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THE BASOLATERAL AMYGDALA REGULATES ADAPTATION TO STRESS VIA β -ADRENERGIC RECEPTOR-MEDIATED REDUCTIONS IN PHOSPHORYLATED EXTRACELLULAR SIGNAL-REGULATED KINASE

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Abstract

The reactivity of physiological systems and behavior to psychological stress is reduced with increasing familiarity with a repeated stressor. This reduced reactivity, termed habituation, is a crucial adaptation limiting negative health consequences of stress and can be disrupted in psychopathology. We hypothesized that the ability to habituate physiologically and behaviorally to previously experienced stressors depends on β -adrenergic receptor activation (β -AR) in the basolateral amygdala (BLA), a specific neural substrate important for the consolidation of multiple types of memories. We observed that administration of the β -AR antagonist propranolol into the BLA after each of 4 daily exposures to restraint stress prevented the normal development of neuroendocrine and behavioral habituation measured during the 5th restraint in adult male rats. In contrast, the β -AR agonist clenbuterol administered into the BLA after each restraint on days 1–4 enhanced neuroendocrine habituation at the lowest dose but attenuated behavioral habituation at high doses. We then explored intracellular signaling mechanisms in the BLA that might be a target of β -AR activation during stress. β -AR activation post restraint is necessary for the alteration in basal pERK levels, as daily post-stress β -AR blockade on days 1–4 prevented repeated stress from leading to decreased pERK in the BLA on day 5. Finally, we examined the effect of blocking ERK phosphorylation in the BLA after each restraint on days 1–4 with the MEK inhibitor U0126, and found that this was sufficient to both mimic neuroendocrine habituation in stress-naïve animals and to enhance it in repeatedly stressed animals during restraint on day 5. Together, the results suggest that an individual's ability to habituate to repeated stress is regulated by activation of BLA β -AR, which may have these effects by transducing subsequent reductions in pERK. Individual variations in β -AR activation and intracellular signaling in the BLA may contribute significantly to adaptation to psychological stress and consequent resilience to stress-related psychopathology.

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Keywords

habituation; stress; amygdala; HPA; ERK; norepinephrine

Adaptation to stress is critical for psychological and physiological health. A crucial form of adaptation to stress is the phylogenetically conserved phenomenon of habituation (Armario, 2006; McEwen, 2008; Nesse et al., 2007). Habituation to stress is defined as decreased reactivity to re-exposure to an increasingly familiar stressor. Habituation is observed in multiple stress-responsive systems, both physiological and behavioral, including activity of the hypothalamic-pituitary-adrenal (HPA) axis as measured by the secretion of the hormones adrenocorticotrophin (ACTH) and glucocorticoids, the autonomic nervous system, behavioral escape or struggling responses, as well as in the underlying neural circuits that coordinate activity of these systems (Grissom et al., 2007; Grissom et al., 2008; Weinberg et al., 2008). Habituation to repeated stress is important because it minimizes the emotional arousal and metabolic costs of reactivity to those stressors that prior experience has shown are not, or no longer, threatening to the organism.

We theorized that the ability of an organism to habituate to previously experienced stressors is reliant on a mechanism underlying the ability to consolidate memories for these previous stressors. One neural substrate important for the consolidation of aversive memories in multiple learning paradigms is the β -adrenergic receptors (β -AR) (McGaugh, 2004) in the basolateral amygdala (BLA) and as such β -AR have been targets for pharmacological intervention in disorders including post-traumatic stress disorder (PTSD) (Strawn and Geraciotti, 2008; Vaiva et al., 2003). Manipulating β -AR function alters activity in the BLA (Buffalari and Grace, 2007; Hurlemann et al., 2010; Silberman et al., 2010). Blocking β -AR in the BLA post-training inhibits memory consolidation and reconsolidation (Debiec and Ledoux, 2004; Roozendaal et al., 2008), while activating β -AR in this region enhances consolidation, though extremely high doses of β -AR agonist are detrimental to consolidation (Roozendaal et al., 2006a; Roozendaal et al., 2008). Therefore, we hypothesized that manipulation of BLA β -AR activation after exposure to a repeated stressor would alter the development of habituation to the familiar stressor. We specifically investigated habituation of HPA activity because elevated corticosterone alters excitation of cells in brain regions critical for mediating emotional responses and memory for salient stimuli (Liebmann et al., 2009; Roozendaal et al., 2006b). Indeed, HPA hyperreactivity is an index of generalized hyperreactivity to stress linked to increased arousal and affective disorders in humans (Armario, 2006; Derijk et al., 2008; Simeon et al., 2007). Intriguingly, β -AR activation regulates extracellular signal-regulated kinase (ERK) signaling. Phosphorylated ERK (pERK) in the BLA is transiently increased during aversive learning, and blocking this ERK phosphorylation via inhibition of MEK (MAPK/ERK kinase) blocks memory consolidation in multiple paradigms (Alonso et al., 2002; Rossato et al., 2004; Schafe et al., 2000). However, to our knowledge this regulation has not been investigated with regards to β -AR effects in the BLA or with respect to repeated stress. Thus, we investigated the role of BLA β -AR mediated changes in ERK signaling in the BLA on habituation to repeated stress. Overall, the results of the current experiments provide evidence that β -AR intracellular signaling in the BLA, possibly via its actions on pERK, is a key regulator of habituation to psychological stress.

Materials and Methods

Animals

Male Sprague-Dawley rats (Charles Rivers, MA) between 225–250 g were individually housed in plastic tub cages with ad libitum food and water access. Experiments occurred

between 0800 – 1200h (lights on at 0600h). All procedures were approved by the UCUC at the University of Michigan or the IACUC at the Children's Hospital of Philadelphia.

Surgery

Guide cannulae were implanted into the BLA. Animals were anesthetized with ketamine-xylazine-acepromazine cocktail (77: 1.5: 1.5 mg/kg), then placed in the stereotaxic apparatus. Bilateral guide cannulae (8mm long, 26 gauge, Plastics One) were directed at the mid-to-posterior BLA (A/P –2.9 mm; M/L +5.0 mm; D/V –7.6 mm) according to coordinates derived from (Paxinos and Watson, 2004). Injector cannulae extended 1mm beyond the tip of the guides. In all experiments, animals were only kept in analyses if their cannulae were placed in the BLA (Figure 1). The criterion used led to a bilateral hit rate between 50 –75%, depending on the experiment.

