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Altered Gene Expression and Spine Density in Nucleus Accumbens of Adolescent and Adult Male Mice Exposed to Emotional and Physical Stress

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Extracellular signal-related kinase 2 · ΔFosB · Dendritic spines · Nucleus accumbens · Depression · Social defeat · Adolescence

Abstract
Stressful early life experiences are implicated in lifelong health. However, little is known about the consequences of emotional stress (ES) or physical stress (PS) on neurobiology. Therefore, the following set of experiments was designed to assess changes in transcription and translation of key proteins within the nucleus accumbens (NAc). Male adolescent (postnatal day 35) or adult (8-week-old) mice were exposed to ES or PS using a witness social defeat paradigm. Then, 24 h after the last stress session, we measured levels of specific mRNAs and proteins within the NAc. Spine density was also assessed in separate groups of mice. Exposure to ES or PS disrupted extracellular signal-related kinase 2 (ERK2), reduced transcription of ΔFosB and had no effect on cAMP response element-binding protein (CREB) mRNA. Western blots revealed that exposure to ES or PS decreased ERK2 phosphorylation in adolescents, whereas the same stress regimen increased ERK2 phosphorylation in adults. Exposure to ES or PS had no effect on ΔFosB or CREB phosphorylation. ES and PS increased spine density in the NAc of adolescent exposed mice, but only exposure to PS increased spine density in adults. Together, these findings demonstrate that exposure to ES or PS is a potent stressor in adolescent and adult mice and can disturb the integrity of the NAc by altering transcription and translation of important signaling molecules in an age-dependent manner. Furthermore, exposure to ES and PS induces substantial synaptic plasticity of the NAc.

Introduction

Stressful early life experiences can have devastating consequences associated with mental illness and behavioral malfunctioning that extend well into adulthood [1]. Individuals with childhood history of abuse, whether physical, emotional or sexual, have increased risk of lifelong negative outcomes [1–3]. These individuals die by suicide more frequently and are more likely to succumb to depression and anxiety disorders [4–7]. However, little is known about the different effects of specific types of

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abuse. Clinical and epidemiological studies in people with history of abuse have largely been unable to tease apart the potentially disparate effects of emotional stress (ES) or physical stress (PS), since these are rarely experienced in isolation [8–10]. Nevertheless, studies that attempt to measure these differences find surprising outcomes. Individuals with childhood history of emotional abuse are at increased risk for developing posttraumatic stress disorder, substance abuse and obesity, as well as for committing suicide, and it appears that emotional maltreatment may predispose a person to developing depression and anxiety symptoms more so than those with physical maltreatment or sexual abuse alone [5, 10–12].

Preclinical models attempting to measure the differences between ES and PS also indicate substantial functional and biochemical changes after exposure to ES. Mice exposed to ES or PS as adults have long-lasting deficits in behavioral tasks designed to assess changes in mood-related behavior [13]. Furthermore, exposure to ES or PS powerfully disrupts the cell biology of ventral tegmental area (VTA) neurons in these mice [13]. The VTA is part of the mesolimbic dopamine system, a brain region that is heavily involved in natural and drug reward [14–16] and is now increasingly being looked at for its role in managing motivation- and mood-related functioning [17–19]. The nucleus accumbens (NAc) is densely innervated by the VTA and also plays a pivotal role in reward and mood processes [17–19]. Therefore, it was of interest to determine whether ES or PS could alter neurobiology and synaptic plasticity within the NAc.

Converging lines of evidence indicate that neurotrophin signaling has a profound influence on emotionality. In particular, extracellular signal-related kinase 2 (ERK2), a downstream target of the neurotrophin BDNF, has emerged as an important mediator of mood. Chronic stress increases – whereas chronic exposure to the antidepressant fluoxetine decreases – ERK2 expression within the VTA [20–22]. Additionally, viral-mediated overexpression of ERK2 results in susceptibility to stress, while knockdown with a dominant negative mutant of ERK2 results in a stress-resistant phenotype in rodents that have had their VTA transfected with the respective viruses [20, 21]. Not surprisingly, then, dysregulation of two of ERK's downstream targets, ΔFosB and cAMP response element-binding protein (CREB), have also been shown to be intimately linked with deficits in mood-related functioning [23–28]. In the NAc, activation of CREB is associated with increased anxiety- and depression-like behavior in adult rodents [24, 29, 30]. Conversely, induction of ΔFosB in the NAc in adulthood appears to be related to resilience to stress [27]. These signaling molecules also appear to be involved in synaptogenesis [14, 31, 32], a type of neural plasticity implicated in susceptibility and resilience to stress [32]. Because adolescence is thought to be a period of increased synaptic plasticity, determining the consequences of exposure to ES and PS on ERK2, ΔFosB and CREB in relation to synaptic plasticity was tantalizing.

While studies assessing the deleterious effects of stress have generally been conducted in adulthood, much less is known about the effects of stressors during adolescence, a critical developmental period of increased sensitivity to stress [33–35]. This is surprising, since adolescence is a period when psychiatric, drug abuse and conduct disorders often emerge [36–38]. Thus, the focus of this study was to assess the effects of ES or PS on NAc biochemistry and synaptic plasticity in adolescent and adult male mice.

