

# Stressor-Specific Regulation of Distinct Brain-Derived Neurotrophic Factor Transcripts and Cyclic AMP Response Element-Binding Protein Expression in the Postnatal and Adult Rat Hippocampus

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Stress regulation of brain-derived neurotrophic factor (BDNF) is implicated in the hippocampal damage observed in depression. BDNF has a complex gene structure with four 5' untranslated exons (I–IV) with unique promoters, and a common 3' coding exon (V). To better understand the stress regulation of BDNF, we addressed whether distinct stressors differentially regulate exon-specific BDNF transcripts in the postnatal and adult hippocampus. The early life stress of maternal separation (MS) resulted in a time point-dependent differential upregulation of BDNF transcripts restricted to early postnatal life (P14-BDNF II, P21-BDNF IV, V). In adulthood, distinct stressors regulated BDNF transcripts in a signature manner. Immobilization stress, administered once, decreased all BDNF splice variants but had differing effects on BDNF I/II (increase) and III/IV (decrease) when administered chronically. Although immobilization stress reduced BDNF (V) mRNA, chronic unpredictable stress did not influence total BDNF despite altering specific BDNF transcripts. Furthermore, a prior history of MS altered the signature pattern in which adult-onset stress regulated specific BDNF transcripts. We also examined the expression of cyclic AMP response element-binding protein (CREB), an upstream transcriptional activator of BDNF, and observed a CREB induction in the postnatal hippocampus following MS. As a possible consequence of enhanced CREB and BDNF expression following MS, we examined hippocampal progenitor proliferation and observed a significant increase restricted to early life. These results suggest that alterations in CREB/BDNF may contribute to the generation of individual differences in stress neurocircuitry, providing a substrate for altered vulnerability to depressive disorders.

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## INTRODUCTION

Building evidence suggests that stress-induced hippocampal damage may play a vital role in the etiology of depressive disorders (McEwen, 2004). Patients suffering from major depression or post-traumatic stress disorder have been reported to exhibit hippocampal volumetric loss (Bremner *et al*, 2000; Smith, 2005). In animal models, sustained exposure to stress is known to induce dendritic atrophy within the hippocampal CA subfields (Magarinos *et al*, 1996; Vyas *et al*, 2002) and to decrease neurogenesis in the

dentate gyrus (DG) (Mirescu and Gould, 2006; Pham *et al*, 2003). The form and extent of stress-induced hippocampal damage is thought to depend upon the timing, type, duration, and frequency of the stressor (Pacak and Palkovits, 2001; Radley and Morrison, 2005). In addition, stress responses are governed by an individual's prior history of stress exposure. Indeed, early life adverse experience is known to alter adult responses to stress (Ladd *et al*, 2000), thus contributing to the generation of individual differences in vulnerability, not only to stress but also to stress-related psychopathology (Heim and Nemeroff, 2002).

Although it is evident that sustained stress, both in early life and adulthood, can adversely affect hippocampal structure and function (Brunson *et al*, 2003; Buwalda *et al*, 2005; Magarinos *et al* 1996; Mirescu and Gould, 2006; Vyas *et al*, 2002), the molecular underpinnings for this remain unclear. Decreased expression of the neuro-

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trophin brain-derived neurotrophic factor (BDNF) has been implicated in stress-induced hippocampal damage and dysfunction (Duman, 2004; Gomez-Pinilla and Vaynman, 2005). BDNF, which is expressed at the highest levels in the hippocampus, plays a vital role in hippocampal development and continues to shape adult hippocampal structure and function (Branchi *et al*, 2004; Lu and Gottschalk, 2000). The rat BDNF gene through the alternate splicing of four distinct 5' exons (I–IV) to a common 3' exon (V), and by the use of two separate polyadenylation sites, can generate eight unique BDNF transcripts (Timmusk *et al*, 1993). The 5' exons, each with their unique promoter, remain untranslated, with only the common 3' coding exon (V) generating the mature BDNF protein. The complex gene structure of BDNF appears to permit the distinct BDNF promoters to be differentially recruited to generate both a region-specific basal expression (Timmusk *et al*, 1993) and a stimulus-evoked regulation during development and in adulthood (Lauterborn *et al*, 1996; Sathanoori *et al*, 2004). Multiple BDNF transcripts are known to be differentially regulated in response to distinct stimuli such as activity, exercise and antidepressant administration (Dias *et al*, 2003; Russo-Neustadt *et al*, 2000; Timmusk *et al*, 1995). Thus far, the stress-dependent regulation of distinct BDNF transcripts is poorly understood. Given the complexity of the BDNF gene, little is known about the transcription factors that regulate exon-specific BDNF promoters to influence gene expression. However, several studies both *in vivo* and *in vitro* implicate the transcription factor cyclic AMP response element-binding protein (CREB) in contributing to the regulation of BDNF expression (Barco *et al*, 2005; Conti *et al*, 2002). In addition, specific stressors in adult life have been reported to regulate hippocampal CREB expression (Alfonso *et al*, 2006; Song *et al*, 2006) and a decline in CREB expression has been observed in depressed patients (Blendy, 2006; Lai *et al*, 2003; Yamada *et al*, 2003). Besides its role in the regulation of BDNF, CREB has been shown to influence both structural and synaptic plasticity in the hippocampus (Josselyn and Nguyen, 2005; Nakagawa *et al*, 2002). Given the crucial role that BDNF and its upstream transcriptional activator CREB have been suggested to play in stress-related hippocampal damage (Blendy, 2006; Duman, 2004), understanding their regulation by distinct stressors in postnatal and adult life is critical.

We hypothesized that the stress regulation of BDNF splice variants, as well as CREB expression, may be dependent upon the timing of stress exposure during the lifespan of an animal, the nature of the stressor, its duration, and frequency. In the present study, we have analyzed the influence of early life stress and of distinct adult-onset stressors on the expression of multiple BDNF transcripts and CREB mRNA in the hippocampus. In addition, we have also addressed whether a history of early life maternal separation (MS) substantially alters the pattern of regulation of specific BDNF splice variants and CREB mRNA following adult-onset stress. Our results raise the intriguing possibility that stressor-specific differences in the regulation of BDNF and CREB, as well as influences on these modulators by early life experience, may contribute to the generation of individual differences in hippocampal vulnerability to stress.

## MATERIALS AND METHODS

### Animals

Sprague–Dawley rats bred in our animal facility were used for all experiments. The animals were group-housed and maintained on a 12 h light/dark cycle (lights on at 0700 hours) with *ad libitum* access to food and water. All experimental procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the TIFR Institutional Animal Ethics Committee.

### Adult Stress Paradigms

The stress paradigms, both acute and chronic, were performed on adult male animals of postnatal day 60 (P60). As previously described (Nibuya *et al*, 1999), acute immobilization stress ( $n = 10/\text{group}$ ) involved placing the animals in plastic restraint stress cone bags (Harvard Apparatus) for 2 h on a single day, and killing them at the end of this 2-h stress period. Animals subjected to chronic immobilization stress paradigm ( $n = 5/\text{group}$ ) received the immobilization stress 2 h daily for a 10 day period, and were killed at the end of the stress period on the final day. In the chronic unpredictable stress (CUS) experiment ( $n = 7\text{--}8/\text{group}$ ), adult males were subjected to two stressors daily for a period of 10 days (Ortiz *et al*, 1996), and killed at the end of the final stressor. The type, order, and time of administration of the stressors were randomly generated, justifying the unpredictable nature of this stress paradigm. The stressors were cage rocking, swim stress, lights on overnight, cold isolation, lights off for 3 h during the light period of the light/dark cycle, overnight social isolation, food and water deprivation overnight, and immobilization for 1 h. Control animals were handled and left in the home cage.

### Maternal Separation

For the MS experiments, pregnant dams were individually housed and randomly allocated to experimental or control groups. Litters in the MS groups were removed from the home cage for a 3-h period (0900 hours–1200 hours), and placed in a beaker lined with soft bedding. The beakers were placed on a euthermic pad maintained at 37°C, and were kept in an isolated room. The dam was placed in a fresh cage for the duration of the separation. After termination of the 3 h separation, the pups were returned to the home cage, followed by the dam. Ac MS involved separating the litters on P7 for 3 or 6 h, and killing the pups at the end of the separation period ( $n = 4/\text{group}$ ). Each MS group had its own control litter. To examine the influence of chronic MS (Chr MS), litters were separated for 3 h daily from P2 to P14, and killed at different time points following the end of the MS on P14. The time points for killing of the litters were: P14 ( $n = 4\text{--}5/\text{group}$ ) (immediately following the end of the 3 h separation period on P14) and P21 ( $n = 3$  for ctrl and 5 for MS) (7 days following the termination of the Chr MS period). Separate age-matched litters served as controls for the two MS groups in the time point experiment. To study the interaction of Chr MS during early life and adult exposure to acute or chronic immobilization stress, two

experiments were performed in which litters were first randomly allocated to one of four cohorts: control, acute/chronic immobilization stress, Chr MS, and Chr MS + acute/chronic immobilization stress ( $n = 4\text{--}5/\text{group}$ ). Although the first two litters were left undisturbed in early life, the Chr MS and Chr MS + acute/chronic immobilization stress litters were subjected to MS from P2 to P14 as described earlier. All animals were weaned at P21, and males from a single litter were group-housed. The animals were left undisturbed until P60, when males of the acute/chronic immobilization stress and Chr MS + acute/chronic immobilization stress groups were subjected to a 2-h immobilization stress for a single day (acute immobilization stress) or daily for 10 days (chronic immobilization stress). Animals from the control and Chr MS groups were left undisturbed until the time of killing. All groups were killed at the end of the final 2-h stress period.