Repeated restraint stress

All restraint exposures occurred in the home room. Repeatedly restrained animals (RR groups) were restrained daily for 30 minutes in Plexiglas cylinders with both ends blocked to prevent egress, as described in (Grissom et al., 2008), followed by bilateral intra-BLA injection of drug for 4 consecutive days. Stress-naïve controls (CTL) received daily handling and injections at the same time of day as RR animals. On day 5 all animals were exposed to a drug-free test restraint. 5 days of restraint is sufficient to demonstrate habituation of ACTH (Grissom et al., 2008), though longer (8+ days) are required for habituation of corticosterone in a daily 30 minute restraint paradigm (e.g. Jaferi and Bhatnagar, 2007). Limiting the experiments to 5 days was necessary to limit the tissue damage produced by daily microinjections into the BLA. Thus, we demonstrate effects on ACTH, with the corticosterone results from the first experiment shown in Figure 2.

Drug Administration

The β -AR antagonist DL propranolol (Sigma) was dissolved in phosphate buffered saline (PBS) to a final dose of 0.3ug/0.2 ul/side. This dose of propranolol prevents consolidation of aversive memories when administered post-training into the BLA (Roozendaal et al., 2006b). The β -AR agonist clenbuterol (Sigma) was dissolved in PBS to a final dose of either 1, 3, or 10 ng/0.2 ul/side, encompassing the dose range shown to enhance memory consolidation (Ferry and McGaugh, 1999; LaLumiere and McGaugh, 2005; McIntyre et al., 2005). 1mg of the MEK inhibitor U0126 (MEKI; Promega) was initially dissolved in DMSO, then mixed with PBS to a final percentage DMSO of 48% and a concentration of MEK inhibitor of 1ug/0.5ul/side (the larger injection volume was necessitated due to solubility of U0126). Vehicle was an equivalent combination of DMSO and PBS. This dilution protocol is similar to that described in (Schafe et al., 2000).

Blood Sampling and Radioimmunoassays

Blood samples were obtained by nicking the tail tip at the start of restraint allowing rapid and repeated sampling. Each blood sample was collected within 60 seconds of the stated timepoint with a volume of 100–250 ul. This is a standard procedure that does not require surgery and provides consistent results (Bhatnagar et al., 2002; Bhatnagar et al., 2005; Grissom et al., 2007). HPA activity from samples obtained via indwelling venous catheters, decapitation or tail nick are comparable and highly correlated (Vahl et al., 2005). Blood samples were obtained from each animal at the start of restraint (0 minutes), 15 minutes, at the end of restraint (30 minutes), and 30 minutes after restraint (60 minutes). Plasma ACTH and corticosterone were measured using double-antibody kits from MP Biomedicals (Orangeburg, NY). The minimum level of detection for ACTH and corticosterone were 5.7 pg/ml and 0.6 ug/dl respectively. Intra and interassay variability were less than 10%.

Behavioral analysis

The duration of struggling/escape behavior in the restrainer was used to assess behavioral habituation to repeated restraint. Struggling was analyzed as described in (Grissom et al., 2008), using EthoVision Pro 3.1 video analysis software (Noldus).

In situ hybridization

Animals were sacrificed at the end of restraint on day 5 and brains flash frozen. Brains were cryostat sectioned at 14 μ m and the sections were stored at -80°C until hybridization. On the day of hybridization, slides were fixed in 4% paraformaldehyde, washed in SSC buffer, treated with 0.1M triethanolamine with 0.25% acetic anhydride, and dehydrated. Antisense and sense RNA probes to the ACTH regulatory peptides corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) (graciously donated by Dr. Audrey Seasholtz) and BDNF (graciously donated by Dr. Stanley Watson) were generated from cut cDNA using ^{35}S labeled CTP and UTP. Probes were dissolved in hybridization buffer containing 50% formamide, 3x SSC, 1x Denhardt's solution, 10% Sodium Phosphate buffer, 10% dextran sulfate, 2% tRNA, and 10mM dithiothreitol. Slides with labeled hybridization buffer were sealed in a container lined with formamide-soaked filter paper and incubated at 55°C for 16 hours. Slides were then washed in 2xSSC, then incubated for 1 hour in RNase A solution, followed by 1 hour incubation in 0.1x SSC at 65°C , followed by ethanol dehydration. Radiolabeled slides were exposed to autoradiography film (Kodak) under darkroom conditions. The developed autoradiographs were scanned and the optical density of brain regions on the films was measured using ImageJ software (NIH). One section per animal was included in final analysis, limited to unilateral BLA (subdivided into lateral and basal nuclei) or PVN (limited to the parvocellular PVN) at bregma -3.1 for BLA and bregma -1.8 for the PVN, and the area analyzed was defined by the boundaries described in (Paxinos and Watson, 2004) at these rostral-caudal levels.

Western blots

Under basal conditions on day 5, brains were rapidly removed and a 2mm coronal section was obtained using an ice-cold rat brain mold (Braintree Scientific). From this section, the bilateral BLA was punched out using a circular 1mm punch (Fine Science Tools), flash frozen, and stored at -80°C as one bilateral pooled sample per animal. Each sample was homogenized in ice-cold lysis buffer including 10mM Tris Base, 5mM EDTA, 1% protease inhibitor cocktail (Thermo) and 0.25% phosphatase inhibitor (Novagen) using a handheld motor-driven pestle (Kimble Kontes). Samples were subjected to standard western blotting procedures. Primary antibodies were to ERK 1/2 in rabbit (Cell Signaling), pERK 1/2 in mouse (Santa Cruz), and beta-actin (Sigma) detected with infrared-dye labeled secondaries (Licor), and optical densities obtained on an Odyssey scanning system. Blots were probed simultaneously for pERK and total ERK, as recommended in the protocols of the secondary antibody vendor (Licor) and pilot experiments confirmed that the primary antibodies did not affect each other's binding. The blots were later probed with β -actin to confirm that total ERK levels did not differ between groups. Each n in the final analysis was thus an individual animal's pERK to total ERK ratio, separated into ERK1 and ERK2, and normalized to a percentage of the mean of the control group for that experiment.

Statistical analyses

All experiments were analyzed as an omnibus two-way or three-way ANOVA, as follows: Stress (usually 2 groups: control (CTL) which received no restraint on days 1–4 but daily intra-BLA injections; or repeated restraint (RR), which received daily restraint on days 1–4 followed by intra-BLA injections) $\times$ Drug (usually 2 groups: vehicle (VEH) or propranolol (PROP)). In Figure 2B and 2D, a third Stress condition was added (RR DELAY) in which

animals received intra-BLA injections 4 hours following daily restraint to test the time dependency of β -AR effects (thus $3(\text{Stress}) \times 2(\text{Drug})$). In Fig. 2E and F, behavior was analyzed as a repeated measures ANOVA ($2(\text{Stress}) \times 2(\text{Drug}) \times 6(\text{Timepoint: 5 minute bins during 30 minute restraint})$). In Figure 5, there were 3 doses of clenbuterol delivered in addition to the vehicle, creating 4 conditions of Drug (thus $2(\text{Stress}) \times 4(\text{Drug})$). All significant effects were followed by Fisher's post hoc tests. The significance levels for all tests were set to $p \leq 0.05$.