Methods

Animals

The mice were male, fed ad libitum, allowed a 1-week habituation period before experimental manipulation, and housed at 23–25°C on a 12-hour light/dark cycle (lights on at 7 a.m.). Adolescent (postnatal day, PD, 35) and adult (8-week old) male C57BL/6J mice (Jackson Laboratory, Bar Harbor, Me., USA) and CD-1 retired breeders (Charles River Laboratories) were used in this study. The mice were housed in clear polypropylene boxes containing wood shavings (4 per cage prior to stress, singly housed following stress) and CD-1 mice (housed 1 per cage immediately upon arrival). Experiments were conducted in compliance with the guidelines for the Care and Use of Laboratory Animals [39] and approved by the Florida State University Animal Care and Use Committee.

Stress Exposure and Experimental Design

ES and PS procedures were performed as previously described [13]. Adolescent mice were randomly assigned to a daily session (10 min per session) of control (CON), ES or PS exposure for 10 consecutive days (PD35–44 for adolescents). Briefly, the home cage (23.5 × 45.5 × 15 cm) of a CD-1 retired breeder was separated into two compartments by a perforated clear Plexiglas divider, allowing olfactory, acoustic and visual signals to be shared between the compartments. The mouse in the ES condition was placed into the empty compartment adjacent to the CD-1 aggressor, while the mouse in the PS condition was placed into the compartment containing the aggressor, as previously described [40–42]. During this time, the PS mouse was subjugated by the CD-1 mouse and adopted a defensive posture. To minimize physical injury and subject attrition, the daily social defeat episode would be terminated immediately in the event that the CD-1 displayed extreme physical aggression (i.e. continuous biting after submissive posturing by the C57BL/6J mouse) [43]. After 10 min, the ES-exposed mouse was transferred to a novel cage, in the compartment adjacent to a novel CD-1, to minimize exposure to any latent stimuli potentially produced by the PS-exposed mouse. The PS-exposed mouse was left overnight in the compartment adjacent to the CD-1 that so-

Table 1. qPCR primers for *Gapdh*, *Mapk1, Creb1* and *ΔFosB*

Gene	Forward sequence	Reverse sequence
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Creb1	AGTGACTGAGGAGCTTGTACCA	TGTGGCTGGGCTTGAAC
ΔFosB	AGGCAGAGCTGGAGTCGGAGAT	GCCGAGGACTTGAACTTCACTCG
Mapk1 (ERK2)	GGTTGTTCCCAAATGCTGACT	CAACTTCAATCCTCTTGTGAGGG

cially defeated it. This process was repeated for 10 consecutive days, such that each day the ES-exposed mouse 'witnessed' the defeat of a novel mouse by a novel CD-1. The term 'witness' in this model refers to all sensory stimuli associated with the ES experience and not visual stimuli alone [13]. CON-exposed mice were housed by pair, 1 on each side of a perforated Plexiglas partition, and handled daily [41]. Behavioral testing began 24 h after the last stress session. All animals (adolescent and adults) were single housed at the initiation of stress treatment and remained single housed for the duration of the study.

Social Interaction Test

The social interaction test is a test of social avoidance [41–43]. Briefly, this is a two-session test. In the first session, a mouse is allowed to explore an open field arena (40 × 40 cm) for 2.5 min. Along one side of the arena is a wire mesh cage that remains empty during the first trial (no target). This mouse is removed and a novel CD-1 male mouse is placed into the wire mesh cage. The test mouse is replaced and the amount of time he spends in the 'interaction zone' (an 8-cm-wide corridor surrounding the cage), as well as the time spent in the 'corners' farthest from the mesh cage, are measured during the 2.5-min trial (target present). Socially defeated mice explore the interaction zone less when another mouse is present and spend more time in the corners. Interestingly, chronic antidepressant treatment alleviates this behavioral phenotype, but acute treatment does not [13, 44, 45]. This makes the social interaction test a highly valid model of antidepressant efficacy and is thus a good model to test for depression-like affect.

Quantitative Real-Time Reversed Transcription-PCR

The mice were sacrificed either 24 h after the last exposure to CON, ES or PS and 1.25-mm punches were taken from the NAc (including both core and shell) and stored at –80°C until use. RNA was isolated using RNEasy Micro kits (Qiagen) and cDNA was created from these samples using iScript cDNA synthesis kit (Bio-Rad, Hercules, Calif., USA). qPCRs were performed in triplicate using 96-well PCR plates and RealMasterMix (Eppendorf) with an Eppendorf Mastercycler RealPlex^2 according to the manufacturer's instructions. Threshold cycle (C_t) values were measured using the supplied software and analyzed with the $\Delta\Delta C_t$ method as described previously [27, 46]. Primer sequences for ERK2, *ΔFosB*, *Creb1* (CREB) and *Gapdh* were generated from the Harvard Primer Bank and are listed in table 1.