### Behavioral Tests

MS animals were put through the novelty suppressed feeding (NSF) test in adulthood to examine anxiety behavior (Santarelli *et al*, 2003). The NSF test was performed in a black plexiglass chamber ( $60 \times 40 \times 30$  cm) for a period of 5 min per animal. All animals, both control and MS were deprived of food, but not water, for 36 h before testing. Animals were placed into the corner of the NSF chamber, which had two food pellets placed on a white platform at the center of the chamber. The platform was well lit, whereas the rest of the test chamber was maintained in the dark. The latency to approach was measured as the time taken from placement in the chamber till the animal chewed on the pellet. Latency scores of 300 s were assigned to animals that had not eaten by the end of 5 min when the test was considered terminated. Immediately after the test the animals were returned to their cages and the amount of food eaten over 2 h was measured (home cage consumption). Home cage consumption was the same in control as well as MS animals (Control =  $14 \pm 0.58$  g, MS =  $14.5 \pm 0.96$  g).

### In Situ Hybridization

All animals were killed by rapid decapitation, the brains removed and frozen on dry ice, before storage at  $-70^\circ\text{C}$ . Sections ( $14\text{-}\mu\text{m}$ -thick) were cut on a cryostat and thaw mounted onto ribonuclease free Probe-on plus slides (Electron Microscopy Services, USA). The slides were fixed, acetylated and dehydrated, and stored at  $-70^\circ\text{C}$ . *In situ* hybridization was carried out as described previously (Dias *et al*, 2003). In brief, rat exon-specific BDNF riboprobes were transcribed using  $^{35}\text{S}$ -labeled UTP (Amersham, Buckinghamshire, UK) from transcription competent plasmids provided by Dr Lauterborn (University of California, Irvine, exons I–IV) and Dr Rattray (Kings College, London, exon V). The CREB antisense riboprobes were generated from a transcription competent plasmid as described previously (Nibuya *et al*, 1996). Slides were incubated with hybridization buffer containing the  $^{35}\text{S}$ -labeled riboprobes ( $1 \times 10^6$  c.p.m./slide) for 20 h at  $60^\circ\text{C}$ . The slides were then treated with Ribonuclease A (RNase A,  $40\text{ }\mu\text{g}/\text{ml}$ ), followed by stringent washes in decreasing concentrations of SSC. Slides were air dried and exposed to Hyperfilm  $\beta$ -max

(Amersham, UK) for 2–6 weeks. RNase A pretreatment ( $40\text{ mg}/\text{ml}$  at  $37^\circ\text{C}$  for 30 min) and competition with excess cold antisense riboprobes for the different BDNF exons or CREB did not yield significant hybridization (data not shown), confirming their specificity.

### BrdU Immunohistochemistry

Neurogenesis in maternally separated animals was studied using bromodeoxyuridine (BrdU; Sigma-Aldrich, USA) as a mitotic marker to label proliferating hippocampal progenitors. Litters were randomly allocated to control and Chr MS groups, the latter being subjected to MS from P2 to P14 as described previously. The animals were left undisturbed until P15, P21, or P60, when both control and MS animals were injected with BrdU ( $100\text{ mg}/\text{kg}$ ) and killed 2 h later ( $n = 4\text{--}6/\text{group}$ ). Animals were perfused with saline followed by 4% paraformaldehyde, and their brains were removed and allowed to sink in 30% sucrose. The brains were cut on a sliding microtome (Leica) and  $50\text{ }\mu\text{m}$  thick sections were collected.

To detect BrdU-positive hippocampal progenitors, every fifth section of the hippocampus was selected and BrdU immunohistochemistry was performed (Nakagawa *et al*, 2002). In brief, following DNA denaturation and acid hydrolysis, sections were incubated overnight with mouse anti-BrdU antibody (1:500, Boehringer Mannheim, USA) and then exposed to secondary antibody (biotinylated anti-mouse IgG, 1:500, Vector Laboratories, USA). Signal amplification was carried out with an Avidin–Biotin complex (Vector) and was detected with diaminobenzidine (Sigma).

### Quantitation and Data Analysis

Levels of BDNF exon-specific transcripts (I–V) and CREB mRNA were determined using the Macintosh-based Scion Image Software (Scion, USA). To correct for nonlinearity,  $^{14}\text{C}$  standards were used for calibration. Equivalent areas of the hippocampal subfields-DG, CA1, CA3, and CA4-were outlined and optical density measurements taken. Hippocampal subfields from both sides of 3–4 sections from each animal were analyzed to obtain a mean value.

Quantitation of the BrdU-positive cells in tissue sections was carried out using a previously described modified unbiased stereology protocol (Nakagawa *et al*, 2002) on a Zeiss Axioskop microscope. Quantitation was performed on coded sections by an experimenter blind to the study code. Sections spanned the rostro-caudal extent of the hippocampus and every fifth hippocampal section was processed for quantitation (8 sections/animal). BrdU-positive cells within DG were counted as being in the subgranular zone (SGZ)/granule cell layer (GCL) when they were directly touching the SGZ or within it. Cells were counted as hilar when they were further than two cell body widths from the SGZ. The total number of BrdU-positive cells per SGZ/GCL or hilus was estimated by multiplying the total number of BrdU cells counted from every fifth section by the section periodicity (5).

Results were subjected to statistical analysis using the Student's *t*-test for experiments with two groups or two-way analysis of variance (ANOVA) followed by the Bonferroni

*post hoc* test for experiments with four groups. Differences were considered statistically significant at  $p$  values  $<0.05$ .

## RESULTS

The influence of acute and chronic stressors on the expression of distinct BDNF transcripts and CREB mRNA levels in the postnatal and adult rat brain was examined using *in situ* hybridization. Densitometric analysis was used to quantify levels of the BDNF splice variants and of CREB mRNA within the DG, CA1, CA3, and CA4 hippocampal subfields.

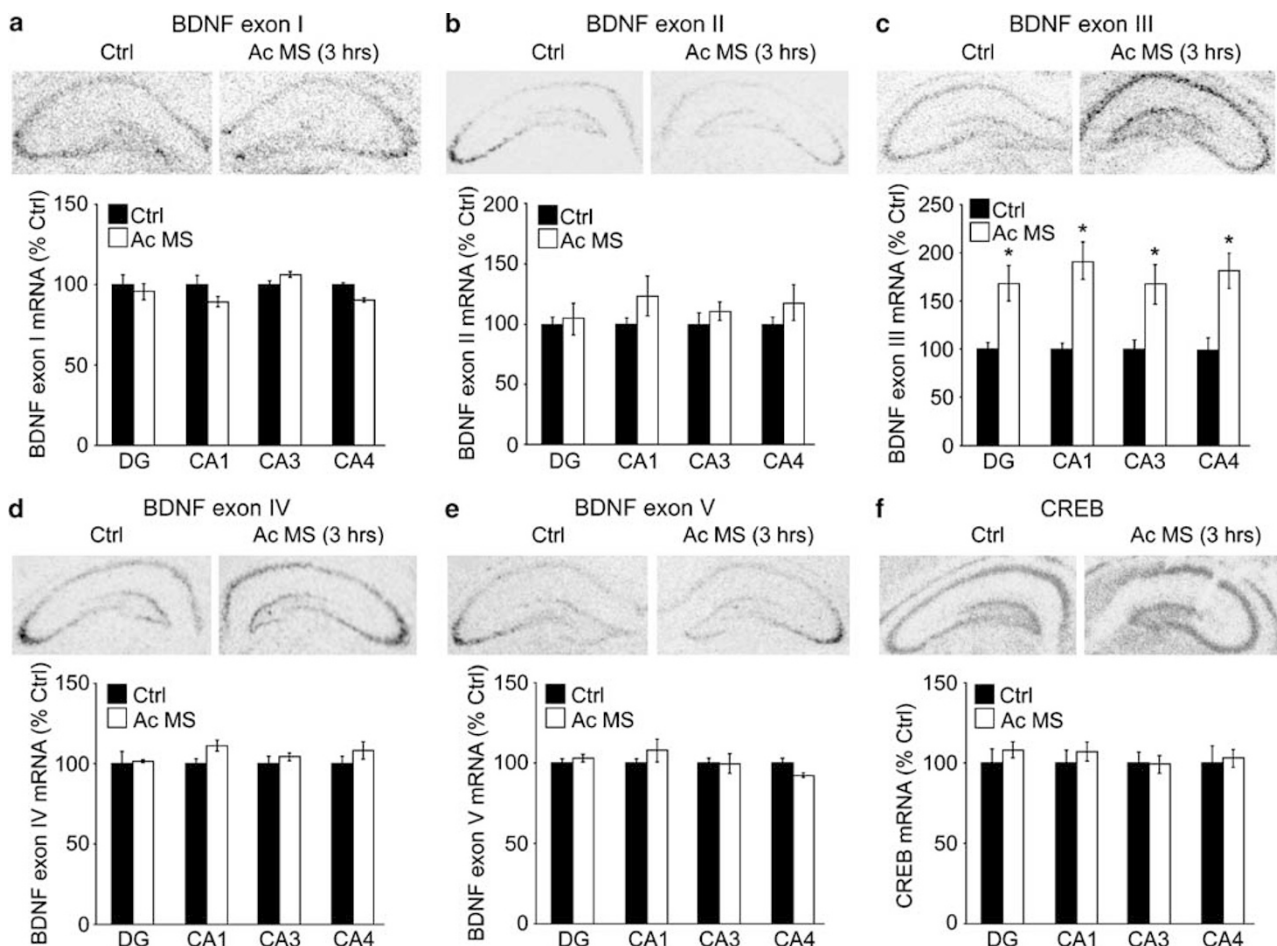
### Influence of Ac MS on the Expression of Specific Exon-Containing BDNF Transcripts and CREB mRNA in the Postnatal Hippocampus

