; Song et al., 2005). Akt has been previously linked to changes in generalized anxiety. For example, previous reports suggest that pAkt has a role in mediating drug-induced and stress-induced changes in anxiety-related behavior (Hauger et al., 2012; Perrine et al., 2008), and it is believed that the regulation of mood and anxiety is through its action on downstream targets (Gould and Manji, 2005). Recently, mice with high trait anxiety show enhanced levels of pAkt (Yen et al., 2012). Alternatively, pAkt has also been associated with trauma-like stress. For example, in another model of PTSD, stress-susceptible mice have increased pAkt levels in the hippocampus and amygdala (Dahlhoff et al., 2010). The increase in pAkt has also been observed in rats exposed to acute restraint and tail shock stress (Yang et al., 2008) and chronic immobilization stress (Lee et al., 2006). Therefore, Akt has been linked to changes in both anxiety and stress, suggesting it may be changed after SPS in brain regions and circuitry that govern mood and emotion. However, the question remains as to whether Akt-related changes are associated specifically with SPS, i.e. GR-driven, or related more to enhanced anxiety after SPS.

While trauma-specific fear is one of the key characteristics of PTSD, generalized (or unconditioned) anxiety is not specific to PTSD, although the two are often comorbid.

However, there have been reports in the literature of SPS producing general anxiety-like behavior in rats. These findings vary across studies using SPS. One study did not find unconditioned anxiety-like behavior after SPS (Harvey et al., 2006), while others observed it only after restress (Brand et al., 2008; Harvey et al., 2004; Liberzon et al., 1997), after other modifications in the procedure, such as the addition of a shock stimulus preceding SPS (Wang et al., 2010a), or when SPS occurred during late adolescence (Imanaka et al., 2006) when animals are more susceptible to stressful events (Romeo and McEwen, 2006). This raises the question of the specificity of potential Akt changes and how they relate to specific SPS behavioral effects or to a general increase in anxiety like behaviors.

The main goal of this study was to determine if SPS-enhanced GR expression accompanies increases in pAkt levels in the hippocampus and amygdala. We hypothesized that GR levels would be increased following SPS and that an increase in pAkt levels would accompany this effect. In addition, the current study examined the specificity of Akt and GR changes in relationship to SPS-induced anxiety-like behavior.

Materials and Methods

The Guide for the Care and Use of Laboratory Animals 7th edition (National Academy Press, Washington D.C.) was followed and all experimental procedures were approved by the Institutional Animal Care and Use Committee at Wayne State University and in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care.

Animals

Male Sprague-Dawley rats (N=32; Charles River Laboratories, Portage, MI) weighing approximately 225–250 g upon arrival were pair-housed in standard microisolator rat (home) polycarbonate cages (45 cm × 26 cm × 21 cm) with bedding. Animals were acclimated to the vivarium for 5–7 days before experimentation during which time the animals were weighed and briefly handled daily. Animals were allowed food and water *ad libitum* in their home cages and housed on a 12 h light-dark cycle with lights on at 7AM. Temperature (~24°C) and humidity (35–40%) were controlled in the vivarium and behavioral testing laboratory.

Single prolonged stress (SPS)

Half of the animals were exposed to the SPS paradigm as previously described (Knox et al., 2010). Rats were restrained for 2 h in Perspex® restrainers (Plas Labs, Inc., Lansing, MI). This was immediately followed by 20 min of group forced swim (n = 8 per swim) in 24°C water in an approximately 75 liter tub (diameter = 45 cm) with a water depth of 28 cm. After the group swim, animals were briefly towel dried and allowed a 15 min recuperation period in new home cages with fresh bedding. Following recuperation, animals were placed in an empty standard rat cage with a wire mesh floor under which two petri dishes filled with diethyl ether anhydrous (50 ml poured into open dishes) were placed. Rats were exposed to ether until loss of consciousness, which took approximately 3–5 min and was as observed visually and confirmed by tail or paw pinch. Rats were placed back into their fresh home cages. The other half of the animals (*i.e.* the control animals) were briefly handled in a separate area of the laboratory during the time of the SPS procedure, and then placed into home cages with fresh bedding. All animals were returned to the vivarium and housed for seven days without disturbance other than to check on health status and replenish food and water if necessary.