Western Blotting

Tissue punches of NAc (1.25 mm) from mice were sonicated in a standard lysis buffer and then centrifuged at 14,000 rpm for 15 min. Each punch contained tissue from both core and shell. Samples (20 μg, estimated through Bradford assay) were treated with β-mercaptoethanol and subsequently electrophoresed on precast 4–20% gradient gels (Bio-Rad), as previously described [47]. Proteins were transferred to a polyvinylidene fluoride membrane, washed in 1× Tris-buffered saline with 0.1% Tween-20 (TBST) and blocked in milk dissolved in TBST (5% weight/volume) for 1 h at 25°C. Blots were probed (overnight at 4°C) with antibodies against ΔFosB (1:1,000), phosphorylated ERK1/2 (1:2,000) and CREB (1:2,000), stripped with Restore (Pierce Biotechnology, Rockford, Ill., USA) and reprobed with antibodies against total ERK1/2 (1:2,000), CREB (1:2,000) and GAPDH (1:2,000). All antibodies were from Cell Signaling (Beverly, Mass., USA) and were used according to the manufacturer's instructions in 5% milk dissolved in TBST. After further washes, the membranes were incubated with peroxidase-labeled goat anti-rabbit IgG or goat anti-mouse IgG (1:5,000; Vector Laboratories, Burlingame, Calif., USA). Bands were visualized with SuperSignal West Dura substrate (Pierce Biotechnology), quantified using ImageJ (National Institutes of Health) and normalized to total protein (optical density phosphorylated protein/optical density total protein) or to GAPDH (optical density target protein/optical density GAPDH). Data are presented as percentage of control expression (test value/CON value × 100). Because ERK1 and ERK2 bands are clearly discernable, only the ERK2 bands were quantified.

Golgi Staining and Spine Analysis

The mice were given an overdose of ketamine/xylazine and perfused transcardially with ice-cold saline. Whole brains were dissected out and stained with FD Rapid GolgiStain kit, as per the manufacturer's instructions (FD Neurotechnologies). After staining, the brains were sliced into 100-μm sections and mounted on gelatin-coated slides, dehydrated with ascending concentrations of ethanol, defatted in xylene, and coverslipped using Permount. Dendritic spines from the NAc core and shell were imaged using Stereo Investigator (MBF Bioscience, Williston, Vt., USA) and spine analysis was performed using ImageJ by two observers blinded to treatment conditions. More specifically, the total number of spines was counted on a given length of dendrite and the spine density was derived (total number of spines/length of dendrite in μm) as described previously [48]. A correlation between values from the two raters demonstrated high concordance.

Statistical Analyses

Data were analyzed using mixed-design (between and within variables) analysis of variance (ANOVA) followed by least significant difference post hoc tests. When appropriate, Student's t tests were used to determine statistical significance of preplanned comparisons. Data are expressed as the mean ± SEM. Statistical significance was defined as $p < 0.05$.

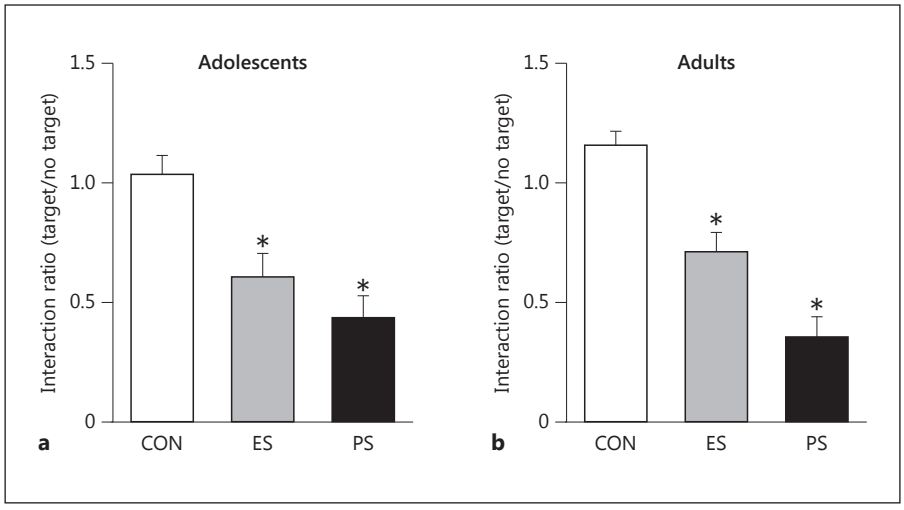

Fig. 1. ES and PS alter social interaction 24 h after the last stress exposure. **a** ES and PS exposure during adolescence reduced the time spent interacting with the CD-1 mouse compared to CON-exposed mice ($n = 30$, $p < 0.05$). **b** ES and PS exposure during adulthood reduced the time spent interacting with the CD-1 mouse compared to CON-exposed mice ($n = 30$, $p < 0.05$). * Significantly different than CON-exposed mice.

Results

Social Interaction following Exposure to CON, ES or PS in Adolescent and Adult C57BL/6J Mice

We first assessed the consequences of 10 days of CON, ES or PS exposure on social interaction 24 h after the last stress session in either adolescent or adult mice. One-way ANOVA revealed that social interaction time varied as a function of stress condition ($F_{2, 27} = 11.7$, $p < 0.001$) in the adolescent mice. Exposure to PS reduced social interaction compared to the CON-exposed mice (fig. 1a). Interestingly, ES-exposed mice also showed social avoidance compared to the CON-exposed mice, indicating that social avoidance can be induced vicariously in adolescent mice ($p < 0.05$). To determine whether stress exposure during adulthood could induce similar changes, a separate group of mice was exposed to CON, ES or PS for 10 consecutive days as adults and then social interaction was assessed 24 h later (fig. 1b). As expected, we found that exposure to ES and PS induced social avoidance in adult mice ($F_{2, 27} = 29.1$, $p < 0.001$). Together, these findings demonstrate that ES and PS exposure induce social avoidance in both adolescent and adult mice.