The levels of the distinct exon-containing BDNF transcripts and CREB mRNA in the hippocampi of animals subjected to an Ac MS for 3 h on P7 are shown in Figure 1. The results show that a single period of separation of pups from the dam for 3 h resulted in a robust and selective upregulation

of exon III-containing BDNF transcripts in all the hippocampal subfields (Figure 1c). The regulation of the BDNF splice variants by a single episode of MS depended upon the duration of separation. Ac MS for 6 h on P7 resulted in a small, but significant, downregulation of BDNF exon I mRNA levels in all the hippocampal subfields examined (Table 1). The upregulation of BDNF exon III mRNA observed following 3 h MS was lost following a longer MS period of 6 h (Table 1). None of the changes in the specific exon-containing BDNF transcripts following Ac MS for 3 or 6 h was accompanied with a concomitant change in the total BDNF expression as determined by *in situ* hybridization for the common BDNF exon V (Figure 1e and Table 1). The expression of CREB was unaltered following an Ac MS for 3 or 6 h (Figure 1f and Table 1).

### Influence of Chr MS on the Expression of Distinct Exon-Containing BDNF Transcripts and CREB mRNA in the Postnatal Hippocampus

Animals were subjected to a daily 3-h MS from P2 to P14, and were killed at two different postnatal ages (P14 and



**Figure 1** Regulation of exon-specific BDNF transcripts (I–V) and CREB mRNA in the hippocampus of P7 pups following an Ac MS. Animals were maternally separated on P7 for 3 h and killed at the end of the 3 h separation period. The levels of the exon-specific BDNF transcripts and CREB mRNA were determined using *in situ* hybridization and quantitation was performed using densitometric analysis. The levels of the different BDNF transcripts (a) exon I, (b) exon II, (c) exon III, (d) exon IV, (e) exon V, and CREB mRNA (f) within the DG, CA1, CA3, and CA4 hippocampal subfields have been shown along with representative autoradiographic images from control and maternally separated animals. The results are presented as percent of control and are the mean  $\pm$  SEM ( $n = 4/\text{group}$ ). \* $p < 0.05$  in comparison to control (Student's *t*-test). The scales of the y-axis differ between the graphs.

**Table 1** Levels of Exon-Specific BDNF Transcripts and CREB mRNA in Hippocampal Subfields

| Probe         | Duration of MS (h) |       | Regions        |                |                |                |
|---------------|--------------------|-------|----------------|----------------|----------------|----------------|
|               |                    |       | DG             | CA1            | CA3            | CA4            |
| BDNF exon I   | 6                  | Ctrl  | 100.00 ± 3.33  | 100.00 ± 3.53  | 100.00 ± 4.82  | 100.00 ± 4.17  |
|               |                    | Ac Ms | 89.05 ± 2.01*  | 86.52 ± 2.21*  | 83.79 ± 1.84*  | 84.58 ± 4.15*  |
| BDNF exon II  | 6                  | Ctrl  | 100.00 ± 6.33  | 100.00 ± 7.10  | 100.00 ± 9.10  | 100.00 ± 5.89  |
|               |                    | Ac Ms | 95.89 ± 13.89  | 95.95 ± 11.37  | 98.00 ± 14.60  | 95.42 ± 12.44  |
| BDNF exon III | 6                  | Ctrl  | 100.00 ± 15.53 | 100.00 ± 15.01 | 100.00 ± 12.48 | 100.00 ± 14.84 |
|               |                    | Ac Ms | 93.65 ± 3.01   | 92.78 ± 3.37   | 102.34 ± 3.96  | 102.81 ± 4.19  |
| BDNF exon IV  | 6                  | Ctrl  | 100.00 ± 3.03  | 100.00 ± 3.05  | 100.00 ± 3.54  | 100.00 ± 4.10  |
|               |                    | Ac Ms | 95.32 ± 5.40   | 102.44 ± 3.65  | 99.29 ± 6.97   | 99.94 ± 7.68   |
| BDNF exon V   | 6                  | Ctrl  | 100.00 ± 8.29  | 100.00 ± 9.42  | 100.00 ± 9.76  | 100.00 ± 9.21  |
|               |                    | Ac Ms | 92.00 ± 5.44   | 99.51 ± 4.31   | 104.60 ± 5.27  | 95.72 ± 4.26   |
| CREB          | 6                  | Ctrl  | 100.00 ± 8.09  | 100.00 ± 9.84  | 100.00 ± 5.30  | 100.00 ± 8.72  |
|               |                    | Ac Ms | 78.76 ± 21.18  | 81.44 ± 20.57  | 91.88 ± 23.29  | 84.18 ± 21.35  |

Effect of 6 h of maternal separation (MS) on the expression of BDNF splice variants and CREB mRNA in the hippocampus. Pups were separated from the dam on postnatal day 7 for 6 h, and killed at the end of the MS. Levels of the exon-specific (I–V) BDNF transcripts and CREB mRNA were determined using *in situ* hybridization and densitometric analysis. Values are expressed as the percent of control and are the mean ± SEM ( $n = 4/\text{group}$ ).

\* $p < 0.05$  as compared to control (Student's *t*-test).

P21) following termination of the Chr MS paradigm. The different BDNF splice variants and CREB mRNA exhibited a time point-dependent regulation following the Chr MS paradigm (Figures 2 and 3). At P14, when animals were killed immediately following the termination of the final 3-h separation, a significant upregulation in BDNF exon II mRNA expression was seen in all the hippocampal subfields (Figure 2b). The expression of BDNF exon III-containing mRNA exhibited a trend towards an increase in the DG ( $p = 0.067$ ), CA3 ( $p = 0.09$ ), and CA4 ( $p = 0.068$ ) in MS animals, but did not achieve significance (Figure 2c). The other BDNF splice variants, as well as the expression of total BDNF mRNA (exon V), remained unchanged in Chr MS animals at P14 (Figure 2). CREB mRNA levels were significantly increased in the DG and CA1, but not the CA3 and CA4, hippocampal subfields in Chr MS animals at P14 (Figure 2f).

At P21, the expression of total BDNF mRNA (exon V) was significantly increased in the DG, CA1, and CA3 hippocampal subfields of Chr MS animals (Figure 3e). In addition, a trend towards significance ( $p = 0.067$ ) was observed with the expression of BDNF V in the CA4 hippocampal subfield. These increases in total BDNF mRNA appeared to be mediated through an induction in BDNF IV mRNA (Figure 3d), but not any of the other BDNF splice variants, which remain unchanged at P21 after Chr MS. In addition, the expression of CREB mRNA was found to be significantly higher in the DG and CA1 of Chr MS animals at P21 as compared to controls (Figure 3f).