Experiment 1 – Locomotor activity

Locomotor activity was measured overnight in a group of SPS ($n = 4$) and control ($n = 4$) rats to determine if SPS increases locomotor activity during the onset or end of the dark cycle. Locomotor activity was measured by an automated monitoring system (Digiscan DMicro, Accuscan Instruments, Columbus, OH), which consists of 16 parallel infrared emitter/detector photocells mounted on a metal assembly into which a standard home cage without bedding was placed. Data were recorded by Accuscan software installed on a PC computer linked to the activity monitors, and activity was measured as total photocell beam break counts. Animals were placed individually into a home cage environment with free access to food and water while locomotor activity was monitored overnight from 5PM to 9AM (16 h total). Animals were tested for baseline activity for three consecutive nights prior to SPS exposure, and then re-tested three nights following the seven day undisturbed period after SPS (Figure 2A). In another group of SPS ($n = 4$) and control ($n = 4$) rats, general locomotor behavior was measured during the day in an OF to determine if SPS increases general locomotor behavior in a novel environment during the light cycle. The OF consisted of a large testing chamber made of black Plexiglas material with no lid and matte black floor (80 cm $\times$ 80 cm $\times$ 36 cm; Formtech Plastics, Oak Park, MI). Animals were started in the center of the OF and spontaneous activity was recorded for 10 min with a digital CCD camera that was connected to a PC computer installed with an automated tracking software package (Ethovision 6.1, Noldus, Inc., Leesburg, VA). The total distance travelled was used as an index to assess behavioral activity. Animals were tested two consecutive days prior to SPS and two days following the seven day undisturbed period (Figure 2B).

Experiment 2 - Unconditioned anxiety-related behavior

In a final group of SPS ($n = 8$) and control ($n = 8$) rats, unconditioned anxiety-like behavior was measured over the course of 3 days following the undisturbed period after SPS exposure using three separate behavioral tests – the open field (OF), light-dark box, and elevated plus maze (EPM) (Figure 3A). Low level illumination at the base of all behavioral equipment was maintained between 1–3 lux using a luxometer (Agriculture Solutions, LLC, Strong, ME) to increase (anxiolytic) behavior in control rats. Behavior was recorded on the three consecutive days following the undisturbed period by a digital CCD camera located above the behavioral apparatus and connected to a PC computer installed with an automated tracking software package (Ethovision 6.1). Automated scoring was analyzed using Ethovision for all behavioral tests of anxiety.

On the first testing day animals were placed into a novel OF to assess anxiety-related behavior. Rats show an unconditioned tendency towards thigmotaxis behavior, or “wall-hugging”, in an OF, which has been demonstrated to be a model for anxiety-related behavior (Pruet and Belzung, 2003). The OF used here was the same used in experiment 1, and the procedures for conducting the study, recording the data and measuring activity were the same. In addition, a virtual center square or “center zone” (approximately 20% of the size of OF arena) was created within Ethovision to assess anxiety-related behavior. The duration of time spent in the OF center zone was measured as an assessment of anxiety-related behavior (*i.e.* thigmotaxis behavior) and total distance travelled was measured as an assessment of locomotor activity.

On the next (second) day, light-dark box behavior was measured in a testing chamber made of Plexiglas with a matte black floor (72 cm $\times$ 30 cm $\times$ 34 cm; Formtech Plastics, Oak Park, MI). The testing chamber was divided into two sides by a black wall with an arched opening (11 cm wide $\times$ 12 cm high) to allow the rat to cross between sides. The testing chamber contained a “light” side (30 cm wide $\times$ 40 cm long) with an open ceiling and white walls and a “dark” side (30 cm wide $\times$ 30 cm long) with a closed ceiling (lid) and black walls. Animals

were placed next to the far wall of the light side facing the archway and behavior was recorded for 10 min. The percentage of time spent in the light side and latency to first entry into the dark side were measured as an assessment of unconditioned anxiety. The total number of transitions was measured to monitor activity levels.