Effects of CON, ES or PS Exposure on ERK2, ΔFosB and CREB within the NAc of Adolescent and Adult C57BL/6J Mice

ERK2, ΔFosB and CREB mRNA levels within the NAc were measured 24 h after either adolescent or adult exposure to CON, ES or PS using qPCR (fig. 2a–f). ERK2 levels varied as a function of stress exposure in both adolescent ($F_{2, 21} = 12.1$, $p < 0.001$; fig. 2a) and adult exposed mice ($F_{2, 21} = 12.9$, $p < 0.001$; fig. 2b) compared to CON-exposed mice. ERK2 mRNA was surprisingly decreased in mice exposed to ES and PS as adolescents, but increased in mice exposed to ES and PS as adults (both $p < 0.05$). ΔFosB mRNA levels also varied as a function of stress exposure in both adolescent ($F_{2, 21} = 5.9$, $p < 0.01$; fig. 2c) and adult ($F_{2, 21} = 8.9$, $p < 0.01$; fig. 2d) exposed mice. Interestingly, ES and PS exposure decreased ΔFosB mRNA in both adolescent and adult exposed mice ($p < 0.05$). Lastly, we also measured the effect of ES or PS on CREB expression. CREB mRNA did not vary with stress exposure in either adolescent (fig. 2e) or adult (fig. 2f) mice.

Effects of CON, ES or PS on Protein Phosphorylation and Expression on ERK2, ΔFosB and CREB within the NAc of Adolescent and Adult C57BL/6J Mice

Since changes in mRNA do not always translate into changes in protein levels, we also assessed the effects of ES or PS on total and phosphorylated levels of protein. Phosphorylated ERK2 protein levels varied as a function of CON, ES or PS exposure in adolescent mice ($F_{2, 20} = 4.50$, $p < 0.05$; fig. 3a). More specifically, we found an increase in ERK2 phosphorylation in the NAc of adolescents compared to the CON-exposed controls ($p < 0.05$) after PS exposure. Similarly, levels of NAc ERK2 phosphorylation also varied as a function of stress exposure in adults ($F_{2, 21} = 4.18$, $p < 0.03$; fig. 3d) after PS exposure compared to the CON-exposed adult mice 24 h after the last stress session ($p < 0.05$). Total levels of ΔFosB varied as a function of stress exposure during adoles-