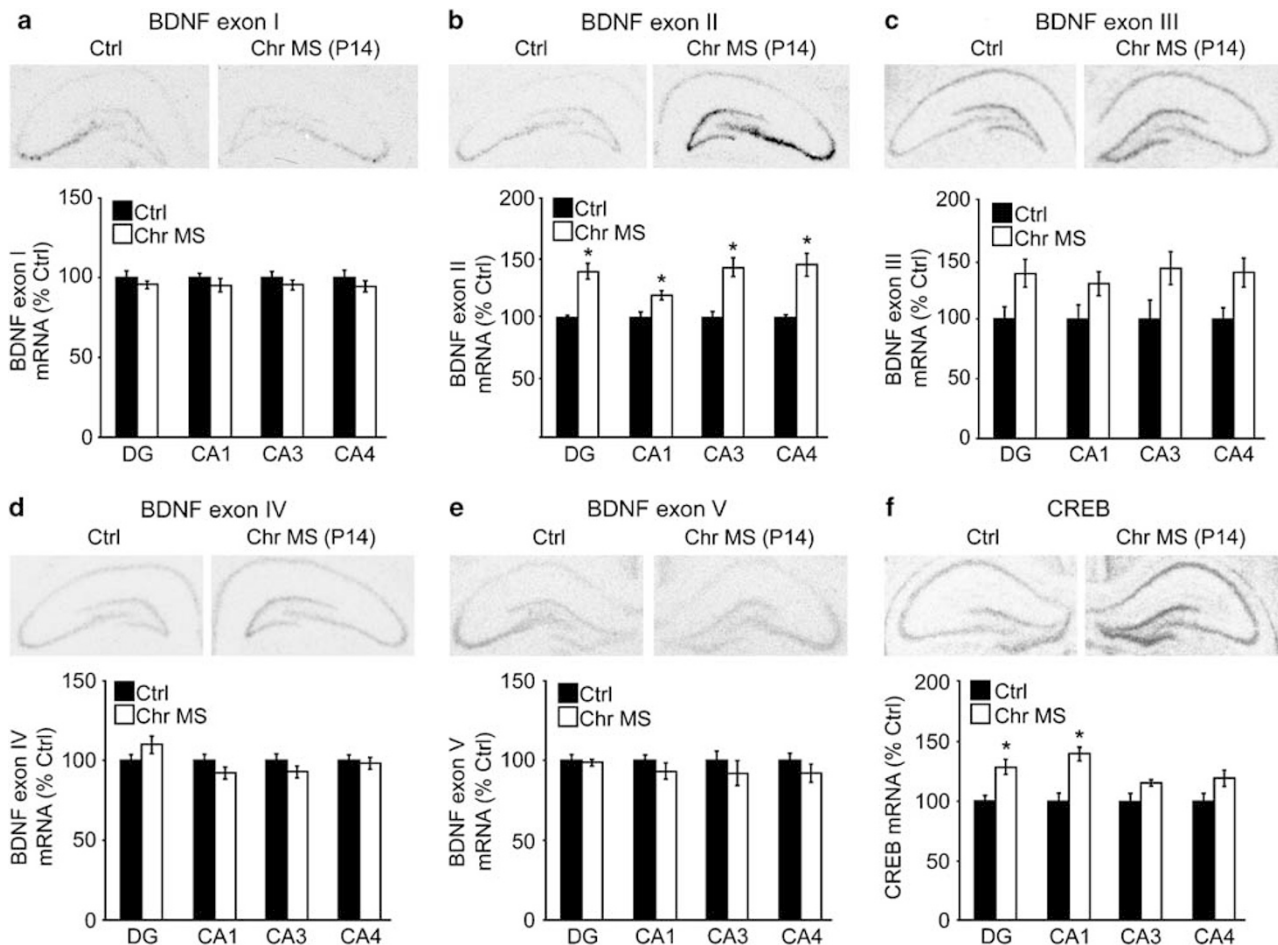
### Stressor-Specific Regulation of BDNF Splice Variants and CREB mRNA in the Adult Hippocampus

To address whether the regulation of BDNF splice variants and CREB mRNA in the adult hippocampus exhibits a stressor-specificity, animals were subjected to acute/chronic

immobilization stress (Figures 4 and 5) or CUS (Figure 6). Acute vs chronic administration of the same stressor, immobilization stress, resulted in a differential regulation of the specific exon-containing BDNF mRNAs. In response to a single administration of immobilization stress (Figure 4) a significant downregulation of all the exon-specific BDNF mRNAs (I–IV) (Figure 4a–d) was observed in the DG, which was accompanied by a significant decline in the levels of total BDNF mRNA (V) (Figure 4e) in the DG. In the CA subfields of the hippocampus, acute immobilization stress selectively reduced the expression of BDNF III mRNAs in the CA3 and CA4 (Figure 4c) and resulted in a decline in total BDNF mRNA expression within the CA4 subfield (Figure 4e).

Although chronic immobilization stress (10 days) induced a significant decrease in the levels of total BDNF mRNA (V) (Figure 5e) in the DG, similar to the effects observed following an acute immobilization stress (Figure 4e), the regulation of the exon-specific BDNF transcripts exhibited dramatic differences dependent upon whether it was a single or repeated exposure to immobilization stress. BDNF exon I- and II-containing transcripts were significantly upregulated in the DG, CA3 and CA4 hippocampal subfields following chronic immobilization stress (Figure 5a and b). In addition, BDNF exon II mRNA was also increased in the CA1 (Figure 5b). In striking contrast, BDNF exon III- and IV-specific mRNAs were significantly decreased in the DG, CA3, and CA4 subfields (Figure 5c and d). The expression of BDNF III mRNA in the CA1 was also significantly reduced and there was a trend towards a decrease ( $p = 0.06$ ) in BDNF IV mRNA levels (Figure 5c and d) in this subfield.

CUS, unlike acute and chronic immobilization stress, did not alter levels of total BDNF mRNA (V) (Figure 6e). However, specific exon-containing BDNF transcripts were found to be regulated by CUS. BDNF III mRNA was



**Figure 2** Influence of Chr MS on the expression of exon-specific BDNF transcripts (I–V) and CREB mRNA in the hippocampus of P14 pups. Animals were maternally separated from P2 to P14 for 3 h daily, and killed on P14 immediately following the termination of the 3 h separation period. Quantitation of the levels of exon-specific BDNF transcripts and CREB mRNA was performed using *in situ* hybridization and followed by densitometric analysis. The levels of exon-specific BDNF transcripts (a) exon I, (b) exon II, (c) exon III, (d) exon IV, (e) exon V, and CREB mRNA (f) in the hippocampal subfields of the DG, CA1, CA3, and CA4 are graphically represented with autoradiographic images from control and maternally separated animals. The results are presented as percent of control and are the mean  $\pm$  SEM ( $n = 4$ –5/group). \* $p < 0.05$  in comparison to control (Student's *t*-test). The scales of the y-axis differ between the graphs.

increased in all the hippocampal subfields (Figure 6c), while BDNF I mRNA was selectively upregulated in the CA regions (Figure 6a). In contrast, BDNF II mRNA was significantly reduced in the DG, CA1 and CA4 (Figure 6b). BDNF exon IV did not appear to be regulated by CUS (Figure 6d).

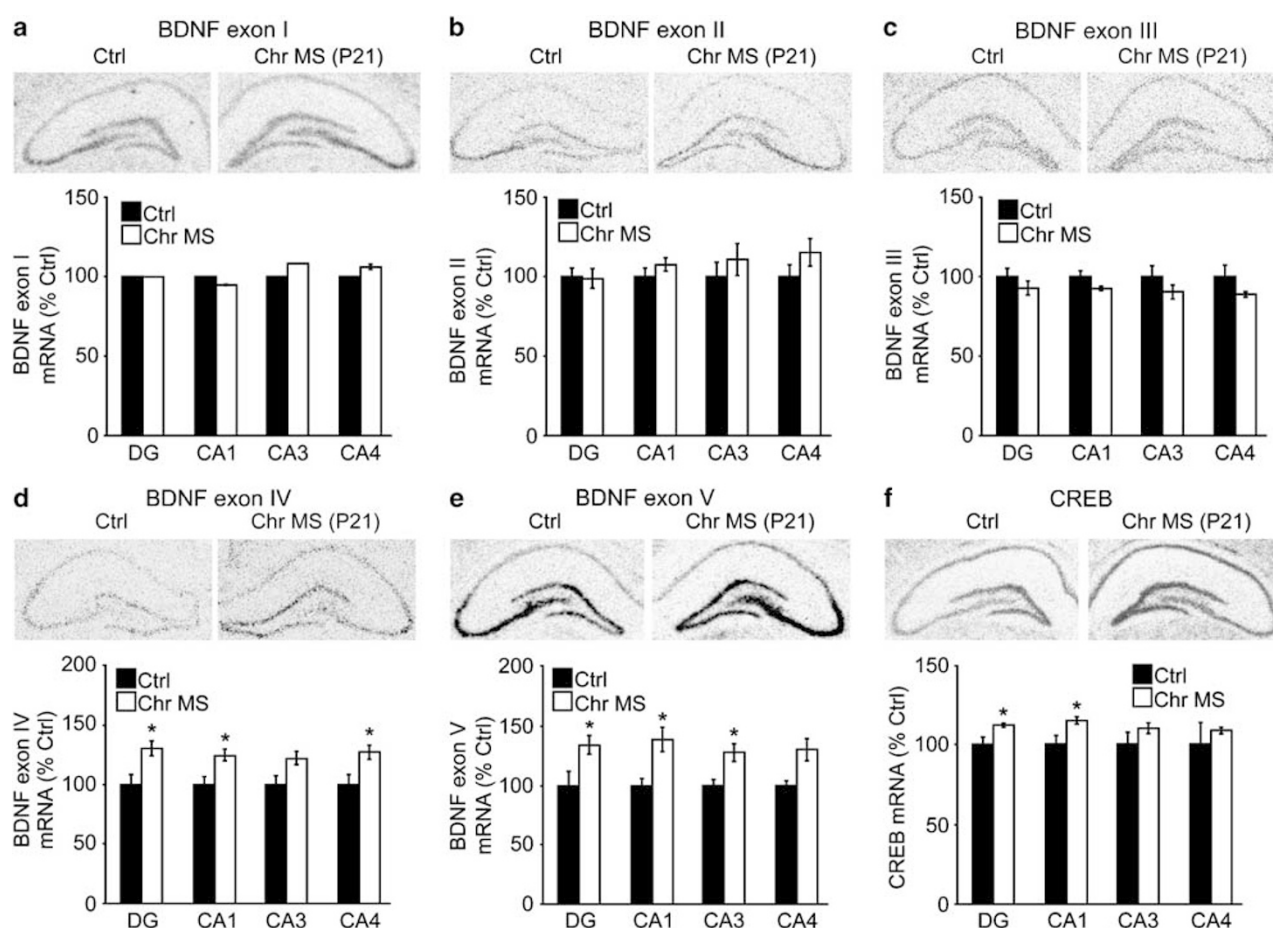
CREB mRNA levels within the different hippocampal subfields were not influenced by exposure to acute (Figure 4f) and chronic immobilization stress (Figure 5f) or CUS (Figure 6f).

