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Brain-region-specific molecular responses to maternal separation and social defeat stress in mice

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Abstract

The association between stress and mental illness has been well documented, but the molecular consequences of repeated exposure to stress have not been completely identified. The present study sought to elucidate the combinatorial effects of early life maternal separation stress and adult social defeat stress on alterations in signal transduction and gene expression that have been previously implicated in susceptibility to psychosocial stress. Molecular analyses were performed in the prelimbic/infralimbic cortex, amygdala, and nucleus accumbens, three brain regions that have been suggested to play critical roles in determining stress responses. The current data reveal that both maternal separation and social defeat significantly impact the expression of genes involved in histone methylation and the β-catenin-, endogenous opioid-, neurotrophin-, and glucocorticoid signaling pathways. Although the effects of maternal separation and social defeat were largely non-overlapping, a subset of genes in each brain region were governed by additive, opposing, or other types of interactions between these stress paradigms, thus highlighting potential molecular mechanisms through which these stressors might coordinately regulate brain function and behavior.

Keywords

maternal separation; social defeat stress; amygdala; nucleus accumbens; frontal cortex

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Contributions:

BDS and MGC designed and supervised the study. BDS and HLT performed the stress paradigms. BDS isolated and processed tissue samples. HLT performed and analyzed the western blotting experiments. EF processed some tissue samples and performed real-time PCR experiments. BDS also performed and analyzed real-time PCR experiments. BDS, HLT, EF and MGC wrote the manuscript.

Introduction

Exposure to stress has been associated with numerous negative consequences, including impaired cognitive performance and the development of neuropsychiatric disorders (Heim and Nemeroff, 2001; Bale et al., 2010). However, the reasons why some individuals remain resilient in the face of stress while others experience significant dysfunction remain largely unknown. Two and three-hit models of stress susceptibility suggest that vulnerability to stress results from a variety of genetic and psychological factors, including prior stress experience (Daskalakis et al., 2013; Bateson et al., 2014; Pena et al., 2017). The duration and developmental timing of early life stress exposure are thought to be critical in determining subsequent stress susceptibility (Lupien et al., 2009; Pena et al., 2017), and gene by environment interactions play key roles as well (Caspi et al., 2003). However, whether various genetic and environmental risk factors for stress susceptibility confer vulnerability to stress through shared or distinct pathways has not been established. The current work seeks to examine the molecular alterations that are associated with and could contribute to the combined effects of early life stress and adult psychosocial stress. This was accomplished using the maternal separation stress (MSS) model of early life stress and the social defeat stress (SDS) model of adult psychosocial stress, which is one of the most commonly used animal models to examine the mechanisms leading to stress vulnerability vs. resilience (Golden et al., 2011). Prior work using the SDS model has shown that exposure to psychosocial stress in adolescence increases vulnerability to SDS in adult Syrian hamsters (Rosenhauer et al., 2017), and published studies have shown that MSS can increase susceptibility to anhedonia in rats (Der-Avakian and Markou, 2010) and social avoidance in c57BL6/J mice (Pena et al., 2017) induced by repeated exposure to SDS. In keeping with these findings, unpublished work from our group has shown that the MSS paradigm employed here also increases susceptibility to social avoidance induced by SDS in adult c57BL6/J mice (B.D. Sachs, unpublished observations).

There are many potential cellular and molecular mechanisms through which MSS could lead to altered susceptibility to SDS in adulthood. For example, the MSS-induced increase in SDS susceptibility in c57BL6/J mice has recently been shown to involve transcriptional alterations in the ventral tegmental area (VTA) (Pena et al., 2017), but the transcriptional changes that occur outside of the VTA in response to combined MSS+SDS have not been reported. In addition to the VTA, the nucleus accumbens (NAc) has also been prominently implicated in the regulation of SDS responses (Berton et al., 2006; Krishnan et al., 2007), and neural circuits outside of mesolimbic reward pathway, including the prelimbic cortex (PLC) and the amygdala (Amyg), have also been shown to play a role in SDS responses (Covington et al., 2010; Kumar et al., 2014) and to exhibit transcriptional alterations in response to SDS (Covington et al., 2010; Bagot et al., 2016;). The dysregulation of multiple signaling pathways has been suggested to influence stress responses, including the Wnt-β-catenin (Wilkinson et al., 2011; Dias et al., 2014; Sachs et al., 2015), glucocorticoid (Wagner et al., 2012; Jacobson, 2014; Arloth et al., 2015; Jochems et al., 2015;), endogenous opioid (Wiedenmayer et al., 2002; Nikulina et al., 2005; McLaughlin et al., 2006; Nocjar et al., 2012; Berube et al., 2013; Donahue et al., 2015;), and brain derived neurotrophic factor (BDNF)-cAMP-response element binding protein (CREB) (Berton et al., 2006; Krishnan et

al., 2007) pathways, as well as transcriptional programming pathways involving the orthodenticle homeobox 2 (Otx2) transcription factor (Pena et al., 2017) and histone methylation enzymes (Murgatroyd et al., 2009; Covington et al., 2011; Saunderson et al., 2016). Here, we evaluated the combinatorial effects of MSS and SDS on the expression of a subset of genes from each of these pathways in the PLC, Amyg, and NAc.

In addition to the many potential transcriptional markers of stress susceptibility that have been identified, several specific protein-level alterations have been reported as being important determinants of SDS responses. For example, c57BL/6J mice that display social avoidance behavior following SDS have been shown to exhibit increased phosphorylation of ERK and Akt in the NAc compared to animals that remain sociable following SDS (Krishnan et al., 2007). In addition, animals that remain sociable following SDS have also been observed to exhibit an increase in the protein levels of β-catenin in the NAc compared to avoidant animals (Sachs et al., 2015). Given that overexpression of β-catenin in the NAc has been shown to promote sociability in mice following SDS, alterations in the NAc levels of β-catenin have been hypothesized to be functionally important in determining resilience to stress (Dias et al., 2014). However, whether any of the protein alterations in the NAc that were found to differentiate socially avoidant from sociable mice following exposure to SDS alone (Krishnan et al., 2007; Sachs et al., 2015) also occur in MSS-exposed vs. control populations following SDS has not been established. Overall, the present study aimed to provide new insight into the molecular adaptations that occur in response to repeated stress exposure, which could have important implications for our understanding of susceptibility vs. resilience to stress.

Experimental Procedures

Subjects

This study used c57BL6 mice that were initially obtained from Jackson laboratories and bred in the Caron lab at Duke University. Mice were housed on a 12 h light-dark cycle and provided food and water ad libitum. Male mice were eight-ten weeks of age at the start of SDS. All experiments were conducted with an approved protocol from the Duke University Institutional Animal Care and Use Committee and were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Maternal Separation and Social Defeat Stress

MSS was performed as described previously (Sachs et al., 2013b). Briefly, the experimental pups were separated from their dams for three hours each day during the light phase (starting between 1–3 hours after the start of the light phase) on PNDs 1–14, and control pups were reared under standard conditions. During the separation period, the pups were placed on a heating pad and remained in contact with their littermates. All animals were weaned on day 21, at which point they were all housed in groups of five per cage. At the time of weaning, both standard facility reared controls and MSS-exposed animals were randomly divided into two additional groups (SDS and control) that were subjected to SDS (or not for controls) starting at 8-10 weeks of age. For the SDS group (both for MSS and non-MSS animals), the SDS paradigm was performed as described previously (Sachs et al., 2015) and consisted of

introducing each experimental mouse into the home cage of a singly housed resident CD1 male for five minutes a day for ten days during the light phase of the light-dark cycle (between 1-3 hours prior to the start of the dark phase). Following each defeat session, each experimental mouse was singly housed for 24 hours. The experimental mice were introduced into the home cage of a new CD1 male mouse each day. Control animals were group housed throughout the experiment and had no exposure to CD1 animals until social interaction testing. On day 12 (~48 hours after the final defeat episode), experimental animals were sacrificed for molecular analyses.

Western Blotting

Mice were killed by cervical dislocation and decapitation, after which their heads were immediately cooled by immersion in liquid nitrogen for five seconds. The brains were rapidly dissected on an ice-cold surface and 1.5 mm tissue punches from the NAc (stereotactic coordinates provided below) were collected and snap frozen in liquid nitrogen. Bilateral punches from the same animal were pooled and considered a single sample. Samples were processed for Western blotting using standard methods that we have described previously (Sachs et al., 2015). Membranes were incubated overnight at 4°C with the following primary antibodies: mouse anti-GAPDH (MAB374, Millipore; 1:1000), mouse anti- β-catenin (#2698, Cell Signaling, 1:300), rabbit anti-phospho-p44/p42 MAPK (Erk1/2; #9101, Tyr202/204, Cell Signaling; 1:500); mouse anti-total ERK (#9107, Cell Signaling, 1:500), rabbit antiphospho-Akt (Ser473, #9271, Cell Signaling, 1:500), and rabbit anti-total Akt (#9272, Cell Signaling, 1:1000). The appropriate AlexaFluor 680 or IRDye 800-conjugated secondary antibodies were incubated at room temperature for ~90 minutes (Molecular Probes and Li-COR; 1:10,000 dilution), and blots were developed using an Odyssey LI-COR system (LI-COR Biosciences, USA). Quantification was performed using densitometry with the Image J program (National Institutes of Health). The relative levels of β-catenin were normalized against GAPDH and the levels of phosphorylated kinase (Akt or ERK) were normalized against total kinase (Akt or ERK) as a loading control.