Results

Blockade of β -AR prevents hormonal and behavioral habituation to repeated stress

HPA activity—Using repeated exposure to 5 days of restraint, the shortest duration that consistently elicits habituation at the central and pituitary level, we demonstrated that blockade of β -AR in the BLA by propranolol after each of the 4 daily restraint exposures prevented the habituation of the ACTH response to the 5th restraint (when no drug was given) (Figure 2A and B). Corticosterone responses, which are downstream of ACTH in the HPA axis, but which is known to sometimes respond non-monotonically to ACTH (Armario et al., 1985; Arnhold et al., 2007; Dallman and Jones, 1973), were not significantly habituated by restraint on day 5 in the repeatedly restrained animals given vehicle after daily restraint (Figure 2C and D). Thus, we focused our subsequent investigations on ACTH as it provides a clear readout of psychological drive to the HPA axis (Armario, 2006).

For ACTH, Stress $\times$ Drug interactions at 15 minutes ($F(2,29)=3.3, p \leq 0.05$) and at 30 minutes ($F(2,26)=3.5, p \leq 0.05$) indicated that, as expected, rats given vehicle into the BLA immediately after 4 daily repeated restraint exposures (RR VEH) exhibited habituated ACTH in response to the 5th restraint compared to control rats that received their first stress on day 5 (CTL VEH) (Figure 2A). However, daily post-restraint intra-BLA β -AR blockade on days 1–4 (RR PROP) prevented habituation to the 5th restraint. Importantly, daily inhibition of β -AR without concurrent stress exposure did not alter HPA responses to acute restraint on day 5 (CTL PROP and CTL VEH were not different). Thus, intra-BLA β -AR blockade immediately after daily restraint prevented habituation of HPA responses to restraint but had no effects on its own. We also examined whether β -AR blockade needed to be delivered within a “consolidation window” after daily restraint. Rats in which propranolol was administered 4h post-restraint on days 1–4 (RR DELAY PROP) habituated to the repeated restraint to a degree similar to vehicle-injected repeatedly stressed rats (RR VEH) (Fig. 2B and D). Thus, β -AR activation in the BLA occurring in temporal proximity to a stress “consolidation window” is necessary for HPA habituation to repeated psychological stress.

Struggling behavior—Habituation to repeated psychological stress occurs in multiple physiological and behavioral modalities. Acutely restrained rats struggle and display escape attempts primarily during the first 5–10 minutes of restraint (Grissom et al., 2008). Struggling during restraint habituates in parallel with HPA activity and McEwen and colleagues have demonstrated that struggling is positively correlated with a state of anxiety (Wood et al., 2008). After 4 days of repeated restraint followed by post-restraint propranolol in the BLA, on day 5 animals were videotaped during the 30 minute restraint to measure struggling, acquired via automated analysis as “strong mobility”. Intra-BLA β -AR blockade immediately after restraint on days 1–4 (RR PROP) prevented the habituation of struggling elicited during restraint on day 5 compared to repeatedly restrained rats that were administered vehicle (RR VEH) (Figure 2E; Stress $\times$ Drug $\times$ Timepoint interaction ($F(5,275)=2.5, p \leq 0.05$). As with HPA activity, inhibition of β -AR without concurrent stress exposure did not affect struggling during acute restraint. We also more closely examined

struggling behavior during the first 10 minutes of restraint on day 5 (Figure 2F) to obtain a more fine-tuned depiction of the differences between groups in this behavior. Strong mobility during the first 10 minutes were divided into 1 minute bins and analyzed in repeated measures ANOVA. This analysis recapitulated our finding that intra-BLA β -AR blockade prevented the habituation of struggling (Stress $\times$ Drug $\times$ Timepoint interaction $F(5, 468)=2.7$, $p<0.01$). During minutes 1, 2, and 4, struggling was significantly higher in RR VEH animals as compared to RR PROP. Thus, intra-BLA β -AR blockade immediately after daily restraint prevented the habituation of struggling behavior, suggesting that post-stress BLA β -AR activation is necessary for the habituation of a spectrum of stress responses.

β -AR blockade in the BLA prevents repeated stress-induced changes in gene expression in the BLA and hypothalamus

While stress responses to a repeated familiar stressor habituate, organisms are able to mount robust responses to novel stressors. Several studies have demonstrated that repeated restraint elevates arginine vasopressin (AVP) mRNA in the paraventricular hypothalamus (PVN) without altering corticotrophin releasing factor (CRF), suggesting that increased AVP in the PVN may underlie the ability of animals to normally respond to novel stress while habituating to familiar stressors (Aguilera et al., 2008; Bhatnagar and Dallman, 1998; Dallman and Jones, 1973; Ma et al., 1997; Vining et al., 2007). We examined the effect of daily post-restraint β -AR blockade on days 1–4 on AVP and CRF mRNA in the medial parvocellular PVN (the HPA-regulating portion of the PVN) at the end of restraint on day 5 (Figure 3). Changes in AVP and CRF mRNA in response to an individual restraint is not apparent until at least 90 minutes after restraint onset; thus the levels measured in these animals at the end of 30 minute restraint represent basal mRNA expression changes as a result of prior repeated stress (Ma et al., 1997). The increase in AVP mRNA in the PVN normally induced by repeated restraint (RR VEH) was prevented by intra-BLA β -AR blockade (RR PROP) (Figure 3A and B, Stress $\times$ Drug Interaction, $F(1,19)=5.4$, $p\leq 0.05$). CRF was not affected by prior stress or drug exposure (Figure 3C and D). Thus, BLA β -AR blockade after daily restraint prevented the increase in AVP mRNA in the PVN induced by repeated restraint. Because the BLA does not directly project to the PVN, these data suggest that β -AR mediated activation of the BLA affects downstream activity in intermediate brain regions, and that signaling from limbic afferents is heavily involved in regulating repeated stress-induced changes in hypothalamic gene expression.