#### Influence of Chr MS in Postnatal Life on the Regulation of Distinct Exon-Specific BDNF Transcripts and CREB mRNA in the Adult Hippocampus by Acute and Chronic Immobilization Stress

We next sought to address whether prior exposure to Chr MS in early postnatal life altered the regulation of BDNF splice variants and CREB transcripts in response to acute and chronic immobilization stress in adulthood. Animals were subjected to the Chr MS paradigm from P2 to P14, and

as adults (P60) were exposed to either 1-day acute immobilization stress, or to 10-day chronic immobilization stress. Chr MS in postnatal life did not alter the basal expression of BDNF splice variants in adulthood (Figures 7 and 8), despite significant anxiety responses exhibited by MS animals in adulthood in the NSF paradigm (Latency to Feed: Control =  $101.67 \pm 10.86$  s, MS =  $230.88 \pm 35.29$  s,  $p < 0.05$ , Student's *t*-test). However, postnatal exposure to Chr MS significantly influenced the regulation of the different BDNF transcripts by acute and chronic immobilization stress.

Acute immobilization stress resulted in a significant decline in total BDNF mRNA (V) (Figure 7e) within the DG and CA4 hippocampal subfields, which was not observed in animals that had been previously exposed to Chr MS in early life. With regards to the acute immobilization stress-mediated changes in specific BDNF splice variants, prior Chr MS experience influenced this regulation in a differential manner. Acute stress resulted in a similar downregulation ( $\sim 50\%$ ) of BDNF I (Figure 7a) and II mRNAs (Figure 7b) in the DG, irrespective of whether



**Figure 3** Influence of Chr MS on the expression of exon-specific BDNF transcripts (I–V) and CREB mRNA in the hippocampus of P21 animals. Animals were maternally separated from P2 to P14 for 3 h daily and killed on P21, a week after the termination of the Chr MS paradigm on P14. *In situ* hybridization and densitometric analysis was utilized to assess the levels of the distinct BDNF splice variants and CREB mRNA. The levels of the different BDNF transcripts (a) exon I, (b) exon II, (c) exon III, (d) exon IV, (e) exon V, and CREB mRNA (f) within the DG, CA1, CA3, CA4 hippocampal subfields have been shown along with representative autoradiographic images from control and maternally separated animals. The results are presented as percent of control and are the mean  $\pm$  SEM ( $n = 3$ –5/group). \* $p < 0.05$  in comparison to control (Student's *t*-test). The scales of the y-axis differ between the graphs.

animals had experienced Chr MS in early life or had been left undisturbed. In striking contrast, the acute stress-mediated downregulation of BDNF III transcripts (Figure 7c) in the DG, CA3, and CA4 hippocampal subfields was completely blocked in animals that had a prior history of Chr MS. Although in the CA1 subfield there was significant difference in BDNF III mRNA levels between stress and Chr MS + stress animals (Figure 7c), stress itself did not achieve significance as compared to controls. Acute stress significantly decreased BDNF IV mRNA only in the DG (Figure 7d) of animals that had not been subjected to Chr MS in postnatal life.

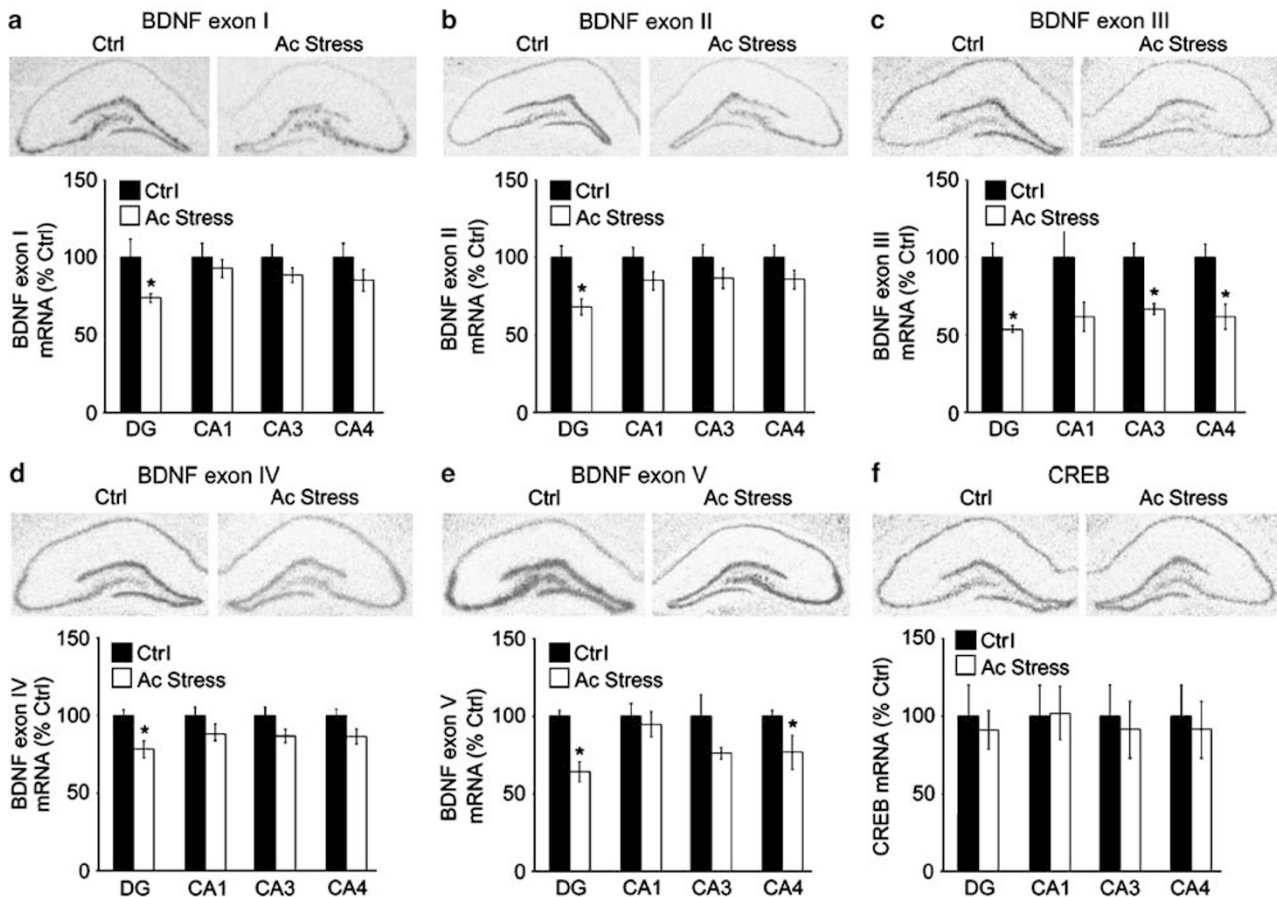
The chronic immobilization stress-induced downregulation of total BDNF (V) mRNA in the DG was completely lost in animals that had a prior exposure to Chr MS in early life (Figure 8e). In addition, the adult-onset chronic stress-mediated regulation of BDNF I (Figure 8a) and IV mRNA (Figure 8d) in the DG, CA3 and CA4, as well as the chronic stress-induced decline observed in BDNF III mRNA levels (Figure 8c) in all the hippocampal subfields was not observed in animals that had undergone Chr MS in postnatal life. The chronic stress-mediated upregulation of BDNF II transcripts in the CA3, CA4 and DG (Figure 8b)

was not observed in the Chr MS + chronic stress group, although there appeared to be an increase in BDNF II in the DG that did not achieve significance. In contrast, the chronic stress-mediated upregulation of BDNF II mRNAs in the CA1 (Figure 8b) was exactly the same in both animals that were separated from dams or were left undisturbed in postnatal life.

The changes observed in CREB mRNA following Chr MS appear to be restricted to postnatal life (Figures 2 and 3) as no differences were seen in the basal levels of CREB mRNA between control and Chr MS animals in adulthood (Figures 7 and 8). In addition, CREB expression in the adult hippocampus was not altered by either acute (Figure 7f) or chronic exposure to immobilization stress (Figure 8f) in either animals that had previously undergone Chr MS in early life or had been left undisturbed with the dam.