Real-Time PCR

Tissue samples were obtained as described above for the Western blotting analysis, except that tissue was isolated from the PLC, Amyg, and NAc. The tissue punches were centered at approximately the following stereotactic coordinates: PLC: A/P: +2.1 mm anterior to Bregma, D/V: −2.2 mm, M/L: at the midline; NAc: A/P +1.1; D/V: −4.5 mm; M/L +/− 0.8 mm; Amyg: A/P: −1.0, D/V: −5.0 mm, M/L: +/− 2.3 mm. A single punch containing PLC from both hemispheres was obtained, but bilateral punches were obtained from NAc and Amyg. RNA was isolated from these tissue punches using RNEasy minikits according to the manufacturer's protocol, and reverse transcription was performed using iScript First Strand Synthesis kits according to the manufacturer's protocol and as described previously (Sachs et al., 2013a). Real-time PCR was performed using a StepOne Plus Real Time PCR System (Applied Biosystems by ThermoFisher Scientific, Waltham, MA, USA) using PowerUp Sybr Green master mix (ThermoFisher Scientific, Waltham, MA, USA). A table describing the primers used is provided in the Appendix.

Statistical analyses

Data were analyzed using two-way ANOVAs with MSS and SDS as between-subjects factors. Shapiro-Wilk W tests were used to verify that data were normally distributed, and Obrien's tests for homogeneity of variance were used to ensure equal variance. When Shapiro-Wilk or Obrien's tests indicated that data were not normally distributed or had unequal variance, data were transformed (log10 or squared) to achieve normalcy and equal variance prior to performing two-way ANOVAs. When significant interactions were observed, Tukey's post-hoc tests, which correct for multiple comparisons, were performed to evaluate potential group differences.

Results

Signaling effects of MSS-SDS

β-catenin signaling in the NAc has been suggested to be a major regulator of susceptibility vs. resilience to SDS (Dias et al., 2014). Given that MSS has been shown to increase SDS susceptibility (Pena et al., 2017), we sought to determine whether MSS might alter levels of β-catenin in the NAc in response to SDS. Our results demonstrate that after ten days of SDS, both control and MSS-exposed mice subjected to SDS exhibit increased β-catenin in the NAc [$F_{(3, 36)} = 5.62$, $p = 0.023$, Fig. 1a], thus suggesting that MSS does not influence the alterations in the protein levels of β-catenin in the NAc induced by SDS.

Other signaling molecules in the NAc that have been implicated in determining SDS responses include Akt and ERK, two kinases that have been shown to be more highly phosphorylated in socially avoidant mice compared to sociable mice following SDS (Krishnan et al., 2007). We sought to evaluate whether MSS-exposed animals would also exhibit increased phosphorylation of these kinases in the NAc following SDS compared to SDS-exposed animals with no history of MSS. However, our results reveal no significant effects of MSS on the phosphorylation of Akt (Fig. 1b) or ERK (Fig. 1c). In contrast, SDS was shown to lead to a significant increase in Akt phosphorylation [$F_{(3,36)} = 12.79$, $p = 0.001$], but MSS did not modify this effect (Fig. 1b). No significant effects of SDS on pERK levels were observed in the NAc (Fig. 1c).

MSS-SDS and Gene Expression

Nucleus Accumbens

We next sought to evaluate potential effects of combined MSS-SDS on gene expression in the NAc. We first evaluated whether the expression of β-catenin target genes was significantly impacted by MSS and/or SDS. Our results demonstrate that MSS led to a significant increase in the mRNA expression of Axin1 [$F_{(3, 28)} = 8.72$, $p = 0.006$, (Fig. 2a)] and Dicer [$F_{(3, 28)} = 5.71$, $p = 0.024$, (Fig. 2c)]. MSS also led to a significant increase in the expression of several genes known to regulate β-catenin that have been previously implicated in SDS responses (Wilkinson et al., 2011), including GSK3β [$F_{(3, 28)} = 12.71$, $p = 0.001$, (Fig 2d)] and disheveled-1 (DVL-1) [$F_{(3, 28)} = 5.84$, $p = 0.022$, (Fig 2e)]. Like MSS, SDS also induced a significant increase in GSK3β mRNA expression [$F_{(3, 28)} = 14.13$, $p = 0.0008$,(Fig. 2d)], thus suggesting that additive effects of SDS and MSS on GSK3β

expression in the NAc could represent one mechanism through which MSS and SDS might coordinately regulate behavior. However, no significant effects of MSS or SDS on the expression of Axin 2 (Fig. 2b), DVL-2 (Fig. 2f), DVL-3 (Fig 2g), or β-catenin (Fig. 2h) were observed in the NAc.

As expected, genes involved in glucocorticoid signaling and histone methylation were significantly impacted by MSS and/or SDS in the NAc. Specifically, SDS led to an up-regulation of the glucocorticoid receptor (GR) [$F_{(3, 28)} = 5.37$, $p = 0.028$, (Fig. 3a)] and the mineralocorticoid receptor (MR) [$F_{(3, 28)} = 8.34$, $p = 0.0074$, (Fig. 3b)] and a significant down-regulation of FK506 Binding Protein 5 (FKBP5) [$F_{(3, 28)} = 5.30$, $p = 0.029$, (Fig. 3d)], which is a known regulator of GR (Binder, 2009). In addition, MSS led to increased expression of serum/glucocorticoid-regulated kinase 1 (SGK1) [$F_{(3, 28)} = 12.02$, $p = 0.002$, (Fig. 3c)] in the NAc. A trend towards an MSS by SDS interaction was observed for the mRNA expression of corticotropin-releasing hormone (CRH) following stress ($p = 0.056$), but none of the individual group comparisons reached statistical significance (Fig. 3e). In addition, MSS increased the expression of the histone methyl transferase, EHMT1, in the NAc [$F_{(3, 28)} = 5.54$, $p = 0.026$, (Fig. 3f)], demonstrating that MSS does influence epigenetic processes, but no significant effects of SDS on EHMT1 or EHMT2 expression were observed in the NAc (Fig. 3g). No significant main or interactive effects of SDS or MSS were observed on the expression of Otx2 in the NAc, although there was a slight trend towards reduced Otx2 expression following MSS ($p = 0.09$, Fig. 3h).

In the NAc, there were no significant main effects of MSS on the expression of any of the components of the endogenous opioid system that we examined. No significant effects of SDS were observed on the expression of the mu opioid receptor (MOR1) (Fig. 4a) or the delta opioid receptor (DOR1) (Fig. 4c), but SDS significantly reduced the levels of kappa opioid receptor (KOR1) [$F_{(3, 28)} = 6.64$, $p = 0.016$, (Fig. 4b)], proenkephalin (PENK) [$F_{(3, 28)} = 23.35$, $p < 0.0001$ (Fig. 4d)], prodynorphin (PDYN) [$F_{(3, 28)} = 13.7$, $p = 0.0009$, (Fig. 4e)], and pro-opiomelanocortin (POMC) [$F_{(3, 28)} = 4.53$, $p = 0.042$, (Fig. 4f)]. In addition, significant interactions between MSS and SDS were observed for KOR [$F_{(3, 28)} = 5.46$, $p = 0.027$, (Fig. 4b)] and PDYN [$F_{(3, 28)} = 6.33$, $p = 0.018$, (Fig. 4e)], with SDS only leading to a significant reduction in PDYN and KOR levels in control mice, not in MSS-exposed animals (Fig. 4b and e, $p < 0.05$ by Tukey's post hoc tests). No significant group differences in BDNF levels were observed in the NAc (Fig. 4g), but SDS significantly increased the mRNA levels of CREB in this brain region [$F_{(3, 28)} = 11.64$, $p = 0.002$, (Fig. 4h)].

Prelimbic/Infralimbic Cortex

Like the NAc, the PLC was highly responsive to both MSS- and SDS-induced changes in gene expression, although the transcriptional alterations observed were largely distinct between the two brain regions. SDS significantly reduced the expression of Axin1 [$F_{(3, 28)} = 4.93$, $p = 0.035$,(Fig. 5a)], Dicer [$F_{(3, 28)} = 11.27$, $p = 0.002$, (Fig. 5c)] and DVL-3 [$F_{(3, 28)} = 19.8$, $p = 0.0001$, (Fig. 5g)] in the FC. In addition, MSS led to significant increases in the mRNA expression of the β-catenin-related genes Axin2 [$F_{(3, 28)} = 4.52$, $p = 0.042$, (Fig. 5b)] and DVL3 [$F_{(3, 28)} = 4.38$, $p = 0.046$, (Fig. 5g)]. No significant alterations in the expression

of GSK3β (Fig, 5d), DVL-1 (Fig. 5e), DVL-2 (Fig. 5f), or β-catenin itself (Fig. 5h) were observed in response to SDS or MSS in the PLC.