On the final (third) day, EPM behavior was measured using a standard, commercially available apparatus (Coulbourn Instruments, Allentown, PA). The maze was made of black Plexiglas, laid out in the shape of a plus sign as viewed from above, consisted of four interconnected runways (10 cm wide $\times$ 45 cm long) with two open and two closed arms, and the runways were elevated off the ground to a height of 52 cm. Closed arms were enclosed on the long sides of the runway by two 30 cm black walls. Open arms had a one cm rim around the perimeter of the entire arm. Animals were placed into the center (10 cm $\times$ 10 cm) of the maze facing an open arm and behavior was recorded for 5 min. The duration of time spent in the open arms (as a percent of total time and compared to closed arm + center zone time) and the percentage of entries into the open arms relative to total (open + closed) arm entries were quantified as assessments of anxiety, and the total number of arm entries was taken as a measure of locomotor activity.

Brain Dissection

All animals from experiments 1 and 2 were euthanized 10 days after SPS treatment (see Figures 1A, 1B, and 2A). Rats were rapidly decapitated using a rodent guillotine in the absence of anesthesia. Brains were immediately extracted, placed into an ice-cold rat brain matrix (Zivic Instruments, Pittsburgh, PA), and sliced into 2 mm coronal sections. Coronal brain tissue slices were placed onto a solid block of CO₂ (dry ice) until frozen. Slices were then transferred to an ice-cold surface and, using a biopsy-punch (1.5 mm diameter), bilateral tissue samples were extracted from the amygdala complex and dorsal hippocampus (relative to bregma, AP -2.3 , ML ± 4.5 , DV -9 and AP -4.8 , ML ± 3.0 , DV -3.25 , respectively) based on the rat brain atlas by Paxinos and Watson (2006). Tissue samples were stored untreated at -80°C until tissue processing for Western Blot assay.

Western Blot analysis

Tissue samples from rats in experiment 1 and 2 were assayed together for Western blot analysis of GR, pAkt-T308, pAkt-S473, total Akt, and β -actin levels. Frozen tissue was placed into homogenization buffer [10% 1 M NaCl, 10% glycerol, 1% 1M Tris, 1% Triton X-100, protease inhibitor EDTA-free cocktail and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL) in dH₂O] and then sonically disrupted (Misonix, Newton, CT). Homogenates were centrifuged at $10,000 \times g$ for 20 min at 4°C and supernatant was extracted and then frozen at -80°C until measurement of protein concentration. Protein concentrations were measured using the Folin-Lowry assay as previously described (Lowry et al., 1951; Perrine et al., 2008).

Equal amounts (30 μg) of protein were loaded into 10% sodium dodecyl sulfate-polyacrylamide Tris-HCl custom-poured gels and electrophoresed in running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS in dH₂O, pH 8.3) using a Mini-PROTEAN Tetra cell system (Bio-Rad, Hercules, CA). After gel electrophoresis, proteins were transferred in buffer (25 mM Tris, 190 mM glycine, 10% methanol in dH₂O, pH 8.3) to polyvinylidene fluoride (PVDF) membranes. Transferred proteins were visualized by Ponceau S Red (general protein dye) to ensure efficient transfer and equal protein loading. Proteins of interest on the PVDF membrane were visualized as follows. PVDF blots were incubated for 2 h in blocking solution [Tris-buffered saline with 0.1% Tween-20 (TBS-T) with 5% non-fat dry milk] and then in primary antibodies overnight at 4°C . Blots were washed in TBS-T and then incubated in a goat anti-rabbit (1:2000; Bio-Rad, Hercules, CA) secondary antibody

conjugated to horseradish peroxidase for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence substrate (West Femto for phosphorylated proteins and West Pico for all others; Thermo Fisher Scientific, Rockford, IL) on a Chemic-Doc XRS+ imaging system (Bio-Rad, Hercules, CA). Blots were stripped between antibodies with RestorePlus Western Blot stripping buffer using standard manufacturer instructions (Thermo Fisher Scientific, Rockford, IL).