### Influence of Chr MS on the Proliferation of Hippocampal Progenitors in the Postnatal and Adult Hippocampus

BDNF and CREB have been previously reported to enhance significantly adult hippocampal progenitor proliferation



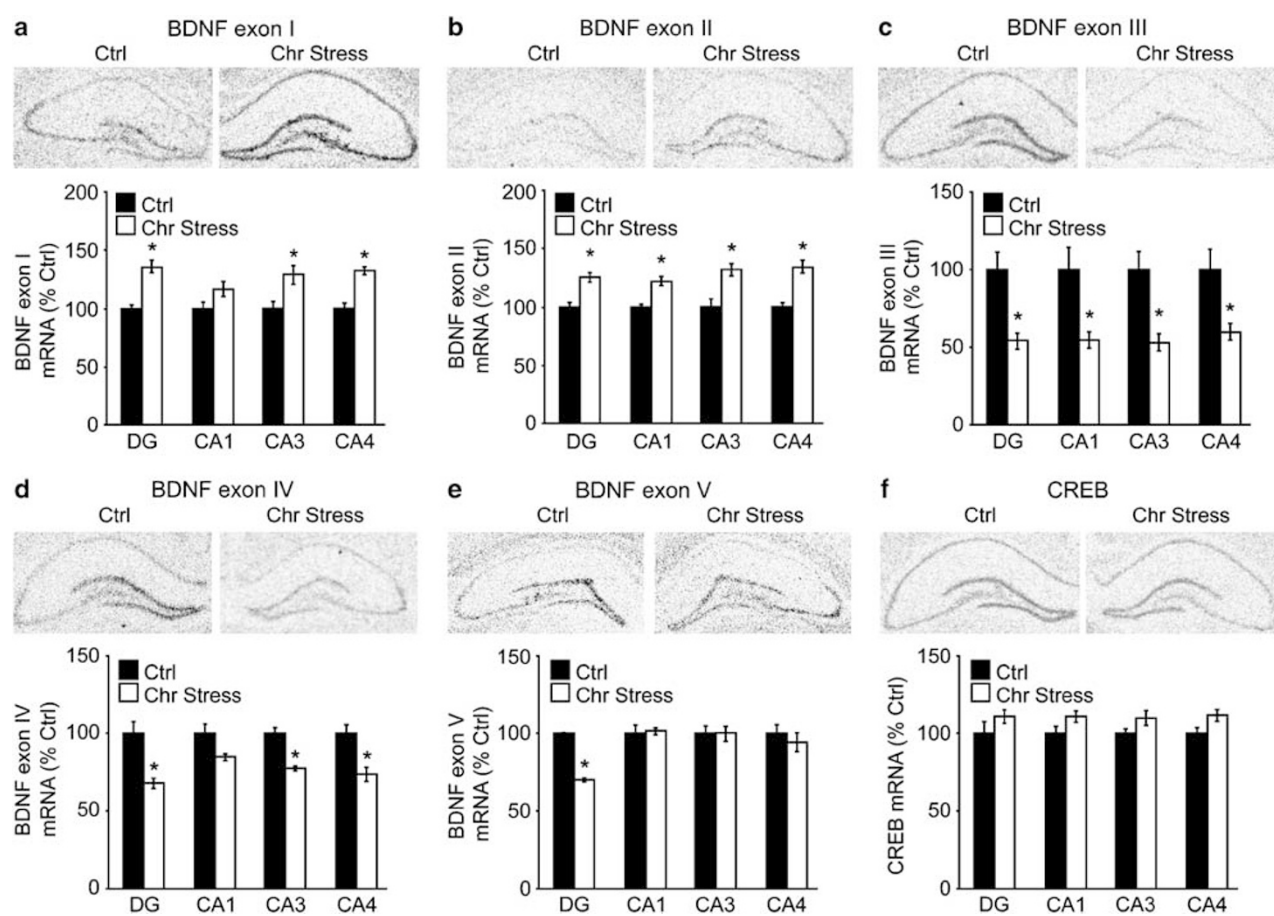
**Figure 4** Regulation of the distinct exon-specific BDNF transcripts (I–V) and CREB mRNA in the hippocampus of adult animals following acute immobilization stress (Ac Stress). Adult (P60) animals were subjected to an acute immobilization stress for 2 h, and killed immediately following the termination of the 2-h stress. Levels of the exon-specific BDNF transcripts and CREB mRNA were determined using radioactive *in situ* hybridization and densitometric analysis. Levels of the different transcripts have been presented for the hippocampal subfields-DG, CA1, CA3, and CA4. Representative autoradiographic images for the control and acute stress animals have also been shown (a) BDNF exon I, (b) BDNF exon II, (c) BDNF exon III, (d) BDNF exon IV, (e) BDNF exon V, and (f) CREB. The results are presented as percent of control and are the mean  $\pm$  SEM ( $n = 10/\text{group}$ ). \* $p < 0.05$  in comparison to control (Student's *t*-test).

(Nakagawa *et al*, 2002; Pencea *et al*, 2001). Given that Chr MS increased the expression of CREB and BDNF mRNAs transiently in postnatal life, we examined whether any change was observed in the proliferation of hippocampal progenitors during postnatal life and in adulthood in Chr MS animals. The effect of Chr MS on the proliferation of dentate granule cell progenitors was determined using the mitotic marker BrdU to label dividing progenitors. Animals were subjected to MS from P2–P14, and were injected with BrdU at different ages subsequent to the termination of the MS paradigm: P15, P21, and P60. All animals were killed 2 h after BrdU injection, and assessed for BrdU immunoreactivity. BrdU-positive cells were found localized to the SGZ at the border of the hilus and the GCL, in both control and Chr MS groups (Figure 9) and were often observed in clusters. At P15, a trend ( $p = 0.07$ ) towards an increase in the number of BrdU-positive cells in the SGZ/GCL was seen in Chr MS animals (Figure 9a). Chr MS animals showed a significant increase in the number of BrdU-positive cells within the SGZ/GCL (Figure 9b) at P21. However, in adulthood the number of SGZ/GCL BrdU-positive hippocampal progeni-

tors in animals that had received Chr MS in early life was similar to that seen in non-separated controls (Figure 9c). As expected, there was an age-dependent decline in BrdU-positive cell number in the SGZ/GCL. The number of BrdU-positive cells in the hilus remained unaltered by Chr MS at all three time points examined.

## DISCUSSION

We provide novel evidence for a stressor-specific differential regulation of BDNF splice variants (Figure 10) and CREB mRNA in the postnatal and adult hippocampus. The early life stress of MS exerts a duration and time point-dependent regulation of distinct exon-containing BDNF transcripts and CREB mRNA. These changes are accompanied by a significant increase in hippocampal progenitor proliferation in MS animals, restricted to postnatal life. Adult-onset stress exhibits heterogeneity in the regulation of BDNF splice variants that is dependent on both the nature and repetition of the stress, as well as the prior