As expected, genes related to glucocorticoid signaling were significantly impacted by both MSS and SDS in the PLC. Specifically, MSS significantly increased the levels of GR [$F_{(3, 28)} = 5.09$, $p = 0.032$, (Fig. 6a)], MR [$F_{(3, 28)} = 5.03$, $p = 0.033$, (Fig. 6b)], and SGK1 [$F_{(3, 28)} = 17.79$, $p = 0.0002$, (Fig. 6c)], and MSS led to a trend towards increased expression of FKBP5 in the PLC ($p = 0.056$, Fig. 6d). SDS led to a significant reduction in CRH [$F_{(3, 28)} = 38.88$, $p < 0.0001$, (Fig. 6e)] and SGK1 [$F_{(3, 28)} = 9.75$, $p = 0.004$, (Fig. 6c)] in this brain region. Similar to the NAc, MSS significantly upregulated EHMT1 mRNA in the PLC [$F_{(3, 28)} = 6.33$, $p = 0.018$, (Fig. 6f)]. EHMT2 expression in the PLC was also dependent on stress exposure, as a significant SDS by MSS interaction was observed in which SDS tended to decrease EHMT2 levels in control animals and increase them in MSS-exposed mice [$F_{(3, 28)} = 9.91$, $p = 0.004$, (Fig. 6g)]. Tukey's post-hoc tests revealed that SDS-MSS-exposed mice had significantly more EHMT2 mRNA in the PLC compared to mice exposed to SDS alone ($p < 0.05$, Fig. 6g). No significant group differences were observed for the PLC expression of Otx2 (Fig. 6h).

Several endogenous opioid-related genes were also differentially regulated by SDS and MSS in the PLC. Although no significant group differences were observed in the expression of MOR1 (Fig. 7a) or DOR1 (Fig. 7c), MSS significantly increased FC POMC mRNA [$F_{(3, 28)} = 4.8$, $p = 0.037$, (Fig. 7f)]. However, a significant MSS by SDS interaction was also observed for POMC expression [$F_{(3, 28)} = 8.11$, $p = 0.008$], and post-hoc tests revealed that all three stress-exposed groups displayed elevated levels of POMC mRNA compared to control animals ($p < 0.05$ by Tukey's, Fig. 7f). Although PENK expression was not affected by MSS or SDS (Fig. 7d), MSS led to a significant up-regulation of PDYN in the PLC [$F_{(3, 28)} = 7.13$, $p = 0.013$, (Fig. 7e)]. In addition, SDS led to a significant increase in KOR1 [$F_{(3, 28)} = 4.89$, $p = 0.035$, (Fig. 7b)] and significant reductions in BDNF [$F_{(3, 28)} = 6.47$, $p = 0.017$, (Fig. 7g)] and CREB mRNA [$F_{(3, 28)} = 8.72$, $p = 0.006$, (Fig. 7h)] in the PLC, but these effects were not modified by MSS.

Amygdala

The stress-induced changes in gene expression in the Amyg were again distinct from those observed in other brain regions. SDS led to several significant main effects on the expression of β-catenin-related genes in the Amyg. For example, SDS significantly increased the levels of Axin2 [$F_{(3, 28)} = 8.38$, $p = 0.007$, (Fig. 8b)] and GSK3β [$F_{(3, 28)} = 5.73$, $p = 0.02$, (Fig. 8d)]. SDS also led to a significant decrease in the expression of DVL1 in the Amyg [$F_{(3, 28)} = 4.53$, $p = 0.042$, (Fig. 8e)]. However, no significant effects of SDS were observed for Axin-1 (Fig. 8a), DVL-2 (Fig. 8f), DVL-3 (Fig. 8g) or β-catenin itself (Fig. 8h). The only β-catenin-related gene for which a significant main effect of MSS was observed in the Amyg was DVL1, the expression of which was reduced by MSS exposure [$F_{(3, 28)} = 6.7$, $p = 0.015$, (Fig. 8 e)].

No significant effects of MSS or SDS on GR (Fig. 9a), MR (Fig. 9b), SGK1 (Fig 9c), CRH (Fig. 9e), or Otx2 (Fig. 9h) were observed in the Amyg, but a trend towards increased GR levels following SDS was observed ($p = 0.052$) (Fig. 9a). SDS was shown to significantly

increase the expression of FKBP5 in the Amyg [$F_{(3, 28)} = 22.23$, $p < 0.0001$], but MSS did not influence this effect (Fig. 9d). While no significant effects on EHMT1 were observed (Fig. 9f), a main effect of SDS [$F_{(3, 28)} = 8.69$, $p = 0.0064$] and a significant MSS by SDS interaction were observed for the expression of EHMT2 in the Amyg [$F_{(3, 28)} = 4.71$, $p = 0.039$]. Specifically, the EHMT2-increasing effects of SDS were potentiated in MSS-exposed animals (~35% increase) compared to controls (~5% increase, see Fig. 9g).

The endogenous opioid system in the Amyg was quite responsive to SDS, with SDS leading to significant increases in the expression of MOR1 [$F_{(3, 28)} = 41.36$, $p < 0.0001$, (Fig. 10a)], KOR1 [$F_{(3, 28)} = 51.4$, $p < 0.0001$, (Fig. 10b)], DOR1 [$F_{(3, 26)} = 64.97$, $p < 0.0001$, (Fig. 10c)], PENK [$F_{(3, 28)} = 16.6$, $p = 0.0003$, (Fig. 10d)], and POMC [$F_{(3, 28)} = 8.04$, $p = 0.008$, (Fig. 10f)]. However, no significant effects of SDS were observed on the expression of PDYN (Fig. 10e), BDNF (Fig. 10g), or CREB (Fig. 10h) in the Amyg. In addition, no significant main effects of MSS and no significant MSS by SDS interactions were observed for CREB, BDNF, POMC, PENK, or PDYN genes (Fig. 10).

Discussion

The current study employs two ethologically and translationally relevant behavioral stress paradigms to investigate the long-term molecular consequences of chronic stress exposure. Given that early life stress has been shown to increase vulnerability to SDS (Der-Avakian and Markou, 2010; Pena et al., 2017; Rosenhauer et al., 2017), this study also provides some insight into the molecular correlates of stress susceptibility. Our results reveal that the molecular alterations induced by combined exposure to MSS+SDS are largely distinct from the ‘hallmark’ molecular adaptations that have been described in other populations of susceptible mice. For example, our data demonstrate that the alterations in protein phosphorylation in the NAc that have been reported to differentiate susceptible from resilient c57BL6/J mice following SDS (Krishnan et al., 2007) do not differentiate MSS+SDS-exposed c57BL6/J mice from animals experiencing SDS alone. Similarly, many of the molecular alterations in β-catenin signaling that have been previously shown to distinguish resilient from vulnerable populations (Wilkinson et al., 2011; Dias et al., 2014; Sachs et al., 2015) were not observed in MSS-exposed animals compared to controls following SDS. Given the numerous procedural differences between these various studies, we do not view the current results as failing to replicate prior findings; rather, we interpret the current results to indicate that the specific molecular adaptations that have been previously associated with stress susceptibility or resilience are simply not generalizable across a wide range of environmental and genetic contexts.

Most of the published studies examining the molecular alterations associated with stress susceptibility have employed a single stress paradigm, SDS, in a single strain of mice, most frequently c57BL6/J. Consequently, many of the ‘hallmark’ gene expression and signal transduction changes that have been implicated in SDS susceptibility have been derived from studies comparing genetically identical animals that differ only with respect to phenotype (Berton et al., 2006; Krishnan et al., 2007). Whether putative genetic and environmental risk factors for psychiatric disease lead to similar molecular alterations has not been established. The current study provided some insight into this question by

evaluating the effects of early life stress on several SDS-induced changes in gene expression and signal transduction. Incorporating a second stress paradigm during a different developmental stage predictably led to multiple changes in gene expression that were not observed in animals exposed to SDS alone, and thus, it is not entirely surprising that the molecular alterations observed comparing SDS-exposed mice to MSS+SDS-exposed mice are unique from those observed comparing socially avoidant mice exposed to SDS alone to mice who remain sociable following SDS. Going forward, it will be important to evaluate genetic influences on MSS and SDS responses by evaluating whether the molecular alterations observed here also occur in other inbred strains of mice or in genetically modified animals that display altered vulnerability to stress.

In addition to the inclusion of MSS, there are other procedural differences that could account for the differing molecular alterations observed in various populations of 'SDS susceptible' animals. For example, the SDS paradigm employed here is distinct from the version that is used by several other groups in that the current paradigm does not involve pair-housing defeated animals in a divided cage with their aggressors. Instead, defeated mice are singly housed between defeat episodes. While single housing is stressful to mice and has been reported to lead to some depression- or anxiety-like phenotypes (Martin and Brown, 2010; Okada et al., 2015; Ieraci et al., 2016), social isolation is likely less stressful than living in continuous sensory contact with aggressors. In addition, the controls in the current study were group housed, whereas some groups report housing their control animals in pairs that are separated by a divider (Golden et al., 2011). Future research will be required to determine which aspects of the stress paradigm (i.e., single housing vs. aggression and social subordination) lead to which molecular and behavioral responses. In addition to differences in housing conditions and the stress paradigm itself, there is also between-lab variability with respect to the timing of behavioral testing. For example, some studies have reported performing SDS and/or behavioral testing during the dark phase (Laman-Maharg et al., 2017), but the current study and many others have performed SDS during the light phase (Krishnan et al., 2007; Sachs et al., 2015). This and other differences in experimental details (e.g., time point of tissue isolation, differences in anatomical location of isolated samples) could also contribute to variability in data between labs.