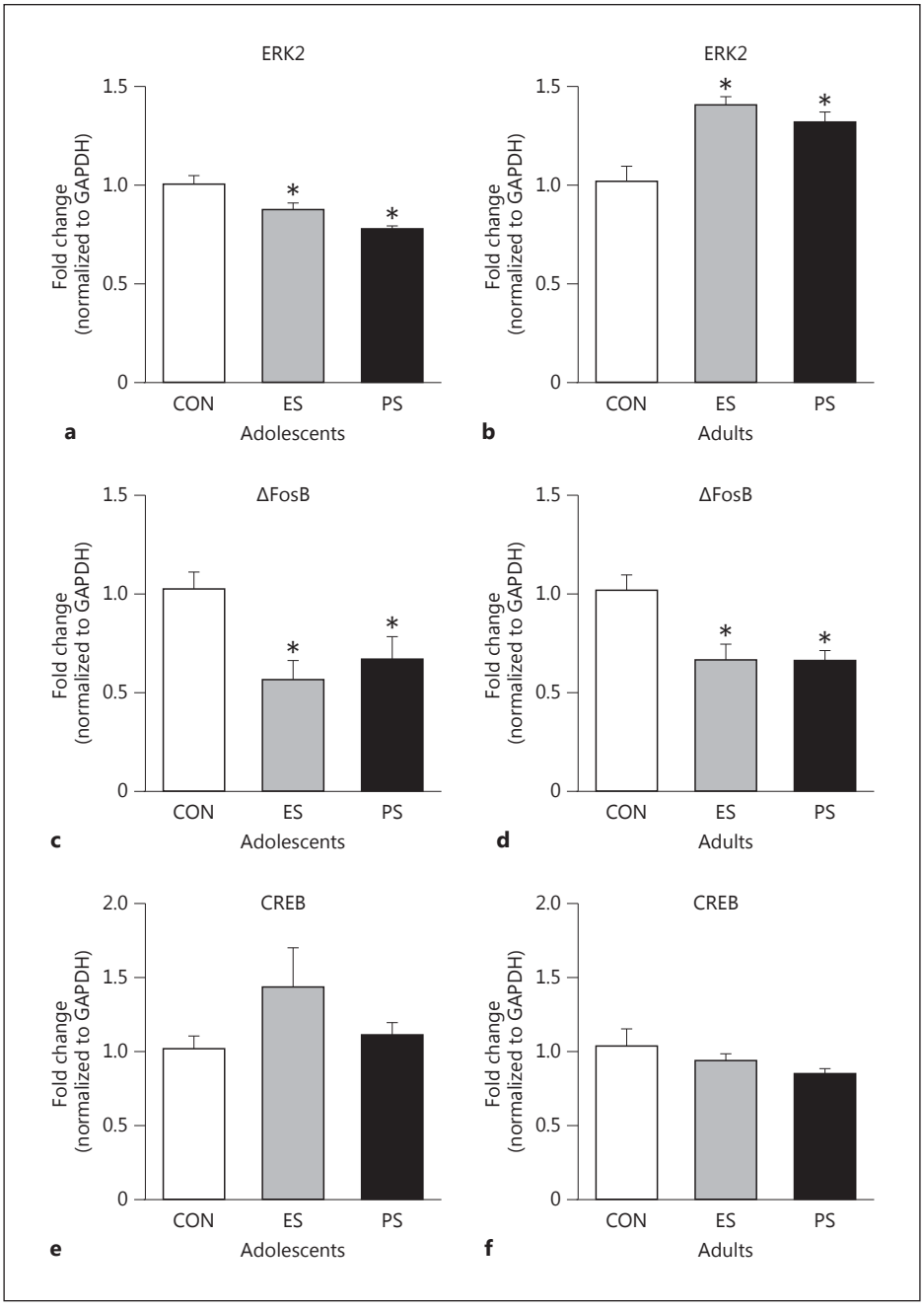

Fig. 2. ES and PS alter mRNA levels 24 h after the last stress exposure. **a** ES and PS exposure during adolescence decreased levels of ERK2 mRNA compared to CON-exposed mice (n = 24, $p < 0.05$). **b** ES and PS exposure during adulthood increased levels of ERK2 mRNA compared to CON-exposed mice (n = 24, $p < 0.05$). **c** ES and PS exposure during adolescence reduced levels of ΔFosB mRNA compared to CON-exposed mice (n = 24, $p < 0.05$). **d** ES and PS exposure during adulthood reduced levels of ΔFosB mRNA compared to CON-exposed mice (n = 24, $p < 0.05$). **e**, **f** ES and PS exposure during adolescence (n = 24, $p > 0.05$) or adulthood had no effect on CREB mRNA compared to CON-exposed mice (n = 24, $p > 0.05$). Data are presented as fold change from CON-exposed mice. * Significantly different than CON-exposed mice.

cence ($F_{2, 20} = 4.65$, $p < 0.05$; fig. 3b) but not in adult mice ($p > 0.05$; fig. 3e). Interestingly, in mice exposed to ES or PS as adolescents, phosphorylation of CREB varied as a function of stress exposure ($F_{2, 20} = 6.10$, $p < 0.01$; fig. 3c), with no effect in the adult mice ($p > 0.05$; fig. 3f) compared to CON-exposed mice. More specifically, exposure to ES or PS during adolescence robustly decreased CREB phosphorylation compared to CON-exposed mice ($p < 0.05$). No significant differences were seen in phosphorylated ERK2, total ERK2 or total CREB in adolescent mice ($p > 0.05$) compared to CON-exposed mice. Phosphorylated CREB was significantly influenced when normalized to GAPDH instead of total CREB in adolescent mice ($F_{2, 20} = 6.1$, $p < 0.01$; data not shown). In adults, only phosphorylated ERK2 was significantly influenced by ES or PS exposure when normalized to GAPDH instead of