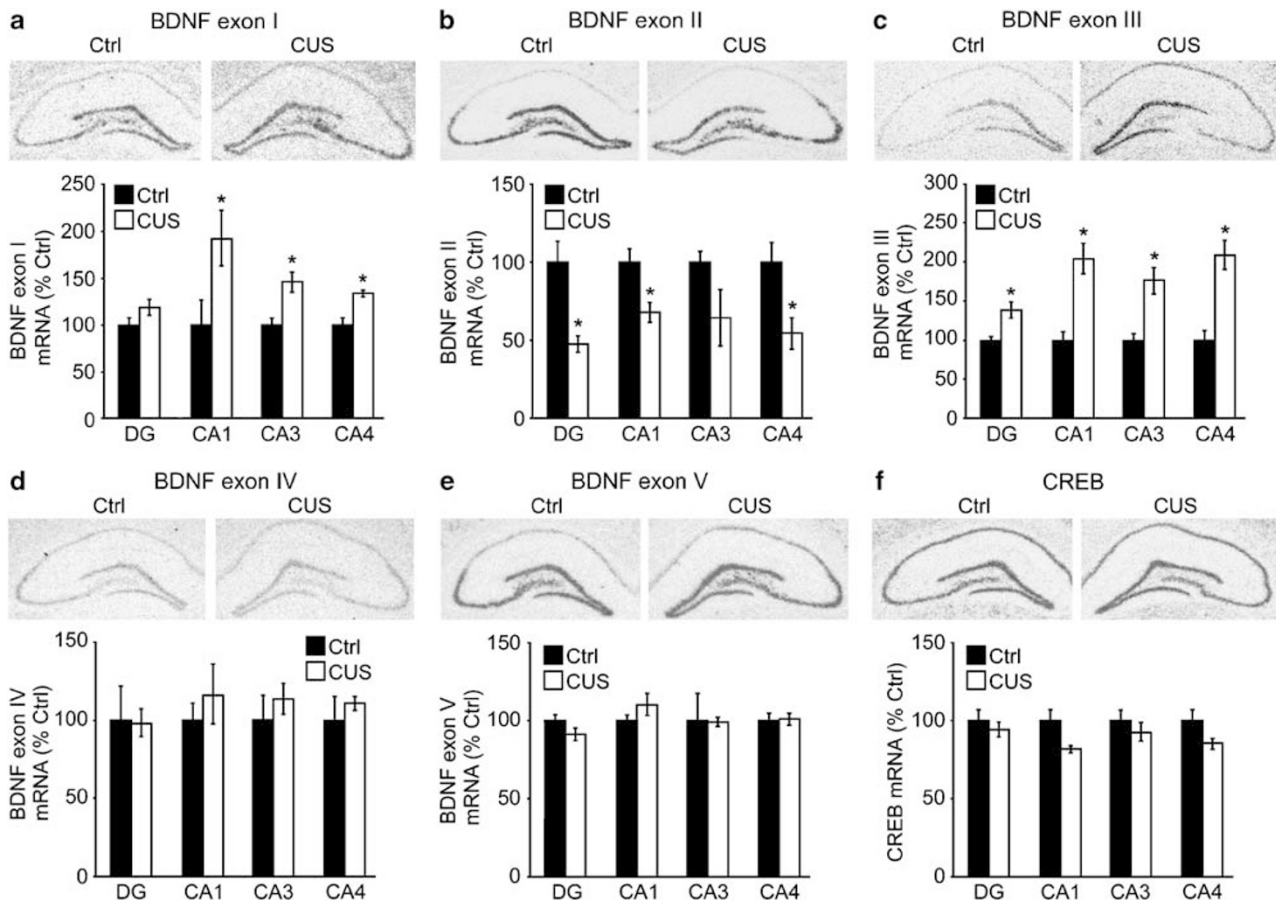
**Figure 5** Regulation of the distinct exon-specific BDNF transcripts (I–V) and CREB mRNA in the hippocampus of adult animals following chronic immobilization stress (Chr Stress). Adult (P60) animals were subjected to a daily 2-h immobilization stress consecutively for a period of 10 days. Animals were killed at the end of the 2-hour stress on the final day, and levels of BDNF exon-containing transcripts and CREB mRNA were determined using *in situ* hybridization. Quantitation was undertaken by densitometric analysis. Levels of the different BDNF transcripts and CREB mRNA for the hippocampal subfields-DG, CA1, CA3, and CA4 have been shown, along with their representative autoradiographic images. (a) BDNF exon I, (b) BDNF exon II, (c) BDNF exon III, (d) BDNF exon IV, (e) BDNF exon V, and (f) CREB. The results are represented as percent of control and are the mean  $\pm$  SEM ( $n = 5/\text{group}$ ). \* $p < 0.05$  in comparison to control (Student's *t*-test). The scales of the y-axis of the graphs for the different exon-specific BDNF mRNAs and the CREB transcript differ.

history of stress exposure of the animal. Indeed, the immobilization stress-induced regulation of BDNF mRNAs is altered in animals that have been exposed previously to Chr MS. Our results raise the possibility that early life adverse experience, through changes to BDNF and CREB regulation, may alter the remodeling of neuronal circuits induced by these molecules thus programming differences in stress responsiveness.

Our results indicate that the stress regulation of BDNF expression is undoubtedly far more complex than revealed by previous reports that did not distinguish among multiple BDNF transcripts (Roceri *et al*, 2004; Smith *et al*, 1995). Although the effects of MS on total BDNF expression (exon V) have been examined (Greisen *et al*, 2005; Roceri *et al*, 2002, 2004), there is no information at present on the regulation of specific BDNF transcripts or the region specificity of changes in BDNF within hippocampal subfields. We find that different durations of a single MS episode result in increased BDNF III mRNA (3 h MS) or a decline in BDNF I expression (6 h MS) in all hippocampal subfields. However, neither total BDNF (V) nor CREB

expression is altered by a single episode of MS. A previous study (Roceri *et al*, 2002) with an acute 24 h MS reported no alterations in total BDNF levels in postnatal life, but resulted in a decline in BDNF expression in the adult hippocampus. Although short duration MS may have an adaptive influence on BDNF splice variants, single MS for long durations appears to cause maladaptive changes in BDNF, and this is likely to reflect both the effects of separation *per se*, as well as disrupted nutritional support and thermal regulation. Collectively, these results suggest differential recruitment of the exon-specific BDNF promoters by Ac MS in a manner dependent upon the severity of the MS (Figure 10).

Chr MS (3 h daily; P2–P14) animals exhibit a time point-dependent regulation (Figure 10) with enhanced BDNF II and CREB mRNA at P14, increased BDNF IV, V, and CREB expression at P21, and normal levels in adulthood. Previous studies (Greisen *et al*, 2005; Roceri *et al*, 2004), using probes that do not distinguish between the BDNF splice variants, find an age-dependent increase in total BDNF expression at P17 with no change at P35 or P90. Our results are in agreement with these reports. Moreover, they implicate the



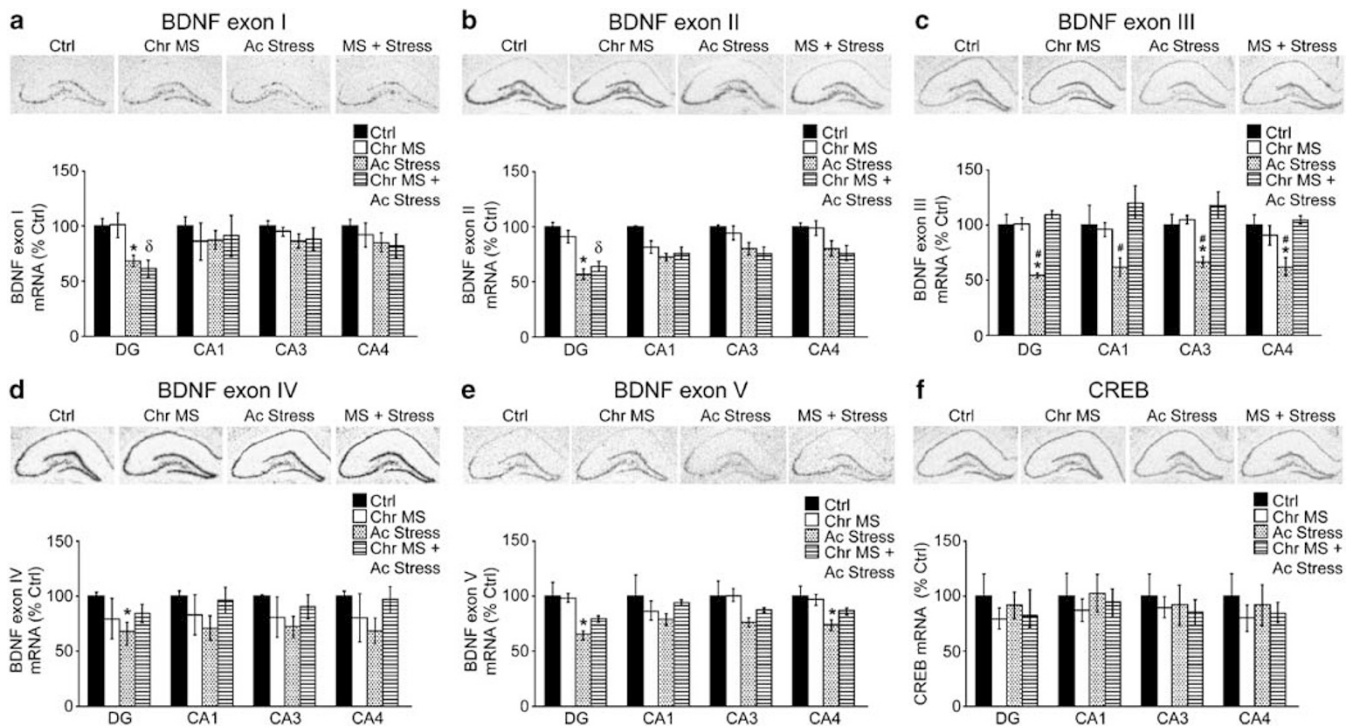
**Figure 6** Regulation of the distinct exon-specific BDNF transcripts (I–V) and CREB mRNA in the hippocampus of adult animals following CUS. Adult (P60) animals were subjected to a CUS paradigm consisting of two random stressors daily for a 10-day period, and killed at the termination of the last stressor. Levels of the exon-specific BDNF and CREB transcripts were determined using *in situ* hybridization and quantitated using densitometric analysis. The expression of the specific BDNF transcripts and the CREB transcript for the hippocampal subfields-DG, CA1, CA3, and CA4 have been shown, along with representative autoradiographic images from control and CUS animals (a) BDNF exon I, (b) BDNF exon II, (c) BDNF exon III, (d) BDNF exon IV, (e) BDNF exon V, and (f) CREB. The results are presented as percent of control and are the mean  $\pm$  SEM ( $n = 7$ –8/group). \* $p < 0.05$  in comparison to control (Student's *t*-test). The scales of the y-axis of the graphs for the different exon-specific BDNF transcripts and CREB mRNA differ.

recruitment of the BDNF exon II and IV promoters in the Chr MS-induced BDNF upregulation seen in postnatal life. While the consequences of enhanced BDNF and CREB following Chr MS are at present unknown, it is possible that they may represent an adaptive response to this early life adverse experience. An alternative possibility is that increased CREB and BDNF expression supports the establishment of a fear conditioned-like state. Indeed, increased fear and anxiety responses are characteristic of this early life manipulation (Kalinichev *et al*, 2002). We speculate that, through the regulation of BDNF and CREB, MS could lead to an 'imprinting' of this adverse early experience on fear and stress neuro-circuitry, such that these animals continue to show enhanced fear and anxiety-like behaviors well after the termination of the MS paradigm.