While the current findings suggest that many of the specific molecular 'hallmarks' of stress susceptibility are not universal, our findings did identify significant dysregulation of many of these previously identified signaling pathways following stress. Overall, of the 72 gene expression analyses that we performed (24 candidate genes in each of three brain regions), 39 yielded statistically significant effects, thus suggesting that the major signaling pathways we chose to investigate on the basis of prior work did in fact become dysregulated following MSS and/or SDS. Future studies aimed at determining the functional significance of these signaling alterations will be required to identify which of these pathways might be effective targets for therapeutic intervention. It will also be important to evaluate which environmental or genetic insults actually lead to dysregulation of these pathways to gain a more comprehensive understanding of the situations in which targeting these pathways might be beneficial. In addition, it will be critical to continue to evaluate molecular changes in multiple brain regions. Indeed, a recent study examining SDS-induced transcriptional changes in multiple brain regions has shown that many SDS-induced alterations in gene

expression are region-specific (Bagot et al., 2016). In keeping with this, the spatial distribution of mRNA changes observed following MSS and SDS in the current study was not uniform across brain regions. For example, while both SDS and MSS significantly impacted the expression of endogenous opioid system genes, SDS primarily impacted these genes in the Amyg and NAc, not the PLC, whereas MSS affected endogenous opioid system genes in the PLC but not the Amyg or NAc. It is also important to note that stress led to opposing effects on the expression of some genes depending on the brain region. For example, SDS increased POMC expression in the Amyg but decreased its expression in the NAc, a finding that could potentially have important implications for how this system could be targeted for therapeutic purposes.

In addition to examining multiple pathways in multiple brain regions following a variety of different stressors, it will also be critical to evaluate different time points following stress exposure. The current data examined only a single time point following MSS and/or SDS exposure, but it is likely that different results would be obtained depending on the age of the animals and how recently they experienced stress. Indeed, prior research has suggested that Otx2-dependent alterations in transcription in the VTA underlie MSS-induced increases in SDS susceptibility (Pena et al., 2017). However, Pena and colleagues reported no significant effects of MSS on the expression of Otx2 in the VTA of adult mice (Pena et al., 2017). Rather, the Otx2-dependent long-term transcriptional alterations induced by MSS were shown to stem from transient MSS-induced down-regulation of Otx2 that recovers by adulthood (Pena et al., 2017). Future research will be required to determine whether transient alterations in the expression of other genes in response to MSS in the NAc, Amyg, or PLC play an important role in long-term changes in behavior following stress.

Of all the significant effects of MSS and SDS that were observed in the current study, only two (MSS- and SDS-induced decreases in DVL-1 in the Amyg and MSS- and SDS-induced increases in GSK3β in the NAc) were additive in the sense that both stressors exhibited qualitatively similar significant main effects on the same gene in the same brain region. Similarly, MSS and SDS had directly opposing effects on gene expression for only two of the genes examined (DVL-3 and SGK1 in the PLC). In contrast to our initial expectations, no synergistic effects were observed. Of course, it is possible that synergistic effects do occur for genes other than those examined here, but future research will be required to identify which genes, if any, exhibit supra-additive responses to the combination of these stressors. Finally, a total of four significant MSS by SDS interactions were observed (KOR1 and PDYN in the NAc, POMC in the PLC, and EHMT2 in the Amyg) in which MSS significantly impacted transcriptional responses to SDS. Taken together, our results suggest that the influence of MSS on SDS responses is likely mediated through complex mechanisms that involve directly additive, antagonistic and interactive effects on gene expression. In addition to these overlapping effects on gene expression, the independent targeting of different genes within the same pathways (or independent targeting of entirely different pathways) by MSS and SDS could provide another mechanism through which these stressors could coordinately affect brain function and behavior.

Overall, the results from the current study are consistent with the idea that various risk factors for stress susceptibility impact brain function and stress responses through distinct

molecular mechanisms. Indeed, the current examination of MSS-exposed animals revealed little evidence of most of the specific transcriptional and signaling alterations that have been previously identified in other 'SDS-susceptible' populations (Krishnan et al., 2007; Wilkinson et al., 2011). However, the fact that both MSS and SDS did lead to significant dysregulation of the histone methylation, endogenous opioid, BDNF-CREB, β-catenin, and glucocorticoid pathways does highlight the potential importance of these pathways in the regulation of stress responses. Continuing to elucidate the precise molecular mechanisms through which various risk factors for mental illness impact the brain may provide new insight into the etiology of mental illness and may allow for the development of rational targets for therapeutic intervention.

Acknowledgments

We would like to thank Wendy Roberts for excellent technical assistance and our funding sources for providing the means to do this work.

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Abbreviations

Amyg	amygdala
BDNF	brain derived neurotrophic factor
CREB	cAMP-response element binding protein
CRH	corticotropin-releasing hormone
DOR1	delta opioid receptor 1
DVL	disheveled
FKBP5	FK506 Binding Protein 5
GR	glucocorticoid receptor
KOR1	kappa opioid receptor 1
MSS	maternal separation stress
MR	mineralocorticoid receptor
MOR1	mu opioid receptor 1
NAc	nucleus accumbens
Otx2	orthodenticle homeobox 2
PNDs	post-natal days
PLC	prelimbic cortex
PENK	proenkephalin

PDYN	prodynorphin
POMC	pro-opiomelanocortin
SGK1	serum/glucocorticoid-regulated kinase 1
SDS	social defeat stress
VTA	ventral tegmental area

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Highlights

- Endogenous opioid signaling is significantly altered by repeated stress exposure.
- SDS and MSS both lead to dysregulation of β-catenin signaling.
- SDS and MSS lead to both additive and antagonistic effects on gene expression.
- Transcriptional stress responses are highly brain region specific.

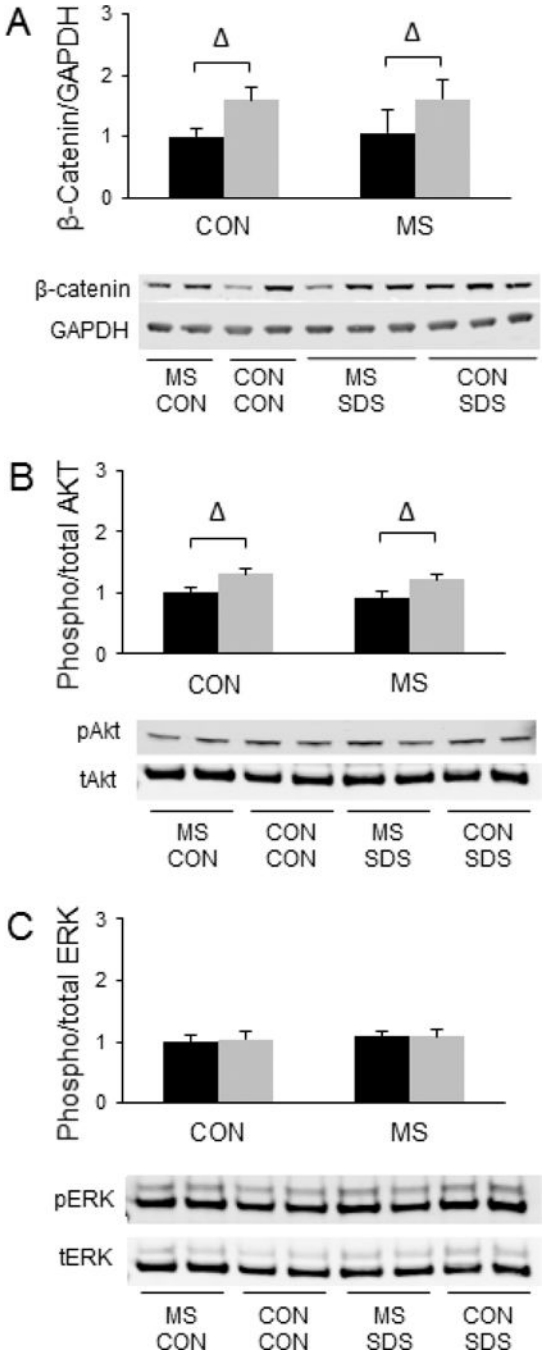

Figure 1.

Combined effects of maternal separation (MS) stress and social defeat stress (SDS) on β-catenin levels in the nucleus accumbens compared to control (Con) animals. (A) Quantification and representative images for Western blot analysis of β-catenin/GAPDH levels in the nucleus accumbens. (B) Quantification and representative images for Western blot analysis of phosphorylated Akt/total Akt levels in the nucleus accumbens. (C) Quantification and representative images for Western blot analysis of phosphorylated ERK2/total ERK2 levels in the nucleus accumbens. Black bars show data from control mice not

subjected to SDS; grey bars show data from mice exposed to SDS. Δ indicates significant main effect of SDS, $p < 0.05$. N = 10 per group.