The BLA exhibits increased activity to a novel stressor after prior repeated restraint and increased dendritic arborization after repeated immobilization. Brain derived neurotrophic factor (BDNF) is known to support increased plasticity. We examined the effect of prior restraint on days 1–4 and daily post-restraint β -AR blockade on BDNF mRNA expression in the BLA at the end of restraint on day 5 (Figure 4). BDNF expression in the BLA was much stronger in the more ventral basal nucleus, and much less intense in the dorsal lateral nucleus in all groups, and thus these regions were analyzed separately. In the basal nucleus of the BLA, repeated restraint increased BDNF mRNA and propranolol prevented this increase (Stress $\times$ Drug Interaction ($F(1,24)=5.8$, $p\leq 0.05$). Neither stress nor propranolol affected BDNF mRNA in the lateral nucleus. Thus, β -AR activation after daily stress is necessary for increased BDNF expression in the basal BLA, which may support previously reported changes in BLA neuronal structure and function and which may have significant consequences for subsequent adaptation to repeated stress.

Intra BLA β -AR agonist administration enhances habituation to repeated stress

Given that inhibition of β -AR prevented stress habituation, we predicted that stimulation of BLA β -AR with the β -AR agonist clenbuterol would enhance habituation. We tested a range of doses of clenbuterol (1, 3, and 10ng) previously shown to enhance memory

consolidation for aversive events when infused in the BLA post-training (Ferry and McGaugh, 1999; McIntyre et al., 2005; Roozendaal et al., 2002; Roozendaal et al., 2008). We modified the experimental design shown in Figure 1 to allow us to capture the development of both HPA activity and struggling behavior in animals given clenbuterol each day after restraint, and to investigate whether clenbuterol accelerated habituation of HPA activity. Restraint and drug administration proceeded as previously described on days 1–4, but repeatedly restrained animals were videorecorded during 30 minute restraint on days 1, 2, and 4 of restraint to investigate behavioral habituation, and on day 3 were tailnicked at the end of 30 minute restraint, immediately prior to drug administration, to interrogate accelerated HPA habituation. On day 5, all control and repeatedly restrained groups were restrained to examine a full HPA response to restraint. No differences were seen between repeatedly restrained groups in struggling behavior on days 1 or 2 (Figure 5A–B). On day 3, prior post-restraint intra-BLA 1ng clenbuterol significantly accelerated habituation of ACTH in comparison to animals given either vehicle or the 10ng dose (ACTH expressed as percent of the mean of RR VEH animals as no CTL animals were sampled on this day) ($F(3,38)=4.2$, $p\leq 0.01$) (Figure 5C). On day 4, repeatedly restrained animals given 3ng clenbuterol (RR 3ng) after each daily restraint struggled significantly more during the first 5 minutes of restraint as compared with RR VEH, and repeatedly restrained animals given 10ng clenbuterol displayed a similar trend towards increased struggling ($p=0.06$) (Figure 5D; Main Effect of Drug on Day 4 $F(3,30)=3.48$, $p\leq 0.05$). On day 5, all RR groups significantly habituated (Main Effect of Stress at 15 minutes ($F(1,63)=8.2$, $p\leq 0.01$; at 30 minutes ($F(1,59)=18.5$, $p\leq 0.0001$) (Figure 5E–G). However, the habituation-enhancing effects of clenbuterol were masked by the large stress effect on day 5. Thus, we focused on ACTH concentrations in all RR groups at the end of restraint (one-way ANOVA at 30 min timepoint), where the largest differences were seen in the previous experiments. At the end of restraint on day 5, prior post-restraint 1ng clenbuterol (RR 1ng) significantly enhanced habituation of ACTH compared to prior post-restraint vehicle (RR VEH) ($F(3,26)=3.0$, $p\leq 0.05$) (Figure 5E). Higher doses were not effective at enhancing habituation (Figure 5F–G). Thus, the 1ng dose of clenbuterol advanced habituation of ACTH to day 3 and enhanced it on day 5, but higher doses were ineffective. In contrast, the 1ng dose of clenbuterol did not affect struggling behavior but higher doses appeared to impair the development of struggling habituation by day 4. In combination, the propranolol and clenbuterol data suggest that the relationship between β -AR activation influences habituation of HPA activity and struggling behavior according to an inverted-U function, similar to what has been described for β -AR in aversive learning (Roozendaal et al., 2008). However, the inverted-U functions regulating HPA activity and struggling behavior appear not to precisely overlap, but rather indicate a more rightward position for the curve describing HPA habituation (Figure 7).

Restraint stress reduces ERK phosphorylation in the BLA which is prevented by β -AR blockade

β -AR activation stimulates extracellular signal-regulated kinase (ERK) pathways (Walters and Weller, 2003; Zheng et al., 2008). Phosphorylated ERK (pERK) in the BLA is transiently increased by Pavlovian fear conditioning and blocking this ERK phosphorylation via inhibition of MEK (MAPK/ERK kinase) blocks the consolidation of both Pavlovian fear conditioning and operant avoidance conditioning (Quevedo et al., 2005; Rossato et al., 2004; Schafe et al., 2000). Thus, we first examined the effect of restraint stress on BLA pERK before and after acute and repeated stress (Figure 6A–C). Compared to the level of pERK in the BLA of nonstressed rats (CTL 0), both pERK 1 and 2 in the BLA were significantly reduced at the end of a first restraint (CTL 30; pERK1 $t(11)=2.49$, $p<0.01$, pERK2 $t(11)=2.51$, $p<0.01$) and at the end of 5th restraint (RR 30; pERK1 $t(14)=1.92$, $p<0.05$; pERK2 $t(14)=2.89$, $p<0.01$), and remained decreased in the BLA of repeatedly restrained

animals prior to restraint on day 5 (RR 0; pERK1 $t(10)=2.28$, $p<0.05$, pERK2 $t(10)=1.51$, $p<0.1$). There was no difference in pERK between CTL 30, RR0, and RR30 animals. Thus, restraint stress leads to reduced pERK in the BLA at the end of restraint, which appears to become maintained basally over the course of repeated restraint.

We then examined whether daily post-restraint β -AR blockade would alter patterns of basal ERK phosphorylation in the BLA. As in the previous experiment, repeated restraint (RR VEH) decreased basal pERK in the BLA as compared to unstressed animals (CTL VEH) and propranolol (RR PROP) prevented this decrease (Stress $\times$ Drug Interaction: pERK 1: $F(1,39)=8.7$, $p \leq 0.005$; pERK 2: $F(1,39)=4.4$, $p \leq 0.05$) (Figure 6D,E,F). Thus, pERK is basally lower in the BLA of animals which have habituated to repeated restraint, but not in stress-naïve animals or animals in which stress habituation was prevented by β -AR blockade. The sum of the results indicates that the rapid reductions in pERK induced by restraint are sustained basally after 4 restraint exposures, and that β -AR activation after restraint is necessary for stress-induced alterations in pERK in the BLA.