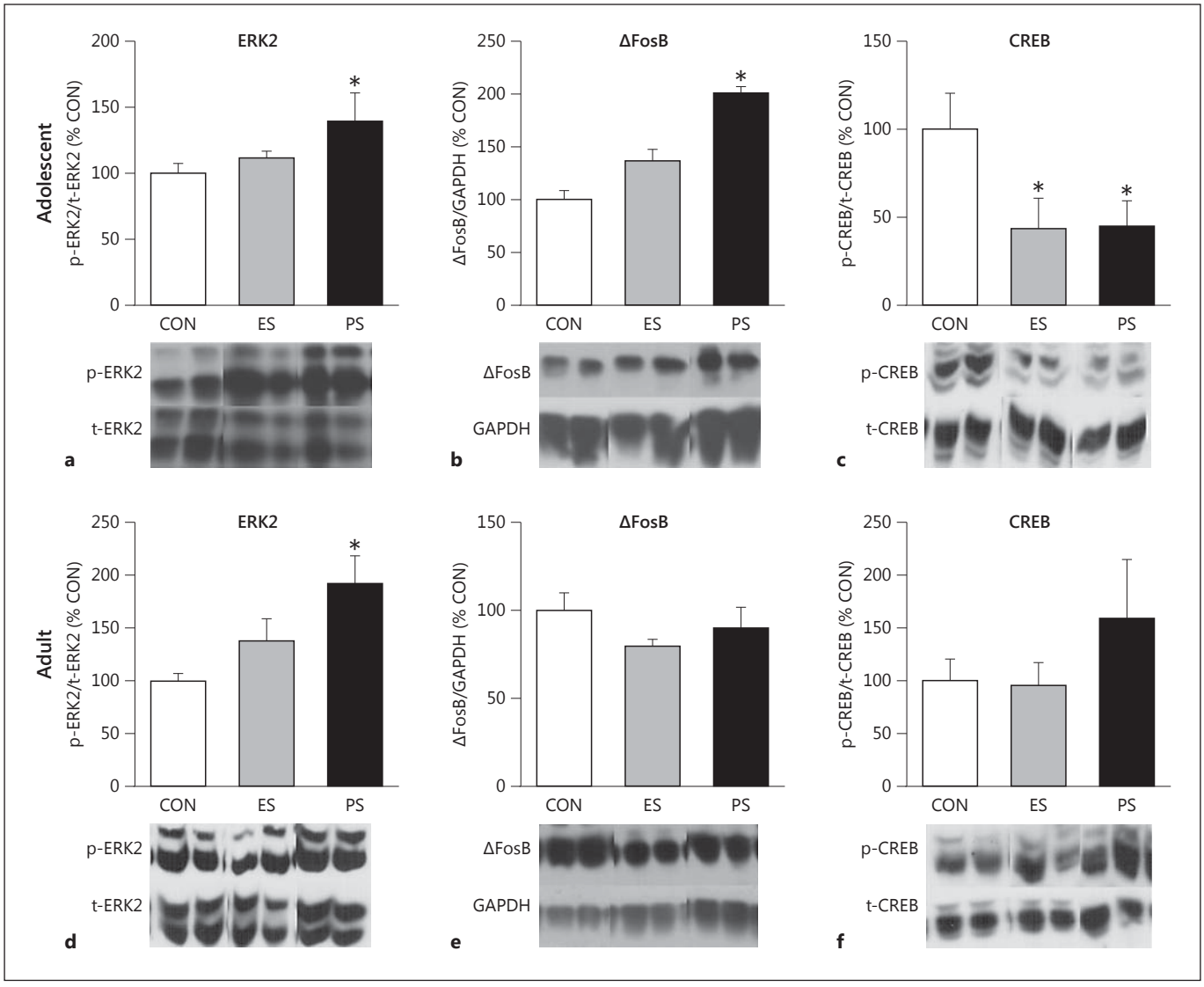

Fig. 3. ES and PS alter protein levels 24 h after the last stress exposure. p = Phosphorylation; t = total. **a**, **b** PS exposure during adolescence increased ERK2 phosphorylation and increased ΔFosB compared to CON-exposed mice ($n = 23$, $p < 0.05$). **c** ES and PS exposure decreased CREB phosphorylation in adolescent mice compared to CON-exposed mice ($n = 23$, $p < 0.05$). **d** In adults, PS, but not ES, exposure increased levels of ERK2 phosphorylation compared to CON-exposed mice ($n = 24$, $p < 0.05$), but had no effect on ΔFosB ($n = 24$; $p > 0.05$) or CREB phosphorylation ($n = 24$, $p > 0.05$) compared to CON-exposed mice (**e**, **f**). Data are presented as percent of CON levels of immunoreactivity. * Significantly different than CON-exposed mice.

total ERK ($F_{2, 21} = 10.88$, $p < 0.01$; data not shown). These differences are expected, since they do not account for changes in total protein levels.

Effects of CON, ES or PS Exposure on Spine Density within the NAc of Adolescent and Adult C57BL/6J Mice

Because changes in intracellular signaling and gene expression often suggest changes in synaptic plasticity, we also assessed the effects of stress exposure on spine density within the NAc. Figure 4 shows the effect of CON, ES or PS on spine density during adolescence (fig. 4a) or adulthood (fig. 4b). In adolescent mice, spine density was found to significantly vary as a function of stress exposure ($F_{2, 26} = 9.7$, $p < 0.001$; fig. 4a). Specifically, mice exposed to ES or PS as adolescents had increased spine density within the NAc, indicating increased synaptic plasticity. In adults, there was also an effect of stress ex-