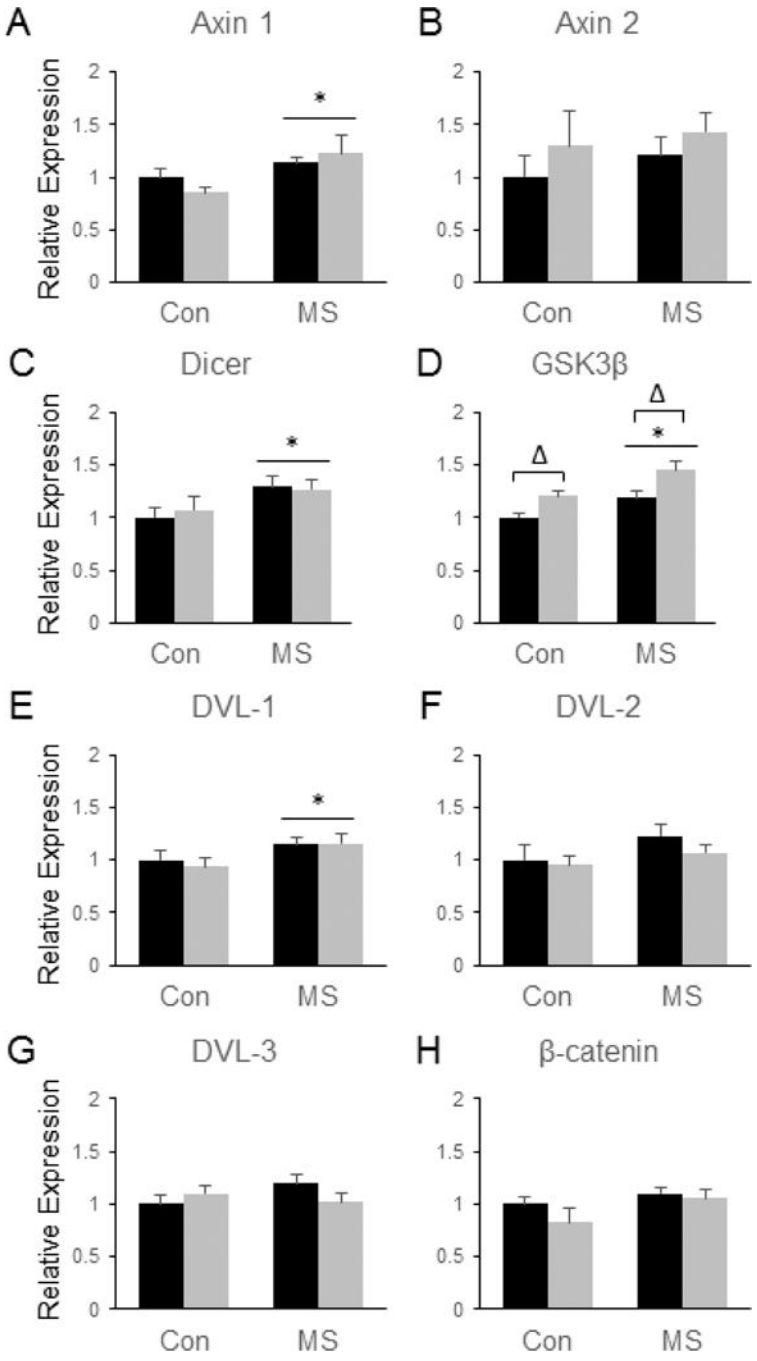

Figure 2.

Combined effects of maternal separation (MS) stress and social defeat stress (SDS) on the transcription of β-catenin-related genes in the nucleus accumbens compared to control (Con) animals. Quantification of real-time PCR data using primers specific for Axin 1 (A), Axin 2 (B), Dicer (C), GSK3β (D), DVL-1 (E), DVL-2 (F), DVL-3 (G), and β-catenin (H). Black bars show data from control mice not subjected to SDS; grey bars show data from mice exposed to SDS. Δ indicates significant main effect of SDS, * indicates a significant main effect of MS. N = 8 mice per group.

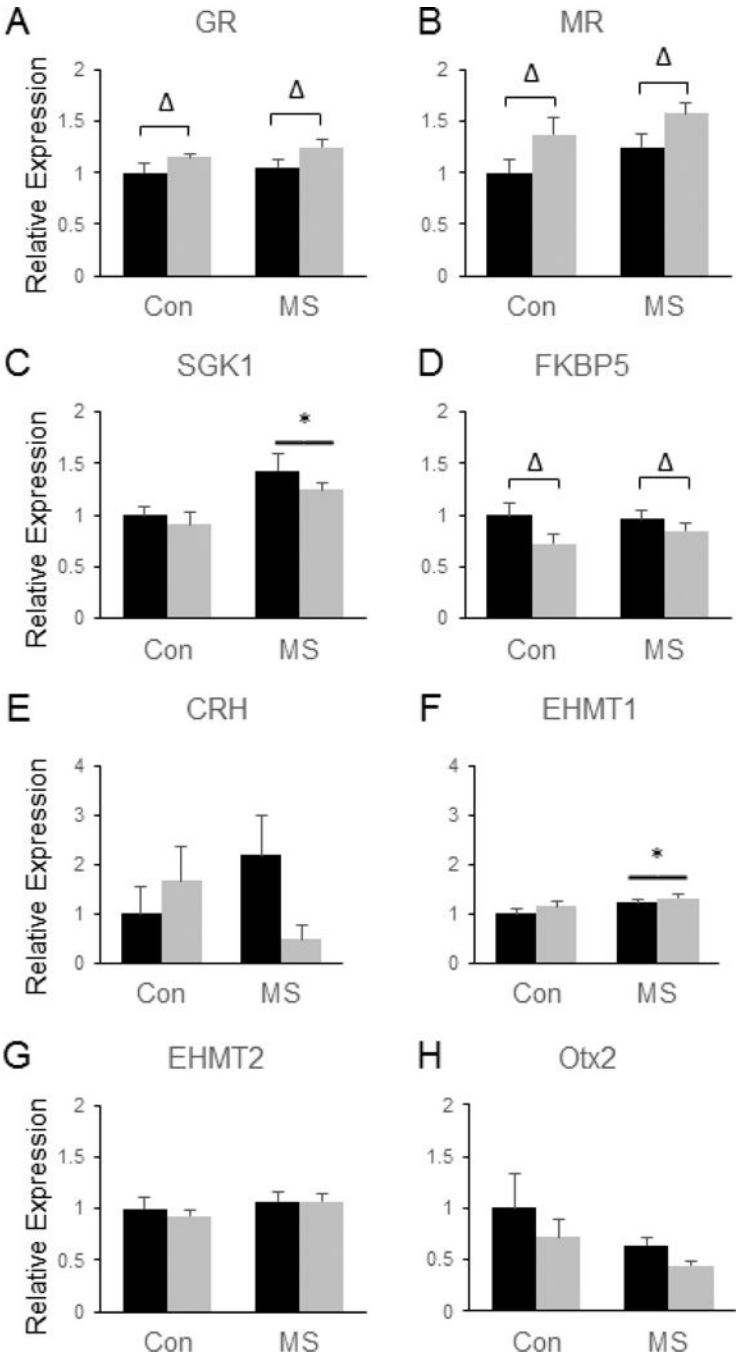

Figure 3.

Combined effects of maternal separation (MS) stress and social defeat stress (SDS) on the transcription of genes involved in glucocorticoid signaling and histone methylation in the nucleus accumbens compared to control (Con) animals. Quantification of real-time PCR data using primers specific for GR (A), MR (B), SGK1 (C), FKBP5 (D), CRH (E), EHMT1 (F), EHMT2 (G) and Otx2 (H). Black bars show data from control mice not subjected to SDS; grey bars show data from mice exposed to SDS. Δ indicates significant main effect of

SDS, * indicates a significant main effect of MS by two way ANOVA. N = 8 mice per group.