Daily blockade of ERK phosphorylation in the BLA is sufficient to reduce HPA activity in stress-naïve and repeatedly stressed animals

The unexpected finding that BLA pERK is basally reduced by repeated restraint raised the question of whether BLA pERK is functionally relevant to habituation. To examine this, we tested whether intra-BLA blockade of MEK using the MEK inhibitor (MEKI) U0126, which reduces ERK phosphorylation, after restraint on days 1–4 would enhance habituation to restraint on day 5. Intriguingly, prior daily restraint and prior daily MEKI injection in the BLA both reduced HPA activity to restraint on day 5 (Figure 6 G,H) (Main Effect of Stress at 15 minutes: $F(1,38)=11.4$, $p \leq 0.001$; at 30 minutes: $F(1,37)=9.4$, $p \leq 0.005$; Trend towards Main Effect of Drug at 15 minutes: $F(1,38)=3.2$, $p \leq 0.07$; at 30 minutes: $F(1, 37)=2.9$, $p \leq 0.09$). The trend towards a Main Effect of MEKI suggested that blocking ERK phosphorylation in the BLA reduced ACTH in drug groups but that the effect was masked by high variability. To test the overall effect of MEKI, we integrated ACTH across the four timepoints. Both repeated restraint and repeated intra-BLA MEKI reduced integrated ACTH concentrations (Main Effect of Stress $F(1,37)=9.3$, $p \leq 0.005$, Main Effect of Drug $F(1,37)=4.6$, $p \leq 0.05$). Thus, inhibition of ERK phosphorylation on days 1–4 mimicked habituation in the stress-naïve (CTL) animals and enhanced it in the repeatedly stressed (RR) animals in response to restraint on day 5. These data suggest that the decrease in pERK induced by repeated restraint activation of β -AR is sufficient to produce habituation of HPA activity to repeated stress.

Discussion

Habituation to stress is an adaptive process involving reduced reactivity of stress systems to a stressor that has been previously experienced and is no longer threatening to the organism. We hypothesized that habituation depends on post-stress β -AR activation in the BLA, as post-experiential β -AR activation in the BLA is an important neural substrate in the consolidation of memories for aversive events. The results indicated that β -AR activation in the BLA during a timeframe proximal to stress exposure regulates habituation of HPA activity, struggling/escape behavior, stress-induced neurotrophin expression in the BLA, and hypothalamic neuropeptide gene expression, indicating that post-stress β -AR activity in the BLA regulates adaptation to stress across multiple domains. Examination of intracellular mechanisms in the BLA revealed that β -AR activation after stress leads to a sustained reduction in ERK phosphorylation. This reduction appears functionally relevant to stress habituation because inhibiting ERK phosphorylation in the BLA was sufficient to mimic habituation of HPA activity in acutely stressed rats and to enhance it in repeatedly stressed animals.

The BLA regulates the adaptation of multiple stress systems

Our primary result was that β -AR blockade in the BLA every day after restraint prevented a number of neuroendocrine and behavioral outcomes that are normal sequelae of repeated stress, including habituated ACTH activity, habituated struggling behavior, and increased AVP mRNA expression in the paraventricular hypothalamus. While we were not able to extend our findings to demonstrate effects on corticosterone habituation, it seems quite likely that the ACTH-driven component of corticosterone activity is similarly affected. Although other laboratories have demonstrated significant habituation of corticosterone to 5 or fewer stress exposures (Dhabhar et al., 1997; Ma and Lightman, 1998; Uchida et al., 2008), these experiments employed longer stress durations and/or longer inter-stress intervals, both of which are known to influence the strength of stress adaptation and habituation processes in general (Gray et al., 2010; Masini et al., 2008).

Also of note, while we demonstrated that the habituation of both HPA activity and struggling behavior are modulated by daily post-restraint β -AR activity, the data suggest a difference in the sensitivity of these two endpoints to β -AR activation in the BLA (schematic in Figure 7A). The habituation of both HPA activity and struggling can be prevented by β -AR blockade, but β -AR activation enhanced HPA habituation at the lowest dose while diminishing struggling habituation at higher doses. Crucially, the 3ng dose of clenbuterol significantly attenuated behavioral habituation, but in the same animals 3ng clenbuterol lead to habituated ACTH activity nearly comparable to the significantly enhancing 1ng dose (Figure 5E and F). We conclude from these data that while both struggling behavior and HPA activity habituate incrementally over the course of repeated restraint exposure, struggling behavior is more sensitive to endogenous β -AR activation by repeated stress. It remains to be seen whether differences in the sensitivity of adaptation of the multiple stress-responsive axes reflects important differences in stress susceptibility or outcomes. However, it was recently demonstrated that prior experience with escapable versus inescapable stress significantly elevated struggling behavior during novel restraint only in the group previously exposed to inescapable stress, though the restraint induced similarly high HPA activity in both groups (Weinberg et al., 2010), suggesting that struggling behavior during restraint may be a key measure of stress sensitivity.

As previously mentioned, habituation to repeatedly experienced, familiar stressors is an adaptation designed to limit hyper-activation of stress systems such as the HPA axis and struggling or escape behaviors to nonharmful stimuli, while simultaneously preparing the animal to remain appropriately responsive to novel stressors, such as by establishing an enhanced AVP signal in the hypothalamus (Aguilera et al., 2008). The current data demonstrate that in repeated stress paradigms the immediate post-stress period is a crucial time of plasticity influencing whether stress habituation occurs at multiple levels, and as such can be considered a stress “consolidation period”. The current results suggest that actions of norepinephrine in the BLA, specifically released in temporal proximity to stress exposure, is an important modulator of stress habituation during this consolidation period. These actions of norepinephrine at BLA β -AR are likely to be influenced by glucocorticoids or CRF which are known to modulate BLA β -AR regulation of aversive learning (Roozendaal et al., 2002; Roozendaal et al., 2008), and by limbic structures which influence stress habituation and/or BLA function, including the paraventricular thalamus and prefrontal cortex (Herman et al., 2005; Jaferi and Bhatnagar, 2006; McGaugh and Roozendaal, 2008).