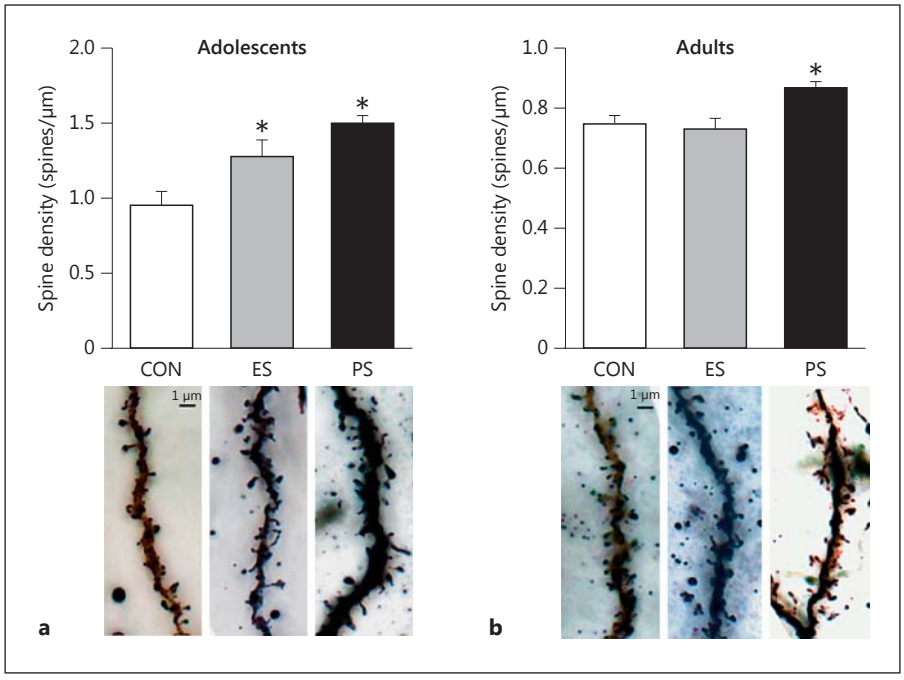

Fig. 4. ES and PS alter spine density 24 h after the last stress exposure. **a** ES and PS exposure during adolescence increased spine density compared to CON-exposed mice ($n = 29$, $p < 0.05$). **b** PS, but not ES, exposure during adulthood increased spine density compared to CON-exposed mice ($n = 17$, $p < 0.05$). * Significantly different than CON-exposed mice.

posure ($F_{2, 14} = 6.3$, $p < 0.05$; fig. 4b); however, only PS exposure increased spine density in the NAc compared to the CON-exposed mice.

Discussion

The present study assessed whether exposure to ES or PS could disrupt the integrity of the NAc of adolescent and adult male mice 24 h after stress exposure. Here, we provide evidence that exposure to ES or PS disrupts social interaction and induces unique, age-dependent adaptations in gene transcription, protein phosphorylation and synaptic plasticity.

Exposure to ES and PS both induced a depression-like state, as indexed in the social interaction test. In this task, a mouse is allowed to interact with a conspecific in an open field arena. This behavioral task is one of the most important tests currently used to assess the behavioral state in socially defeated mice [40, 41]. We found that mice exposed to ES or PS during adolescence or adulthood spent significantly less time interacting with a social target than the CON-exposed mice. This is interesting because it suggests that exposure to ES can induce social avoidance even in young mice. Furthermore, simply witnessing the physical stressor appears to be enough to induce social avoidance in ES-exposed mice. Exposure to social defeat, as in the PS-exposed mice, is known to induce a robust and reliable social avoidance in adult mice [13, 21, 40–44]. Here, we show that PS exposure can produce this effect in adolescent mice as well, and further extend these findings to also demonstrate that it can be induced vicariously through ES exposure [13]. In contrast to previous reports showing a distribution of behavioral response to social defeat (i.e. PS) from 'resilient' to 'susceptible', we found relatively few resilient mice. Because these resilient mice were not statistical outliers from the mean, they were included with susceptible mice in the PS group. Given the relatively small number of resilient mice, it is unlikely that susceptible versus resilient effects could have been found. Nevertheless, we believe that future studies using more mice may be able to uncover differences in gene expression and spine density by susceptibility.

While there is substantial evidence demonstrating that the NAc plays a role in mediating depression-like behavior in adults [23, 27, 30, 41, 46, 49], significantly less is known about its role in adolescence, a period of increased synaptic plasticity [50, 51]. The NAc receives dopaminergic input from the VTA and, together, these brain areas form part of the mesolimbic dopamine system [52, 53]. This circuit is widely believed to be responsible for mediating the effects of natural- and drug-rewarding behavior and, more recently, in mediating responses to emotion-eliciting stimuli [17, 54–56]. Here, we show that the NAc

undergoes substantial changes in response to ES and PS exposure both in adolescence and adulthood. First, exposure to ES and PS differentially regulated ERK2 mRNA in adolescent and adult mice. ERK2 mRNA was significantly reduced following ES and PS in adolescent mice, but elevated in the NAc of adults. Surprisingly, we found that the decreases in ERK2 mRNA were accompanied by increases in ERK2 phosphorylation in the NAc of adolescent mice. In addition, while both ES and PS influenced mRNA levels of ERK2, only PS influenced ERK2 phosphorylation. ERK2 is a protein kinase that must be phosphorylated to become active, and therefore it is expected that changes in ERK2 expression might accompany changes in ERK2 phosphorylation. However, this is not always the case [57, 58]. The relationship between the activity of signaling proteins and the expression of their mRNA can be complex, and it is likely that there are several factors controlling the expression of ERK2 beyond its activation. The stress-induced decreases in ERK2 mRNA and increased phosphorylation were both robust and unique to adolescence, making it particularly interesting. The mechanism underlying this effect is unknown. Given the role ERK2 plays in drug abuse processes [59–61] and that drugs of abuse and stress can cross-sensitize [62, 63], we expected that ES or PS exposure would have resulted in increased ERK2 expression and activity in the adolescent mice. This finding, in particular, is surprising because we have reported increases in ERK2 phosphorylation in the VTA of adult rodents exposed to chronic unpredictable stress [21]. The paradoxical finding between adolescent and adult mice is intriguing. It is likely that these effects may be due, at least in part, to uniquely age-dependent responses to stress often seen in periods prior to adulthood [33, 34, 64, 65].