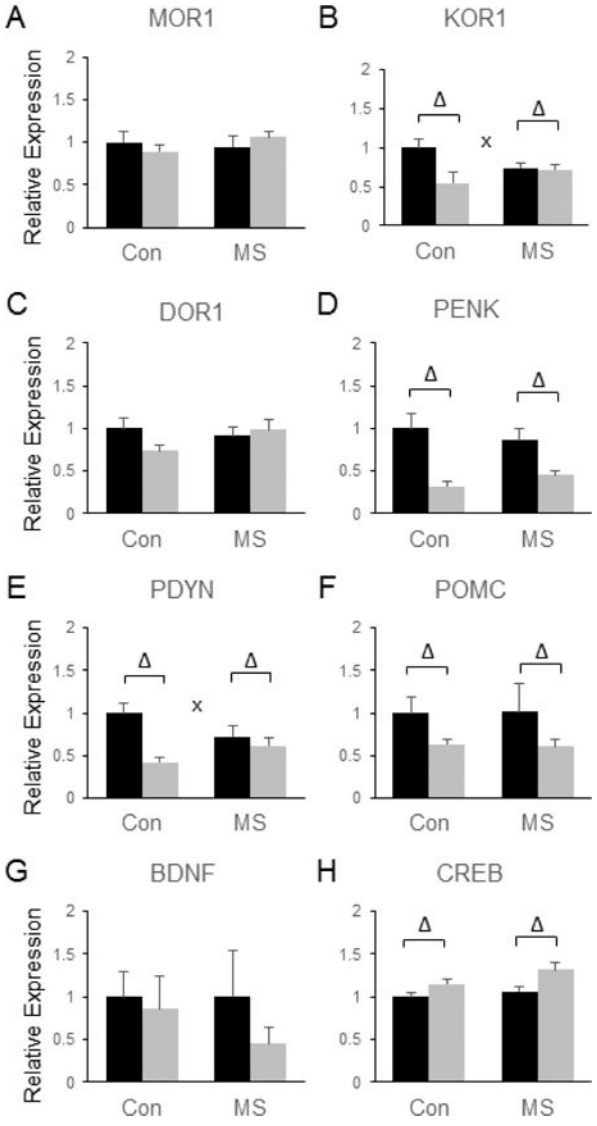

Figure 4.

Combined effects of maternal separation (MS) stress and social defeat stress (SDS) on the transcription of genes involved in endorphin and neurotrophin signaling in the nucleus accumbens compared to control (Con) animals. Quantification of real-time PCR data using primers specific for MOR1 (A), KOR1 (B), DOR1 (C), PENK (D), PDYN (E), POMC (F), BDNF (G), and CREB (H). Black bars show data from control mice not subjected to SDS; grey bars show data from mice exposed to SDS. Δ indicates significant main effect of SDS, * indicates a significant main effect of MS, 'x' denotes significant MS by SDS interaction by two way ANOVA. N = 8 mice per group.

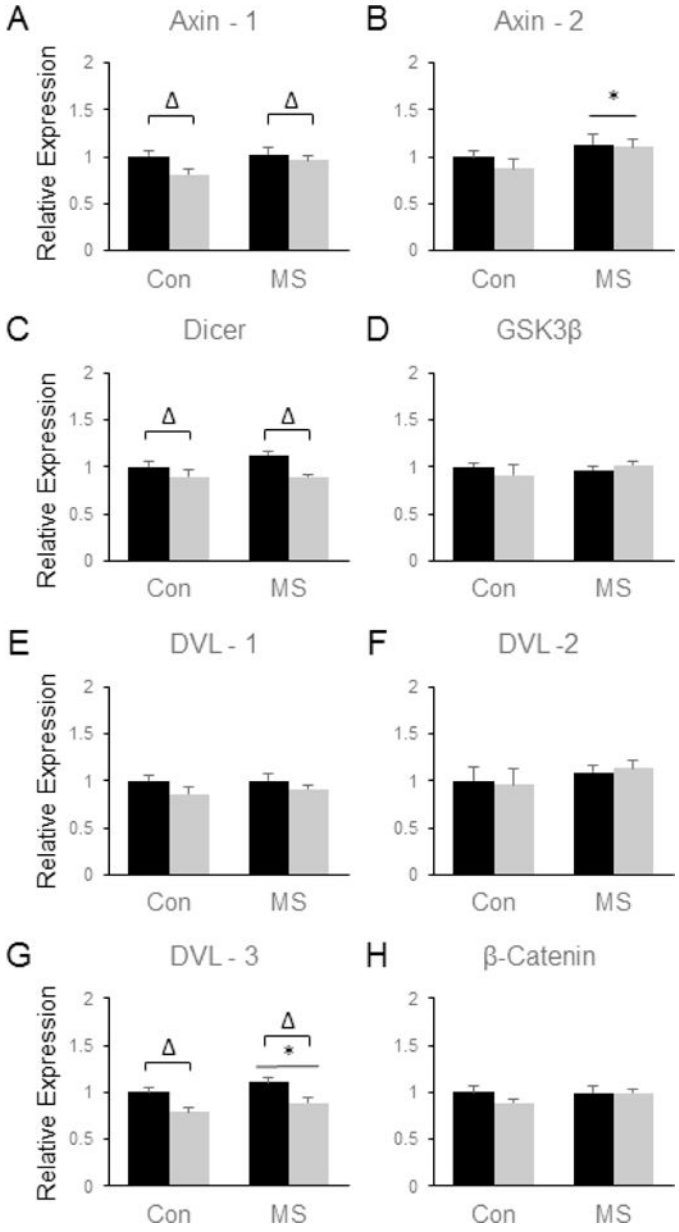

Figure 5.

Combined effects of maternal separation (MS) stress and social defeat stress (SDS) on the transcription of β-catenin-related genes in the prelimbic/infralimbic cortex compared to control (Con) animals. Quantification of real-time PCR data using primers specific for Axin 1 (A), Axin 2 (B), Dicer (C), GSK3β (D), DVL-1 (E), DVL-2 (F), DVL-3 (G), and β-catenin (H). Black bars show data from control mice not subjected to SDS; grey bars show data from mice exposed to SDS. Δ indicates significant main effect of SDS, * indicates a significant main effect of MS, 'x' denotes significant MS by SDS interaction by two way ANOVA. N = 8 mice per group.

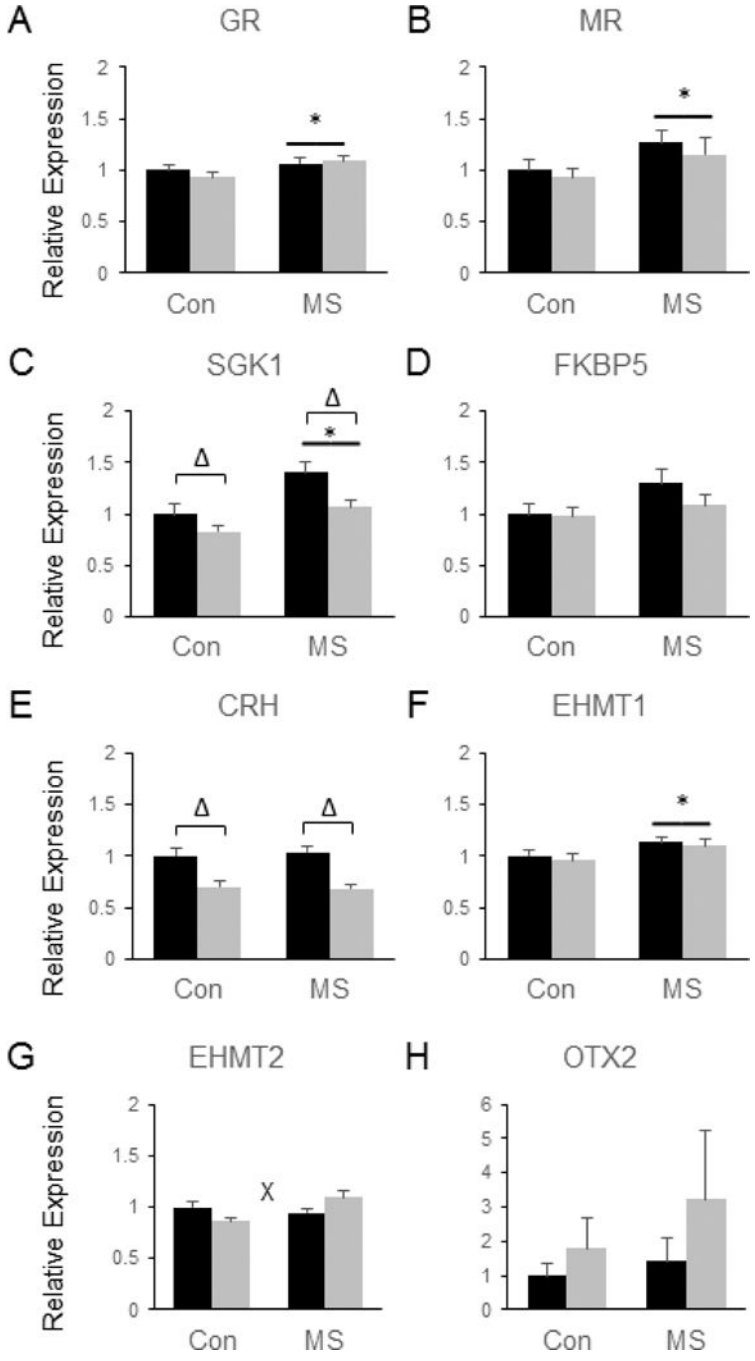

Figure 6.

Combined effects of maternal separation (MS) stress and social defeat stress (SDS) on the transcription of genes involved in glucocorticoid signaling and histone methylation in the prelimbic/infralimbic cortex compared to control (Con) animals. Quantification of real-time PCR data using primers specific for GR (A), MR (B), SGK1 (C), FKBP5 (D), CRH (E), EHMT1 (F), EHMT2 (G), and Otx2 (H). Black bars show data from control mice not subjected to SDS; grey bars show data from mice exposed to SDS. Δ indicates significant

main effect of SDS, * indicates a significant main effect of MS, ‘x’ denotes significant MS by SDS interaction by two way ANOVA. N = 8 mice per group.