All blots were probed for pAkt S473 (pAkt-S473, 1:1000, Cell Signaling, Beverly, MA), pAkt T308 (pAkt-T308, 1:1000, Cell Signaling, Beverly, MA), total Akt (Akt(pan), 1:1000, Cell Signaling, Beverly, MA), GR (GR-20, 1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and β -actin (1:5000, Sigma-Aldrich, St. Louis, MO). Bands of interest were confirmed by comparison to molecular weight standards (Protein Plus Western C Standards, BioRad, Hercules, CA).

Statistical Analysis

For experiment 1, locomotor activity data were analyzed by two-way mixed subjects, repeated measures analysis of variance (ANOVA) with the independent factor being SPS treatment (SPS vs. control) and the repeated measure being day relative to SPS (−3 to 9 for home cage locomotion; −2 to 9 for OF locomotion). When appropriate, significant F-tests for all ANOVAs were followed by individual comparisons using independent samples *t*-tests, with Bonferroni corrections for multiple comparisons. For experiment 2 all measure of anxiety-like behavior and locomotor activity were subjected to independent samples *t*-tests (SPS vs. control).

For the analyses of protein levels from all animals, the optical density of each protein band (i.e. chemiluminescence) was quantified using ImageLab 3.0 software (Bio-Rad, Hercules, CA). Non-phosphorylated proteins were normalized relative to β -actin (e.g. GR/ β -actin) to correct for potential differences in protein loading. Phosphorylated proteins were normalized relative to the non-phosphorylated form of the protein (e.g. pAkt/Akt). Phosphorylation of Akt has been previously demonstrated to be highly correlated with Akt enzymatic activity (Ma et al., 2008), therefore relative levels of pAkt/Akt can be used as a proxy for Akt function. These relative scores were then expressed as a percent change of control levels and subjected to *t*-test (SPS vs. control).

Results

The current study sought to examine the role of pAkt and GR in the hippocampus and amygdala after SPS. The experimental timeline and behavioral design are shown in Figures 2A–B and 3A. Tissues from all animals of each experiment were used for protein measurement and analysis. Representative protein bands for hippocampal GR, Akt, pAkt (T308 and S473 residues), and β -actin are illustrated in Figure 1A. Independent samples *t*-test revealed that SPS significantly enhanced GR levels in the hippocampus ($t(24)=3.39$, $P<0.01$). Even though total Akt levels in the hippocampus were not affected by SPS exposure ($t(24)=1.65$, $P>0.05$), SPS increased pAkt levels at the T308 ($t(24)=2.46$, $P<0.05$) and S473 residues ($t(24)=2.40$, $P<0.05$) in the hippocampus (Figure 1B). Representative protein bands for amygdala GR, Akt, pAkt (T308 and S473 residues), and β -actin are illustrated in Figure 1C. Independent samples *t*-tests revealed that SPS had no effect on GR levels ($t(27)=0.92$, $P>0.05$), Akt levels ($t(27)=0.09$, $P>0.05$), or pAkt levels (T308 - $t(27)=0.31$, $P>0.05$ and S473 - $t(27)=0.38$, $P>0.05$) in the amygdala (Figure 1D).

To explore potential specificity of Akt changes we also examined locomotor activity (experimental design Figures 2A–B) and general anxiety-like behavior (Figure 3A) after SPS. Analysis of beam breaks in the home cages of rats during the dark cycle revealed no

significant main effect of SPS, ($F(1,6)=0.49$, $P>0.05$), or SPS $\times$ day interaction ($F(5,30)=0.60$, $P>0.05$), however there was a significant main effect of day ($F(5,30)=2.78$, $P<0.05$), which reflected the finding that locomotor activity decreased from the first day of testing (Figure 2C). For analysis of exploratory activity in an OF during the light cycle, there was no significant main effect of SPS ($F(1,6)=0.03$, $P>0.05$), or SPS $\times$ day interaction ($F(3,18)=0.74$, $P>0.05$), however there was a significant main effect of day ($F(3,18)=18.2$, $P<0.001$), which again reflected the finding that locomotor activity decreased following the first day of testing (Figure 2D).