Exposure to ES and PS reduced expression of ΔFosB mRNA in both adolescent and adult mice. This finding is surprising, since one might expect to see an increase in ΔFosB, especially given previous reports that exposure to social defeat and other stressors increases ΔFosB in the NAc [27, 66–68]. Recently, it was demonstrated that ΔFosB may play a role in resilience to social defeat stress, rather than in susceptibility: social isolation reduced ΔFosB levels in the NAc, thus rendering mice susceptible to stress, and lower levels of this protein were found in postmortem NAc tissue from depressed patients, whereas mice with a larger induction of ΔFosB following exposure to social defeat appeared more resilient than those with a smaller induction [27]. It is possible that, because we did not look until 24 h after the last stress session, the levels of ΔFosB transcription had decreased to compensate for higher levels of stress-induced Fos transcription in the NAc. However, within this framework, one might still expect to see elevated levels of ΔFosB protein in the NAc, given its high level of stability [69]. Interestingly, we did see elevated levels of ΔFosB protein in the NAc, which lends support to this hypothesis. Because of the relationship between ΔFosB and resilience, it is somewhat surprising to see increased levels of this transcription factor, given the robust social avoidance demonstrated by our mice. However, given the increased ERK2 phosphorylation, it is not surprising that ΔFosB, a downstream target of ERK2, is increased. In addition, this increase could be because we did not distinguish between core and shell during dissection, as differential expression between these two NAc subregions is often found [27, 67, 70, 71]. Future studies are clearly necessary to assess the specific features of this hypothesis within the context of differential gene expression across the life span.

We also assessed potential changes in the expression of NAc CREB to gain a deeper understanding of the molecular changes taking place in ES- and PS-exposed mice. Here, we found decreased CREB phosphorylation in mice exposed to ES and PS as adolescents. It is not surprising that we found changes in CREB expression, since CREB is usually induced in the NAc in response to stress [23, 24, 29, 30]. However, in cases of prolonged stress, such as social isolation, CREB is inhibited and induces an anxiogenic- and anhedonia-like state [30]. Although speculative, since these mice were exposed to a 10-day social defeat paradigm, it is possible that the chronic stress of social instability and constant threat could be acting on CREB in a similar way to prolonged mild stress. Another possibility is that our experimental manipulations activate inducible cAMP early repressor (ICER), a potent repressor of CRE-mediated transcription, thus serving as an important negative-feedback mechanism for shutting off CREB-induced transcription [72, 73]. Because our biochemical analysis was performed 24 h after behavioral testing, it is plausible that CREB induction was already reversed at this time point. Within this context, it is likely that CREB protein levels would be downregulated or brought back to baseline. However, given previous reports that social defeat increases CREB activity in the shell of the NAc [45], decreases in CREB signaling after ES or PS were not anticipated. The relationship between induction of CREB and functional outputs within the NAc is complex [23, 24, 74, 75] and future studies should examine these possibilities.

Because changes in ERK2, ΔFosB and CREB can each result in changes in synaptic plasticity [76–79], and because spine density changes are known to accompany ad-

olescence, we were also interested in determining whether exposure to ES or PS would impact synaptic plasticity by assessing spine density within the NAc. Here, we show that exposure to ES or PS during adolescence does increase spine density in the NAc 24 h after the last stressor. As a positive control, we also assessed spine density in adult exposed mice. Not surprisingly, adult PS-exposed, but not ES-exposed, mice had elevated spine density in the NAc [46, 71, 80]. Increased spine density after adolescent PS exposure was expected, given previous reports showing that stress during postnatal development increases spine density within the NAc [81]. Interestingly, our results also show that exposure to ES during adolescence induced changes similar to those observed in the PS-exposed mice, indicating that ES is a potent stressor [13]. This suggests that the adolescent NAc undergoes substantial restructuring, even after more subtle stressors. Perhaps this is not unexpected, since the developing brain goes through substantial overproduction and pruning of synapses and relatively high levels of dendritic spine turnover [34, 51]. Indeed, it has been hypothesized that different levels of synaptic turnover in discrete brain areas could reflect critical developmental periods for these brain regions [34, 50, 51]. Given that adolescence is a period of increased risk of initiating drug use, increased sensitivity to reward and increased risk taking [37, 82, 83], it is plausible that the NAc could be undergoing a particularly sensitive period of turmoil during adolescence. This could explain why we see higher levels of spine plasticity in adolescents and could partially be responsible for the increased sensitivity of adolescents to stress. However, it is currently unknown whether exposure to stress increases different subtypes of spines. Due to inherent limitations surrounding the methodologies used, determination of spine subtype was unreliable. Future experiments will be needed to fully elucidate the effects of stress during adolescence on synaptogenesis of discrete spine types. Furthermore, this study did not differentiate between the effects of stress on core versus shell. It is plausible, given reports of contrasting functioning between these NAc anatomical distinctions, that there may be different effects in these two NAc subregions. Nevertheless, these data suggest that considerable synaptic plasticity is taking place in the NAc, and future studies will be aimed at determining potential unique effects of ES and PS on core versus shell of the adolescent NAc.

In summary, we show that exposure to ES or PS induces different neurobiological effects in the NAc depending on the developmental stage of the mice. Adolescent and adult male mice exposed to ES or PS demonstrated aberrant behavioral reactivity to social stimuli, altered NAc gene expression, protein phosphorylation, and dendritic spine plasticity. Importantly, we show that mice that witness, but do not physically experience, stress have almost identical changes as those subjected to PS. Taken together, exposure to ES and PS are potent stressors in adolescent and adult mice capable of disrupting the integrity of the NAc. Ongoing studies are assessing whether these stress-induced biochemical changes are long-lasting and how they may affect functional behavioral outputs.

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