The current data provide ample evidence that the BLA is a key regulator of stress adaptation and HPA activity, paralleling other recent demonstrations of the importance of this region in the regulation of stress responses (Hill et al., 2009; Ulrich-Lai et al., 2010). However, a previous experiment (Carter et al., 2004) investigating the role of the amygdala complex in

stress habituation found that lesioning the entire amygdala complex delayed, but did not prevent, stress habituation, demonstrating that the BLA is not necessary for stress habituation. The apparent discrepancy between the findings of Carter et al. and the current data is paralleled by findings in other literatures, wherein non-BLA dependent tasks (i.e., able to be learned and recalled in animals without a functioning BLA) can nevertheless be modulated by BLA drug administration. While the BLA has been shown to be necessary for particular tasks such as Pavlovian fear conditioning (Rabinak and Maren, 2008), a wider variety of cognitive processes are modulated by BLA activity, including for tasks dependent on hippocampal and prefrontal cortex memory consolidation (Roosendaal et al., 2003; Roosendaal et al., 2004), a list to which stress habituation should also be added. Continuing research from many laboratories will more thoroughly define the psychological and biological mechanisms linking the roles of the BLA in Pavlovian learning and its neuromodulatory functions in other paradigms, including stress adaptation.

Decreased ERK activity in the BLA regulates stress adaptation

An important contribution of the current work is the demonstration of intracellular changes occurring in the BLA that may participate in its neuromodulatory functions by β -AR activity. We observed both increased BDNF mRNA and decreased ERK phosphorylation in the BLA of repeatedly stressed animals, both of which were prevented by daily post-restraint intra-BLA β -AR blockade. While we do not know whether the increased BDNF message in the BLA of repeatedly stressed animals is functionally relevant to habituation, it dovetails with previous demonstrations of long-lasting increases in dendritic arborization in the BLA of repeatedly stressed animals (Mitra et al., 2005; Vyas et al., 2002; Vyas et al., 2004; Vyas et al., 2006) that may be supported by the increased neurotrophic activity observed in our experiments. These data suggests that stress-related activation of β -AR in the BLA may be an important regulator of long-term changes in plasticity in this region by repeated or chronic stress experiences.

We pursued pERK as a candidate signaling molecule that might mediate key downstream effects of β -AR activity. MEK/ERK signaling is activated by β -AR (Waltereit and Weller, 2003; Zheng et al., 2008) and MEK/ERK activation is also a key intracellular signaling pathway involved in BLA-dependent memory consolidation (Rossato et al., 2004; Schafe et al., 2000). However, the role of the MEK/ERK pathway in mediating β -AR effects in the BLA on behavior has not been established. We found that stress decreased ERK phosphorylation in the BLA immediately post stress in animals exposed to their first or 5th restraint, and that this decrease in pERK was maintained basally in repeatedly stressed animals. Furthermore, the decrease in basal pERK in the BLA of repeatedly restrained animals was prevented by β -AR blockade given each day after restraint. The current data indicate that pERK activity in the BLA is an inverse regulator of habituation to stress (schematic in Figure 7B).

Initially, we expected repeated stress to increase, rather than decrease, pERK in the BLA, due to the importance of pERK in aversive learning paradigms. It is possible that the time points at which animals were sacrificed in the current experiments did not capture a critical period of increased pERK that is important to stress habituation. However, we found that intra-BLA administration of a MEK inhibitor, which reduces the ability of MEK to phosphorylate ERK, mimicked habituation in stress-naïve animals and enhanced it in repeatedly stressed animals. This finding suggests that the decreased pERK that we measured in the BLA of repeatedly stressed animals contributes to stress habituation, although we cannot know from these data at what times during 5 days of repeated stress decreased pERK is important for habituation to occur. We elected to administer the MEK inhibitor post-restraint on days 1–4 because β -AR blockade during this window prevented repeated stress induced basal decreases in pERK. However, it remains to be seen whether

stress habituation is regulated by immediate post-stress reductions in pERK which are maintained 24 hours later by post-stress β -AR activation, or whether habituation is regulated by the decreased basal pERK 24 hours following stress, which may be induced by post-stress β -AR activation and be a separate phenomenon from the reduction in pERK seen immediately post stress. Further sampling at various timepoints post-stress would be necessary to tease apart the precise dynamics of the regulation of BLA pERK by stress and β -AR. However, our data establish that immediate post-stress β -AR activation in the BLA of normal animals is required for reduced basal pERK in this region after a repeated stress regimen, and this may be a key mechanism in the BLA modulation of habituation. More fundamentally, these data indicate that the immediate post-stress period is a key time of intracellular activity in the BLA involved in modulating whether and how quickly habituation to the just-experienced stressor will occur.

As mentioned above, our initial prediction was that pERK would be increased in the BLA of restrained animals, but indeed we found decreased pERK in the BLA after acute and repeated restraint. This is in contrast with the transient increases in pERK in the BLA after aversive conditioning (Duvarci et al., 2005; Rossato et al., 2004; Schafe et al., 2000). These results suggest that decreased reactivity to stressors associated with habituation is a fundamentally different phenomenon than aversive conditioning, on both a psychological and biochemical level. Indeed, there is suggestive evidence that reductions in pERK in the BLA are related to reduced anxiety, in contrast to the temporary elevations in pERK which are associated with increased fear and avoidance responses (Rossato et al., 2004; Schafe et al., 2000). First, infusions of d-cycloserine into the amygdala increased ERK phosphorylation in the amygdala and concurrently increased anxiety on the elevated plus maze (Wu et al., 2008). Furthermore, in animals well-trained in a cued avoidance response, exposure to the cue reduces amygdala pERK (Botreau and Gisquet-Verrier, 2006) and cue exposure is known to reduce anxiety in animals well-trained in avoidance (Mineka and Gino, 1980). pERK activation in the amygdala is highest when animals are uncontrollably stressed but is reduced in animals that learn to control the stressor and are also less anxious (Ilin and Richter-Levin, 2009). Therefore, an intriguing possibility is that the reduction in pERK in the BLA that we observed may be necessary for reductions in anxiety, as evidenced by decreased struggling to repeated restraint, and for habituation to psychological stressors.