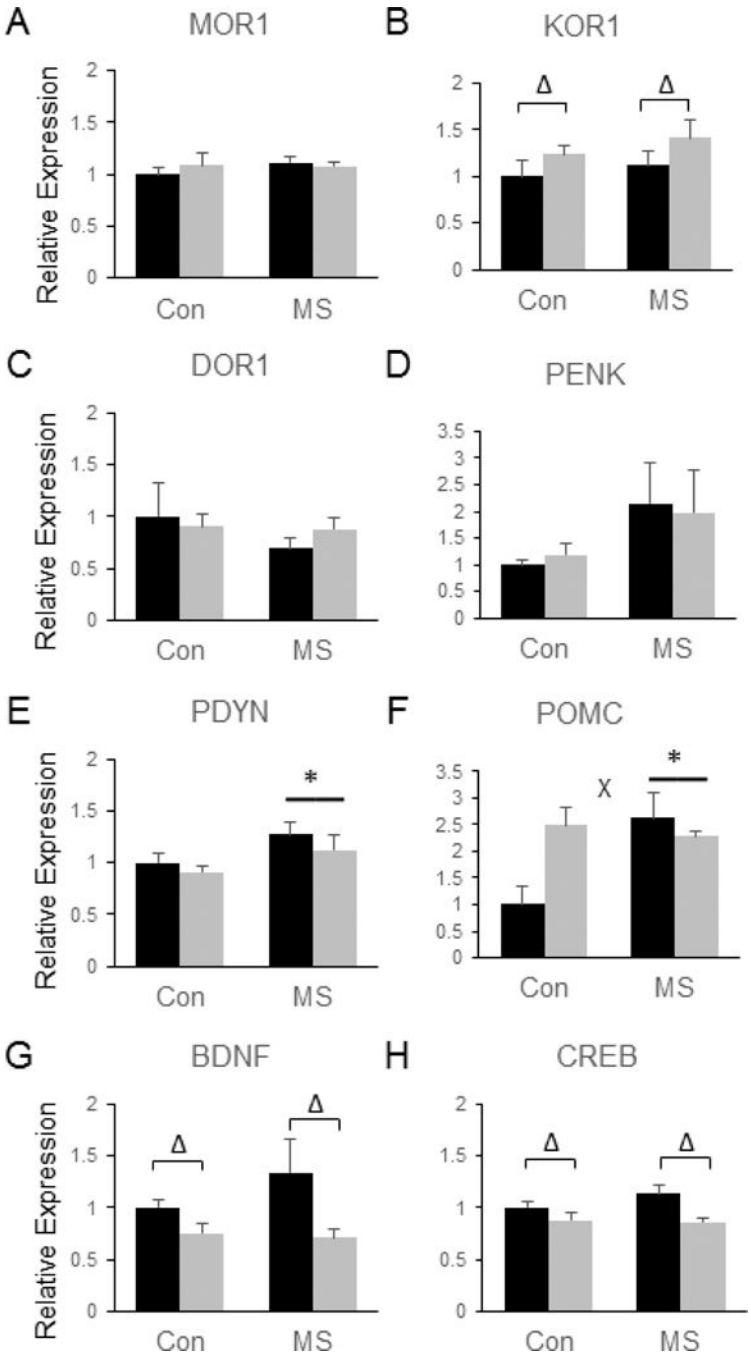

Figure 7.

Combined effects of maternal separation (MS) stress and social defeat stress (SDS) on the transcription of genes involved in endorphin and neurotrophin signaling in the prelimbic/infralimbic cortex compared to control (Con) animals. Quantification of real-time PCR data using primers specific for MOR1 (A), KOR1 (B), DOR1 (C), PENK (D), PDYN (E), POMC (F), BDNF (G), and CREB (H). Black bars show data from control mice not subjected to SDS; grey bars show data from mice exposed to SDS. Δ indicates significant main effect of

SDS, * indicates a significant main effect of MS, ‘x’ denotes significant MS by SDS interaction by two way ANOVA. N = 8 mice per group.

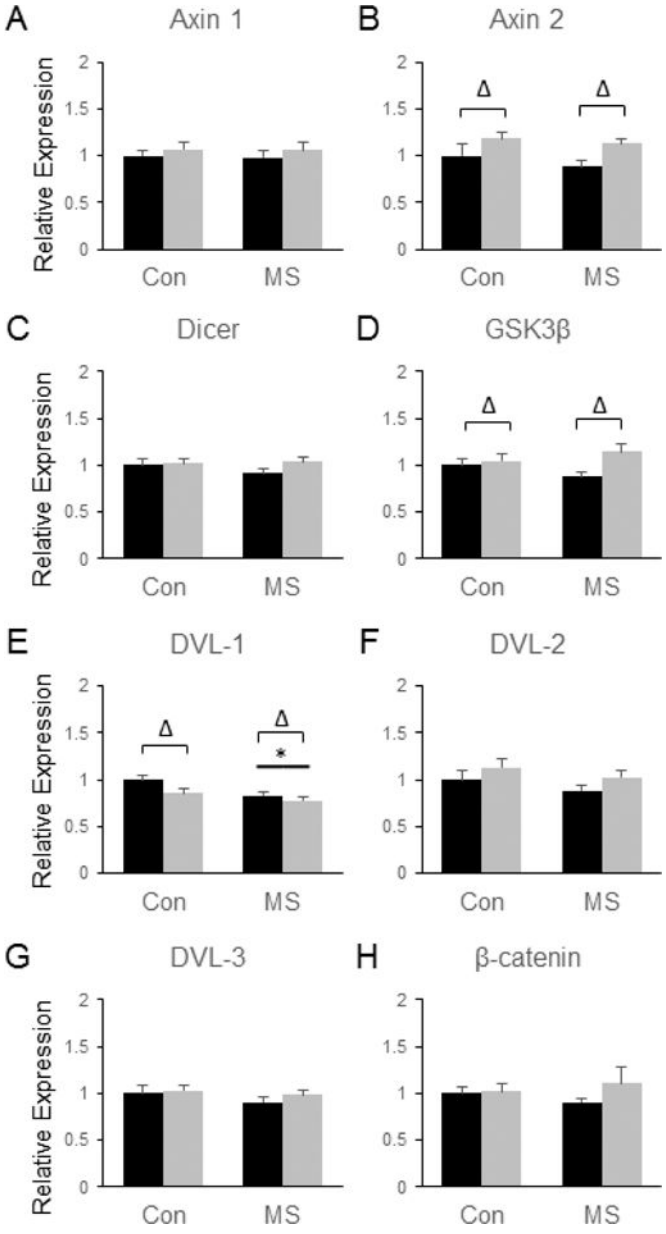

Figure 8.

Combined effects of maternal separation (MS) stress and social defeat stress (SDS) on the transcription of β-catenin-related genes in the amygdala compared to control (Con) animals. Quantification of real-time PCR data using primers specific for Axin 1 (A), Axin 2 (B), Dicer (C), GSK3β (D), DVL-1 (E), DVL-2 (F), DVL-3 (G), and β-catenin (H). Black bars show data from control mice not subjected to SDS; grey bars show data from mice exposed to SDS. Δ indicates significant main effect of SDS, * indicates a significant main effect of MS, 'x' denotes significant MS by SDS interaction by two way ANOVA. N = 8 mice per group.