Independent samples *t*-tests did not reveal any differences between SPS and control rats for behavioral measures in the OF. These included center zone duration ($t(14)=0.26$, $P>0.05$; Figure 3B) and locomotor activity ($t(14)=0.18$, $P>0.05$; Figure 3C). Independent samples *t*-tests of behavior in the light-dark box did not reveal differences between SPS and control rats for time spent in the light side of the light-dark box ($t(14)=0.79$, $P>0.05$, Figure 3D), latency to enter the dark side ($t(14)=0.97$, $P>0.05$, Figure 3E), and the total number of transitions between the light and dark sides ($t(14)=0.49$, $P>0.05$, Figure 3F). Independent samples *t*-tests of behavior in the EPM did not reveal differences between SPS and control rats for any behavioral measure, which included time spent in the open arms ($t(14)=0.61$, $P>0.05$, Figure 3G), open arm entries ($t(14)=0.91$, $P>0.05$, Figure 2H), and total activity in the EPM ($t(14)=0.82$, $P>0.05$, Figure 2I).

Discussion

In our study, SPS-enhanced GR expression in the hippocampus accompanied enhanced phosphorylation of Akt at both the S473 and T308 residues without affecting total Akt. The effects of SPS on GR and pAkt were restricted to the hippocampus in that neither protein was changed in the amygdala. This is the first study to show that SPS produces a change in the phosphorylation state of Akt and confirms previous findings that SPS enhances GR protein and mRNA in the hippocampus (Knox et al., 2012b; Liberzon et al., 1999; Wang et al., 2009). Taken together, the results are consistent with the notion that SPS-enhanced GR expression leads to enhanced phosphorylation of Akt in the hippocampus.

The current study thus suggests a putative mechanism to explain the consistent finding that SPS in rats, and presumably PTSD in humans, produces changes in fear conditioning (Kohda et al., 2007; Yamamoto et al., 2009) and extinction retention (Knox et al., 2012a; Knox et al., 2012b, in press). Although causality cannot be established in the present set of experiments, previous research supports a link between GR and PI3k-Akt activity (Hafezi-Moghadam et al., 2002; Karst et al., 2002). The mechanism by which SPS-enhancement of GR leads to phosphorylation of Akt is currently unknown, but could involve a number of genomic and non-genomic mechanisms, such as transcriptional activity on calcium entry (Karst et al., 2002) or GR's direct interactions with PI3K (Hafezi-Moghadam et al., 2002). The increase in pAkt levels may reflect a compensative response to increase GR. Corticosterone induces cell death in hippocampal cell cultures, which is reversed by inhibition of PI3k/Akt signaling (Moosavi et al., 2008). Munhoz et al., (2010) also demonstrated that glucocorticoids exacerbate lipopolysaccharide-induced expression of pro-inflammatory markers, including nuclear factor κ B, which in turn is known to increase pAkt to activate cell survival pathways (Song et al., 2005). In addition, evidence may suggest a relationship between genome-activated GR signaling on Akt via increased calcium entry (Yano et al., 1998). Furthermore, the relatively new findings of non-genomic actions of GR on downstream Akt (Limbourg and Liao, 2003; Löwenberg et al., 2008) indicate that GR may have direct effects on PI3K, an upstream activator of Akt. While any of these mechanisms are plausible, further research is needed to clearly identify whether a link between GR and pAkt in SPS exists. Importantly, GR activity induces phosphorylation of

Akt, via PI3k, in the immune and cardiovascular systems, but the results of this study are the first to suggest a possible relationship between GR and Akt in the brain; specifically the hippocampus.

Phosphorylation of mitogen-activated protein kinases (MAPK), such as extracellular signal-related kinase (ERK), could conceivably be attributed to changes in either GR or pAkt levels, since SPS produces increase in pERK in the amygdala (Xiao et al., 2011). In addition, MAPKs are well-known for their involvement in mediating memory formation of stressful events (Reul and Chandramohan, 2007), possibly via glucocorticoids. For example, MAPK expression and activity, which mediates stress-related behaviors, has been shown to be increased by GR signaling (Revest et al., 2005). Given that *hippocampal* pERK levels following SPS (within a similar time-frame) do not change (Kohda et al., 2007), it is unlikely that increased pAkt levels in the hippocampus are associated with the MAPK/ERK signaling pathway, yet this remains to be addressed experimentally.