Translational implications

BLA β -AR activity has been repeatedly shown to influence memory consolidation according to an inverted U-shape function, such that extremely low or extremely high β -AR activation is detrimental to memory consolidation relative to moderate activation, which is beneficial. Our results indicate that the relationship between BLA β -AR activity and habituation to repeated stress is also best described by an inverted U-shape. Repeated restraint produced habituation that was enhanced by pharmacologic stimulation of β -AR (by low doses of clenbuterol), while higher doses of clenbuterol did not further enhance habituation, suggesting that further increasing β -AR activation could reduce or prevent habituation. Conversely, blocking BLA β -AR receptors resulted in an inability to adapt to a familiar psychological stressor. Additional doses and the use of more selective pharmacological agents in future experiments will be important in reinforcing the findings here. However, one implication of the current results is that individual differences in β -AR activity may influence the magnitude of habituation to stress significant to psychopathology. That is, either increasing β -AR activity in a hypoactive noradrenergic state or decreasing it in a hyper-noradrenergic state should promote habituation. A confluence of evidence suggests that PTSD, characterized by hyperactivation of stress responsive systems including noradrenergic activity (Yehuda and LeDoux, 2007), involves deficits in habituation that are

relieved by targeted propranolol administration. PTSD can only be diagnosed one month following trauma as experiencing PTSD-like symptoms for the first days and weeks is normal (Yehuda and LeDoux, 2007), suggesting stress habituation processes occur in individuals not later diagnosed with PTSD. Propranolol reduces reported symptoms of PTSD and the autonomic stress response elicited when recalling the traumatic memory, but only when drug delivery is paired with the traumatic event or memory for the event (Brunet et al., 2008; Pitman et al., 2002; Vaiva et al., 2003) suggesting that habituation was enhanced by memory-paired propranolol administration. Based on the current results, it is possible that PTSD sufferers given targeted propranolol treatment may experience a reduction of β -AR activity to more moderate levels during memory recall, leading to a reduction in symptoms via enhanced habituation to stressful aspects of the traumatic stimulus. More broadly, the results of the current studies suggest that an individual's ability to habituate to repeated stress is determined, at least in part, by BLA β -AR activity and related changes in pERK. Further understanding individual differences in this mechanism may be important for developing individualized treatments for stress-related psychopathology and for identifying individuals resilient to stress-related psychopathology.

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Figure 1.

A) General experimental design. All experiments were conducted over 5 days in male rats with cannulae bilaterally implanted into the BLA, with the exception of the data in Figure 6 A-C which was conducted over 5 days in intact animals. All drugs were administered during the first 4 days only, after each daily 30 minute restraint (RR) or at the same time of day in unstressed control animals (CTL). On day 5, all animals received the same treatment under drug free conditions. One of four endpoints was measured on day 5: 1) HPA activity during restraint was assessed by repeated blood sampling at 0, 15, 30, and 60 minutes after onset of 30 minute restraint; 2) struggling behavior was video acquired during undisturbed 30 minute restraint; 3) animals were sacrificed at the end of 30 minute restraint to measure gene expression; or 4) sacrificed at 0 min or at the end of 30 minute restraint for protein measures. B–D) Representative cannulae placement in the BLA in the current experiments. B) plate from the rat brain atlas by Paxinos and Watson depicting the BLA, which is outlined in C and D. C) representative photomicrograph of a cannula directed at the BLA from the current experiments. D) representative photomicrograph of a misplaced cannula directed outside of the BLA from the current experiments.

Figure 2.

Daily post-restraint administration of β -AR antagonist in the BLA prevents habituation of ACTH activity to restraint. A) The β -AR antagonist propranolol (PROP) given immediately after daily restraint prevented HPA habituation to repeated restraint (RR) on day 5. ACTH habituated in RR VEH on day 5 compared to CTL groups but did not habituate in repeatedly restrained animals given propranolol in the BLA (RR PROP). n's: CTL VEH=7-8, CTL PROP=5-9, RR VEH=4, RR PROP=5-9. B) PROP delivered at a 4h delay after restraint did not affect habituation of HPA activity in the same manner as PROP delivered immediately after restraint. The experiment in 2A included 2 additional groups where intra-BLA injections of VEH or PROP were delayed until 4 hours post-restraint (RR DELAY); these groups are graphed in (1B) and (1D) with RR PROP and RR VEH from 1A and C. Both RR DELAY VEH and RR DELAY PROP demonstrated habituation similar to RR VEH. n's: RR DELAY VEH=5-9, RR DELAY PROP=5-9. C and D) Corticosterone from the same animals depicted in 2A and 2B. In contrast to ACTH, corticosterone did not significantly habituate to 30 minute restraint by day 5 in the current experiments, preventing us from analyzing whether PROP prevented habituation of HPA activity downstream of ACTH. E) β -AR blockade in the BLA given after daily restraint prevented the habituation of struggling

behavior to restraint. Animals previously exposed to repeated restraint struggle significantly less during the first 5–10 minutes of restraint compared to animals undergoing a first restraint, providing a behavioral measure of habituation to repeated restraint. Intra-BLA propranolol after daily repeated stress (RR PROP) prevented behavioral habituation as seen in repeatedly restrained animals given vehicle (RR VEH). F) Struggling behavior from the first 10 minutes of restraint depicted in 2E, depicting the minute-by-minute changes in behavior during the first third of restraint. n's: CTL VEH=12, CTL PROP=17, RR VEH=15, RR PROP=15. * signifies $p \leq 0.05$ between RR VEH and RR PROP. # signifies $p \leq 0.05$ between RR VEH and CTL VEH. All data is mean $\pm$ SEM.

Figure 3.

Intra-BLA β -AR blockade each day after stress prevented the increased AVP mRNA in the paraventricular hypothalamus (PVN) elicited by repeated restraint. A) Representative autoradiographs of AVP mRNA in the PVN, outlining the medial parvocellular region of the PVN centered at approximately bregma -1.8 , which regulates HPA activity. The non-outlined area in the images is the posterior magnocellular AVP-expressing cells of the PVN, which are not involved in HPA regulation. B) RR VEH animals demonstrated a significant increase in AVP mRNA in the PVN compared to CTL VEH. However, daily post-restraint propranolol prevented stress-induced increases in AVP message in the hypothalamus. C) Representative autoradiographs of CRF mRNA in the PVN, outlining the medial parvocellular region of the PVN centered at approximately bregma -1.8 . D) There were no effects of stress or drug administration on CRF expression in the PVN. * signifies $p \leq 0.05$. All data is mean $\pm$ SEM.

Figure 4.

Intra-BLA β -AR blockade each day after stress prevents repeated-stress induced changes in brain-derived neurotrophic factor (BDNF) gene expression in the basal BLA. A) Representative autoradiographs of BDNF mRNA in the BLA at approximately bregma -3.1 . The BLA is comprised of two nuclei: the lateral nucleus (outlined in the figure as the dorsal triangular shape) and the basal nucleus (outlined in the figure as the ventral rectangular shape). Because we observed that BDNF mRNA expression in the BLA was not uniformly distributed but appeared to be located primarily in the basal nucleus, we subdivided these regions for our analyses. B) RR VEH animals demonstrated a significant increase in BDNF mRNA in the basal BLA as compared to CTL VEH animals, but β -AR blockade after each daily restraint prevents this increase in neurotrophin message in the BLA. C) No differences in BDNF mRNA expression were seen in the lateral BLA as a result of stress or drug administration. ns: CTL VEH=6, CTL PROP=6, RR VEH=4, RR PROP=7. * signifies $p \leq 0.05$. + signifies $p \leq 0.1$. All data is mean $\pm$ SEM.