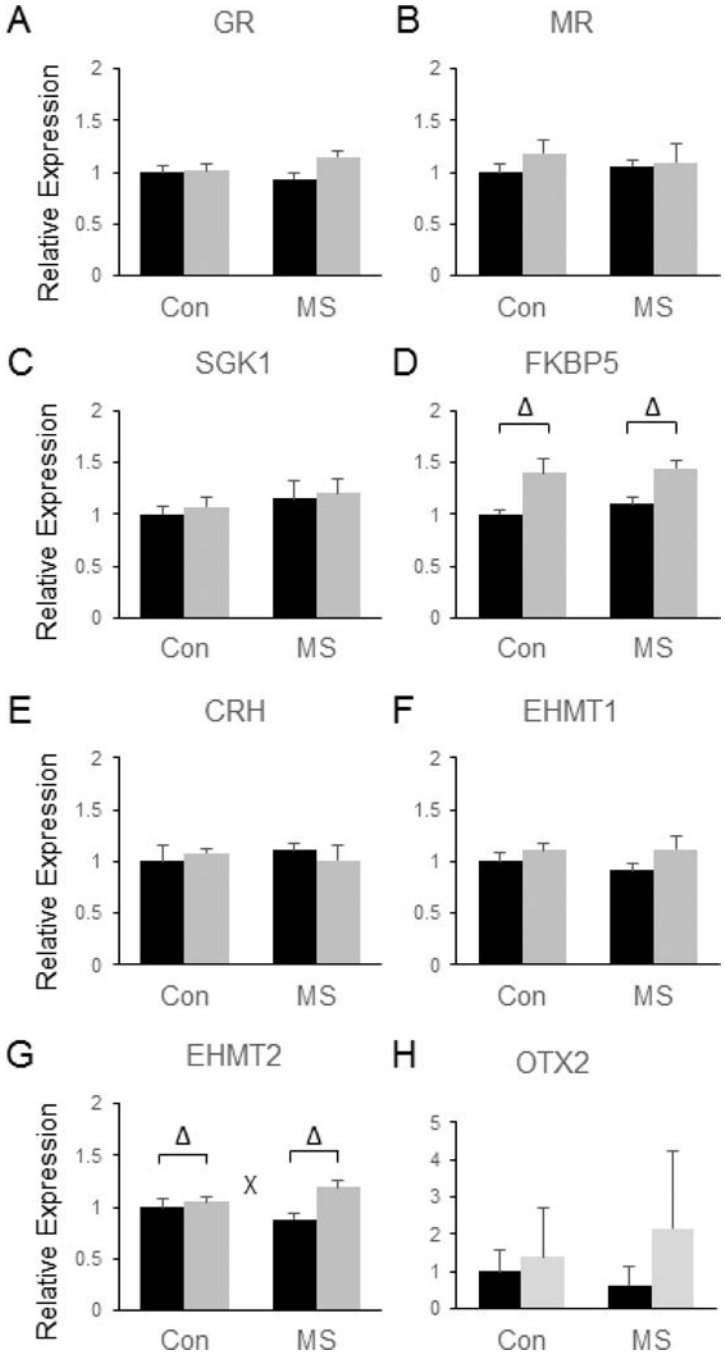

Figure 9.

Combined effects of maternal separation (MS) stress and social defeat stress (SDS) on the transcription of genes involved in glucocorticoid signaling and histone methylation in the amygdala compared to control (Con) animals. Quantification of real-time PCR data using primers specific for GR (A), MR (B), SGK1 (C), FKBP5 (D), CRH (E), EHMT1 (F), EHMT2 (G), and Otx2 (H). Black bars show data from control mice not subjected to SDS; grey bars show data from mice exposed to SDS. Δ indicates significant main effect of SDS,

* indicates a significant main effect of MS, ‘x’ denotes significant MS by SDS interaction by two way ANOVA. N = 8 mice per group.

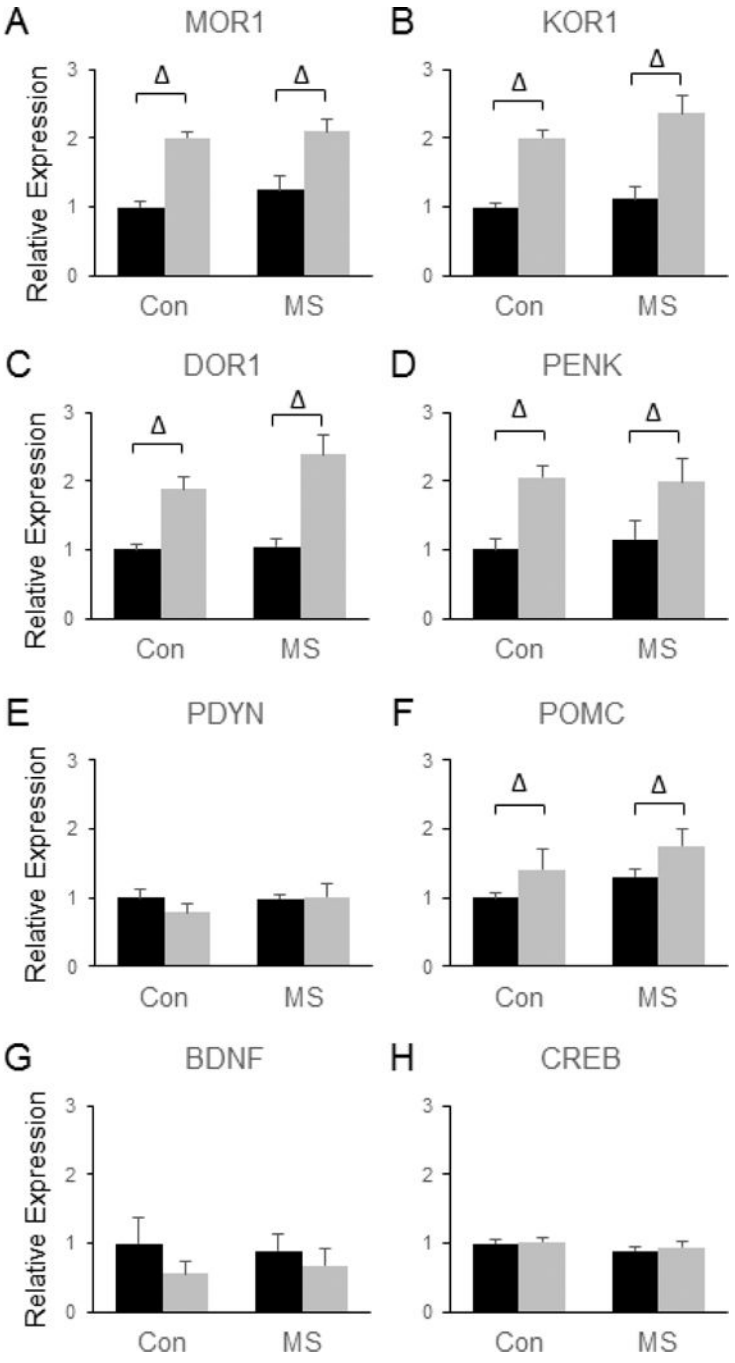

Figure 10.

Combined effects of maternal separation stress and social defeat stress on the transcription of genes involved in endorphin and neurotrophin signaling in the amygdala. Quantification of real-time PCR data using primers specific for MOR1 (A), KOR1 (B), DOR1 (C), PENK (D), PDYN (E), POMC (F), BDNF (G), and CREB (H). Black bars show data from control mice not subjected to SDS; grey bars show data from mice exposed to SDS. Δ indicates

significant main effect of SDS, * indicates a significant main effect of MSS, ‘x’ denotes significant MSS by SDS interaction by two way ANOVA. N = 8 mice per group.

Gene Name	Forward Primer	Reverse Primer
Axin 1	CCACCACCATGTTCACCATA	TGGCATGACCATGTGTTTCT
Axin 2	AAC CTA TGC CCG TTT CCT CTA	GAG TGT AAA GAC TTG GTC CAC C
BDNF	CAA TGC CGA ACT ACC CAA	AAC ATA AAT CCA CTA TCT TCC CC
beta catenin	TATTGACGGGCAGTATGCAA	CCCTCATCTAGCGTCTCAGG
CREB	AGC CGG GTA CTA CCA TTC TAC	GCA GCT TGA ACA ACA ACT TGG
CRH	CCT CAG CCG GTT CTG ATC C	GCG GAA AAA GTT AGC CGC AG
Delta Opioid Receptor 1	GCTGGTGGACATCAATCGG	GCGTAGAGAACCGGGTTGAG
Dicer	GCCAAGAAAATACCAGGTTGAGC	GCGATGAACGTCTTCCCTGAG
DVL1	AGT GGA GCC TCA GAT CAG GA	GGT CCT GGG TAC TGG TAG GG
DVL2	TGA CAA TGA CGG TTC CAG TG	GCG CTG GAT ACT GGT AGG AG
DVL3	CTA CAC GCA GCA GTC TGA GG	CAT AGC TTG GGT GTG TGT GG
Ehmt 1	TAAAACAGAGGACGGTGATTGAG	AGGGCACTATCATCTAAGGCTT
FKBP5	TTTGAAGATTCAGGCGTTATCCG	GGTGGACTTTTACCGTTGCTC
Ehmt 2 (G9a)	CGAGCCCGGAAAACCATGT	TCATGCGGAAATGCTGGACTT
GAPDH	CAT GTT CCA GTA TGA CTC CAC TC	GGC CTC ACC CCA TTT GAT GT
GR	AGC TCC CCC TGG TAG AGA C	GGT GAA GAC GCA GAA ACC TTG
GSK3b	ACAGGCCACAGGAGGTCAGT	GATGGCAACCAGTTCTCCAG
Kappa Opioid Receptor 1	GAATCCGACAGTAATGGCAGTG	GACAGCGGTGATGATAACAGG
MR	GAA AGG CGC TGG AGT CAA GT	TGT TCG GAG TAG CAC CGG AA
Mu opioid receptor 1	CCAGGGAACATCAGCGACTG	CATGGGTCGGACTGGTTGC
Otx2	TATCTAAAGCAACCGCCTTACG	AAGTCCATACCCGAAGTGGTC
POMC	ATGCCGAGATTCTGCTACAGT	CCACACATCTATGGAGGTCTGAA
Prodynorphin	AGGTTGCTTTGGAAGAAGGCT	GACGCTGGTAAGGAGTCGG
Proenkephalin	GAGAGCACCAACAATGACGAA	TCTTCTGGTAGTCCATCCACC
SGK1	TCCGCCAAGTCCCTCTCAACAAAT	TGCCTAGCCAGAAGAACCTTTCCA