The results of several studies suggest that SPS phenotypes are related to enhancement of hippocampal GR expression. Extinction retention deficits in the SPS model do not manifest unless there is a robust enhancement in GR expression in the hippocampus and prefrontal cortex (Knox et al., 2012b, in press). Antagonism of GRs during SPS blocks the enhancement of hippocampal GR expression and contextual fear conditioning induced by SPS (Kohda et al., 2007). Enhanced GR expression induced by SPS exposure requires a post-stress incubation period to manifest (Liberzon et al., 1999), and enhanced fast negative feedback of the HPA axis and deficits in extinction retention induced by SPS exposure also require a similar post-stress incubation period to manifest (Knox et al., 2012a; Liberzon et al., 1999). Together, these strongly support the link between these SPS effects and enhanced GR expression induced by SPS exposure. Interestingly, enhanced phosphorylation of Akt may also contribute to phenotypes associated with SPS. The PI3K-Akt signaling cascade and its downstream targets within hippocampal cells have been previously implicated in memory consolidation (Dash et al., 2002). Mice that have been sensitized with footshock presentation show enhanced pAkt levels in the hippocampus and enhanced contextual fear conditioning (Dahlhoff et al., 2010). Intrahippocampal infusion of a PI3K inhibitor also impairs contextual fear memory retrieval (Chen et al., 2005). Given that SPS exposure can enhance contextual fear conditioning (Kohda et al., 2007), increased hippocampal pAkt levels induced by SPS may contribute to this phenotype. However, further studies are required to more definitively explore the relationship between GR, pAkt and SPS-induced phenotypes.

The current study also found that locomotor activity was unaffected by exposure to SPS, which is consistent with previous reports (Iwamoto et al., 2007; Takahashi et al., 2006). No change in unconditioned anxiety-related behavior was observed in the current study, and upon close inspection of literature on unconditioned anxiety-like behavior after SPS, our findings agree with the SPS literature (Harvey et al., 2006; Peng et al., 2010; Wang et al., 2010a; Wang et al., 2010b; Wang et al., 2008) and are consistent with the clinical finding that people with PTSD often do not report generalized anxiety although the two forms of anxiety disorder can be comorbid. Previous research has demonstrated that modifications of SPS can increase anxiety-related behavior (Harvey et al., 2006; Imanaka et al., 2006; Khan and Liberzon, 2004; Liberzon et al., 1997; Wang et al., 2010a; Wang et al., 2008), and a few reports show unmodified SPS increases anxiety-like behavior (Peng et al., 2010; Wang et al., 2010b). This could be due to modifications of the SPS procedure, i.e. Harvey et al., (2006) used a modified SPS-re-stress paradigm to show that SPS does not increase anxiety-related behavior alone, but when rats are re-stressed by re-exposure to the forced swim component of SPS (i.e., a cue), they exhibit enhanced anxiety-related behavior in the EPM. Thus, re-exposing SPS-treated rats to a component of the SPS procedure may serve as a

trauma-related cue that enhances anxiety-related reactivity. Furthermore, using a modified SPS protocol that included footshock presentation 30 min after exposure of the SPS stressors, an increase in anxiety-related behavior in the EPM with SPS exposure has been observed (Wang et al., 2010a; Wang et al., 2008). The introduction of footshocks at the end of SPS differs from the standard SPS protocol employed in this study. Indeed, shock alone, has been demonstrated to be enough to produce long lasting enhanced anxiety-like behavior (Korte et al., 1999).

Using the standard SPS protocol, increases in some general anxiety-related behavior have been observed in other studies. For example, Peng et al., (2010) and Wang et al., (2010b) observed a decrease in open arm time in an EPM with SPS exposure. However, in these studies the EPM test was conducted one hour after rats were exposed to an OF. In the present study, the EPM was conducted two days after exposure to the OF, and all behavioral tests were separated by a one-day interval. Thus, in Peng et al., and Wang et al., the shortened interval between the OF and the EPM may have rendered behavior in the EPM more sensitive. Finally, our data and the results of previous reports (Harvey et al., 2006; Khan and Liberzon, 2004), suggest that exposure to the standard SPS protocol does not consistently enhance unconditioned (or non-cued) anxiety-related behavior. Therefore these findings collectively suggest that modifications of the SPS procedure may lead to anxiety-like behavior that closely reflects (cue-induced) trauma-specific anxiety observed in clinical PTSD.