Figure 5.

Intra-BLA clenbuterol, a β -AR agonist, accelerates HPA habituation to repeated restraint at the lowest dose but attenuates behavioral habituation at higher doses. We tested a dose-response curve of clenbuterol (1 ng, 3 ng, 10 ng) injected after daily restraint as different doses of clenbuterol have been found to be effective at enhancing memory consolidation for different paradigms. Because we hypothesized that clenbuterol might accelerate the development of habituation, we analyzed struggling behavior on days 1, 2, and 4 in repeatedly restrained groups only, and we took a single blood sample at the end of restraint on day 3 in repeatedly restrained groups only. A and B) There were no differences between groups in struggling behavior on day 1 (A) or 2 (B). C) Compared to RR VEH animals on day 3, RR 1ng animals exhibit significantly further reduced ACTH activity on day 3. n's: RR VEH=12, RR 1ng=10, RR 3ng=11, RR 10ng=12.). D) On day 4, animals given 3ng clenbuterol after restraint on days 1–3 struggled significantly more during the first 5 minutes than repeatedly restrained animals given vehicle, and animals given 10ng clenbuterol tended ($p=0.06$) to struggle more than repeatedly restrained vehicle animals during the first 5 minutes. 1ng clenbuterol had no effect. E) Intra-BLA administration of 1ng clenbuterol enhanced HPA habituation to restraint on day 5. We observed a Main Effect of Stress indicating significant habituation of all RR groups compared to all CTL groups; comparison within RR groups given vehicle, 1 ng, 3 ng, or 10 ng clenbuterol revealed that RR 1ng animals enhanced ACTH habituation at 30 minutes as compared to RR VEH animals. F and G) On day 5, there were no differences in HPA habituation between repeatedly restrained animals given daily post-restraint vehicle and either 3ng clenbuterol (F) or 10ng clenbuterol (G). n's: CTL VEH=12–14, CTL 1ng=7–8, CTL 3ng=8–9, CTL 10ng=8–10, RR VEH=8–12, RR 1ng=7–10, RR 3ng=7–11, RR 10ng=8–12. * signifies $p \leq 0.05$. + signifies $p \leq 0.1$. All data are shown as mean $\pm$ SEM.

Figure 6.

ERK phosphorylation (pERK) in the BLA is reduced in response to β -AR activation by repeated stress, and inhibition of pERK is sufficient to reduce HPA activity to repeated stress. A) Representative western blots from two different experiments, comparing levels of phosphorylated ERKs 1 and 2 in the BLA of naïve (CTL 0) animals, animals at the end of a first restraint (CTL 30), animals at the end of their 5th restraint (RR 30), and separately, CTL 0 and repeatedly stressed animals under basal conditions (RR 0). B) pERK 1 levels normalized to CTL 0. In the BLA, pERK 1 is significantly decreased in response to a first restraint (CTL 30) and after 4 prior restraint exposures remains significantly decreased under basal conditions on day 5 (RR 0) as well as after 5th restraint (RR 30). C) pERK 2 levels normalized to CTL 0. ERK 2 phosphorylation is significantly decreased in response to first restraint (CTL 30) and in response to a 5th restraint (RR 30), and tends ($p=0.07$) to be decreased under basal conditions on day 5 (RR 0). ns: CTL 0' = 7, CTL 30' = 6, RR 0' = 5, RR 30' = 9. D) Representative western blots, comparing levels of phosphorylated ERKs 1 and 2 in the BLA of unstressed (CTL) or repeatedly stressed animals sacrificed basally (RR) on

day 5 which received intra-BLA injections on days 1–4. E and F) pERK1 (E) and pERK 2 (F) normalized to CTL VEH (100%). Phosphorylation of both ERKs in the BLA was significantly reduced in RR VEH animals compared to CTL VEH, similarly to the effect shown in 6A–C. Intra-BLA propranolol delivered after daily restraint (RR PROP) prevented repeated restraint-induced reductions in basal pERK levels seen in RR VEH. ns: CTL VEH=11, CTL PROP=11, RR VEH=10, RR PROP=11. G–H) Daily post-restraint intra-BLA infusion of the MEK inhibitor U0126 (MEKI), which prevents MEK from phosphorylating ERK, is sufficient to reduce HPA activity to restraint regardless of prior stress experience. G) Animals received MEKI or vehicle after restraint (RR) or under unstressed (CTL) conditions each day on days 1–4, and ACTH responses to restraint were measured on day 5. RR groups demonstrated significant habituation in ACTH responses to restraint at 15 and 30 minutes as compared to CTL groups, and there was a trend ($p \leq 0.1$) towards reduced ACTH in both MEKI groups. ns: CTL VEH=11–12, CTL MEKI=8–9, RR VEH=10–12, RR MEKI=12–13. H) Integrating ACTH values, to obtain a weighted average HPA response to restraint on day 5, revealed that overall ACTH activity was reduced in RR groups regardless of drug treatment, indicating significant habituation of HPA activity, and in MEKI groups regardless of prior stress, indicating that inhibition of ERK phosphorylation in the BLA on days prior to restraint on day 5 reduced HPA activity to this test stress regardless of prior stress history. ns: CTL VEH=11, CTL MEKI=8, RR VEH=10, RR MEKI=12. * signifies $p \leq 0.05$. + signifies $p \leq 0.1$. All data are shown as mean $\pm$ SEM.

Figure 7. Schematic of the role of BLA β -AR activity and pERK activity on habituation or adaptation to repeated stress. A) The current findings support an inverted-U function to describe the effects of BLA β -AR activation immediately post-stress on the habituation of HPA activity and struggling behavior. However, the data indicate that the curve for HPA activity should be placed rightward of the curve for struggling behavior, as the habituation of HPA activity can be enhanced by the administration of a low dose of β -AR agonist, but struggling behavior cannot be enhanced beyond the levels induced by endogenous β -AR activation. A remaining question regards whether stress habituation can be completely prevented by intra-BLA administration of a high dose of β -AR agonist after stress. B) The current data indicate that reductions in pERK in the BLA below that seen in control animals is a key mechanism by which β -AR activation regulates stress adaptation. Daily administration of the MEK inhibitor U0126, which prevents ERK phosphorylation, significantly reduced HPA activity, whereas blockade of β -AR by propranolol prevented habituation and prevented decreased pERK in the BLA. However, it is not known how β -AR agonist such as clenbuterol affects pERK in the BLA, or how intracellular signaling in the BLA might be altered as a result of high levels of β -AR activation.