While it is clear that the increase in pAkt is not specific to a change in unconditioned anxiety-related behavior, it may be that the molecular changes associated with SPS may lead to greater susceptibility to anxiety-related behavior. It is well known that alterations in GR expression confer susceptibility to anxiety, stress, and impairment in hippocampal-dependent memory (Conrad, 2011; Kim and Diamond, 2002). For example, mice overexpressing forebrain GR, including hippocampal regions, show enhanced susceptibility to anxiety (Wei et al., 2004). As previously mentioned, restress to the forced swim component produces increases in anxiety (Harvey et al., 2006). The molecular changes, either increased GR or pAkt levels, thus may have induced a susceptibility to the restress-induced anxiety.

Conclusions

The current study shows that SPS-enhanced GR expression accompanies increased pAkt levels in the hippocampus of SPS rats and that these biochemical changes are not accompanied by enhanced unconditioned anxiety behavior. These findings suggest specificity of pAkt findings linking it to SPS-induced, PTSD-specific changes, rather than to a general increase in anxiety like phenomena. The molecular mechanisms by which SPS-enhanced GR expression results in SPS-enhanced pAkt levels and the functional consequences of these neurochemical effects remain to be determined. Ultimately, establishing the causal link between GR, Akt, and the maladaptive behaviors of PTSD could lead to the development of novel therapeutics for PTSD as evidence is indicating potential benefits of GR antagonism, as well as modulation of the PI3K-Akt pathway, in reducing some of the symptoms of PTSD.

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Abbreviations

PTSD	posttraumatic stress disorder
SPS	single prolonged stress
EPM	elevated plus maze
OF	open field
GR	glucocorticoid receptor
Akt	protein kinase B
pAkt	phosphorylated Akt
PI3K	phosphoinositide 3-kinase
mTOR	mammalian target of Rapamycin
GSK3	glycogen synthase kinase 3
S473	serine residue 473
T308	threonine residue 308
MAPK	mitogen-activated protein kinases
ERK	extracellular signal-related kinase

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Highlights

- Single prolonged stress (SPS) enhances hippocampal glucocorticoid receptor (GR) levels.
- SPS increases phosphorylated Akt in the hippocampus coincident with GR increases.
- SPS does not produce unconditioned anxiety-like behavior or disrupt spontaneous locomotor activity.

Figure 1.

Single prolonged stress (SPS) enhances glucocorticoid receptor (GR) expression and phosphorylated Akt (pAkt) levels in the hippocampus but not amygdala. A) Representative blots of GR, pAkt (T308 and S473 residue sites), Akt, and β -actin protein bands from hippocampal tissue. B) SPS induced a significant increase in GR expression and enhanced pAkt levels in the hippocampus. Quantified protein levels from hippocampal tissue normalized to either total Akt or β -actin levels are shown relative to controls (as %; mean $\pm$ SEM; control - open bars; SPS - shaded bars, $n=13$ /group). C) Representative blots of GR, pAkt (T308 and S473 residue sites), Akt, and β -actin protein bands from amygdala tissue. D) No SPS-induced changes in levels of GR, pAkt, or total Akt levels were observed in the amygdala. Quantified protein levels from amygdala tissue normalized to either total Akt or β -actin levels are shown relative to controls (as %; mean $\pm$ SEM; control - open bars, $n=15$; SPS - shaded bars, $n=14$). (* $P<0.05$; ** $P<0.01$)

Figure 2. Single prolonged stress (SPS) does not alter locomotor activity. A–B) Experimental design. SPS exposure had no effect on C) overnight locomotor activity in their home cages or D) locomotor activity during the day in an open field (control - open bars; SPS - shaded bars).

Figure 3.

Single prolonged stress (SPS) does not enhance unconditioned anxiety-like behaviors. A) Experimental design. SPS had no effect on any behavioral measure in the B–C) open field (OF), D–F) light-dark box, or G–I) elevated plus maze (control - open bars; SPS - shaded bars).