Another question that arises is whether CREB plays an important role in priming changes in BDNF transcripts following MS. Interestingly, enhanced CREB expression in Chr MS animals does not translate into increased BDNF I- or III mRNAs, the promoters of which are known to contain CRE sites (Shieh *et al*, 1998; Tabuchi *et al*, 2002). This

suggests that CREB may not mediate the MS effects on BDNF promoters, and that the changes observed in its expression may actually be a consequence of enhanced BDNF signaling, which can itself upregulate CREB expression (Xing *et al*, 1996). Given that MS overlaps in time with a period of developmental modulation of the BDNF splice variants (Sathanoori *et al*, 2004; Timmusk *et al*, 1994), this may directly alter the availability of specific BDNF promoters for regulation by early life stress. This could then set up the age-dependent differences seen in the stress responsiveness of different BDNF transcripts. MS through perturbations of the developmental profile of BDNF transcripts and CREB (Bender *et al*, 2001; Timmusk *et al*, 1993) may then pattern changes in hippocampal circuitry by altering its developmental trajectory.

The development of the hippocampal DG subfield continues well into postnatal life. Postnatal hippocampal development involves both the addition of substantial numbers of neurons (Schlessinger *et al*, 1975) and their subsequent maturation and integration into hippocampal circuitry (Zhao *et al*, 2006). BDNF and CREB have been strongly implicated in these processes (Fujioka *et al*, 2004;

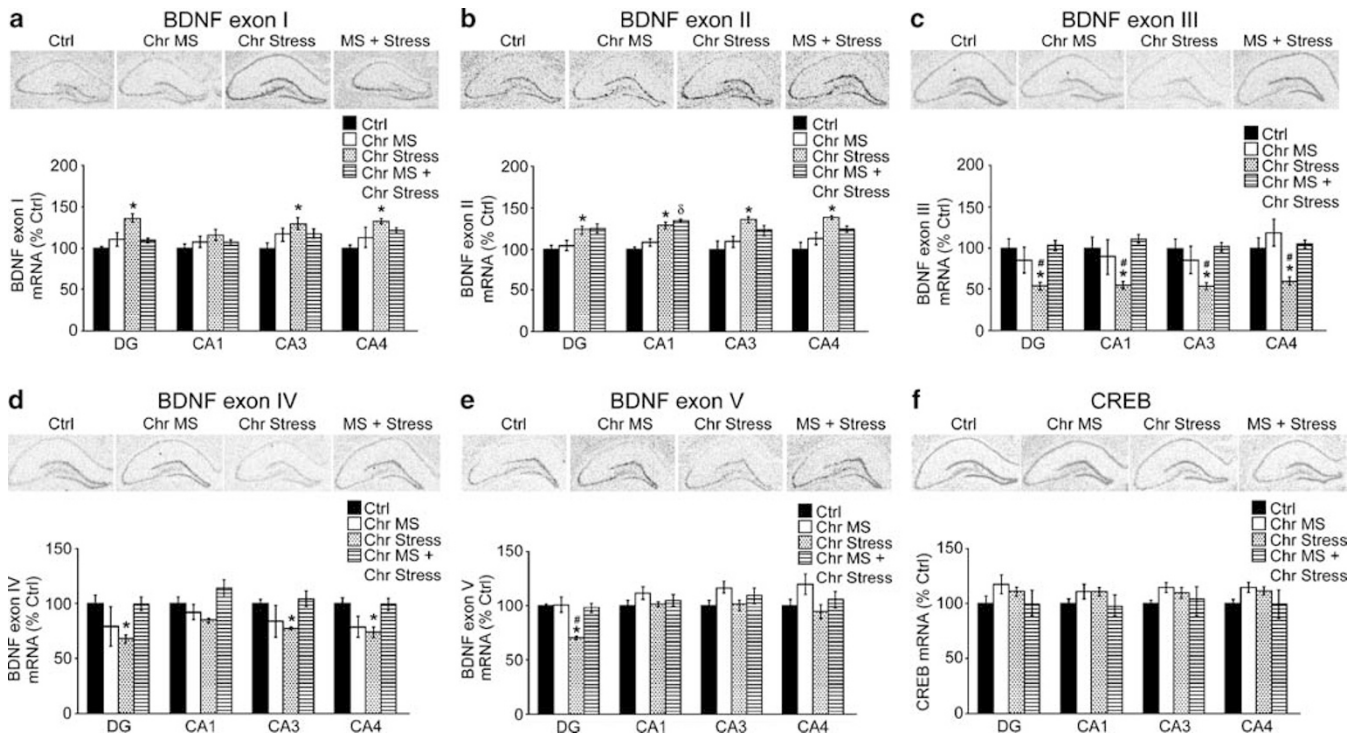


**Figure 7** Influence of prior exposure to Chr MS on the acute immobilization stress (Ac Stress)-mediated regulation of the exon-specific BDNF transcripts (I–V) and CREB mRNA in the hippocampus of adult animals. Animals were maternally separated from P2 to P14 for 3 h daily. As adults (P60), these animals were subjected to a single 2-h acute immobilization stress and killed at the end of the stress. *In situ* hybridization followed by densitometric analysis was used to estimate the levels of the different exon-specific BDNF mRNA and the CREB transcript. The results for the different transcripts have been shown for the different subfields of the hippocampus, namely the DG, CA1, CA3, and CA4. Representative images for the four cohorts: 'control', 'Chr MS', 'acute stress', and 'Chr MS + acute stress' have been shown for the distinct exon-containing BDNF transcripts and the CREB transcript (a) BDNF exon I, (b) BDNF exon II, (c) BDNF exon III, (d) BDNF exon IV, (e) BDNF exon V, and (f) CREB. The results are presented as percent of control and are the mean  $\pm$  SEM ( $n = 4$ –5/group). \* $p < 0.05$  in comparison to control,  $\delta p < 0.05$  in comparison to Chr MS, # $p < 0.05$  in comparison to Chr MS + acute stress (ANOVA, *post hoc* Bonferroni test).

Nakagawa *et al*, 2002; Pencea *et al*, 2001; Sairanen *et al*, 2005). Given that the time course of CREB and BDNF transcript upregulation following MS exhibits a remarkable coincidence with this period of development and functional maturation in the DG, we hypothesized that there may be consequences on the normal hippocampal developmental trajectory. We provide the first evidence of a dramatic increase in hippocampal progenitor proliferation in MS animals during postnatal life. Previous studies indicate that Chr MS results in cell death in the postnatal DG (Zhang *et al*, 2002). The idea that the death and birth of new neurons is coordinated has been suggested by several studies (Gould *et al*, 1991; Silva *et al*, 2006). This raises the interesting possibility that increased cell death is associated with an increase in neurogenic modulators like CREB and BDNF, thus promoting enhanced cell birth in the postnatal DG following MS. We find that the Chr MS-induced increase in hippocampal progenitor proliferation is restricted to postnatal life, much like the changes in BDNF and CREB transcripts. This is particularly intriguing given that we find that Chr MS animals in adulthood exhibit behavioral changes on the NSF test clearly associated with animal models of depression, but these are not accompanied by decreased BDNF, CREB, or adult neurogenesis. Previous studies (Greisen *et al*, 2005; Mirescu *et al*, 2004) have reported conflicting results on the regulation of hippocampal neurogenesis in adulthood in MS animals. Although our

results are in agreement with a previous report showing no change in adult neurogenesis following MS (Greisen *et al*, 2005), they differ from other studies that report a decline in hippocampal progenitor proliferation in adulthood (Mirescu *et al*, 2004; Park *et al*, 2002). It is possible that the discrepancy in these findings arise from differences in the BrdU dosing regimens and in the MS paradigms. Our results provide impetus to further explore the role of CREB and BDNF in the increased progenitor proliferation observed during postnatal life in MS animals, and to examine the consequences of enhanced cell turnover to hippocampal circuitry and function.

High maternal care and paradigms like postnatal handling (PNH), which induce increased maternal behavior (Pryce *et al*, 2001), have been previously reported to enhance the hippocampal expression of BDNF (V) and CREB (Garoflos *et al*, 2005; Liu *et al*, 2000). Here, we provide evidence that adverse experience in early life also alters the expression of CREB and BDNF transcripts. While PNH programs decreased anxiety-like behavior in adulthood (Vallee *et al*, 1997), Chr MS results in a phenotype characterized by increased anxiety responses (Kalinichev *et al*, 2002; Ladd *et al*, 2000). Paradoxically, both paradigms increase hippocampal levels of BDNF and CREB in postnatal life. A possible explanation may involve the differential recruitment of BDNF exon-specific promoters by these two diverse early life experiences resulting in

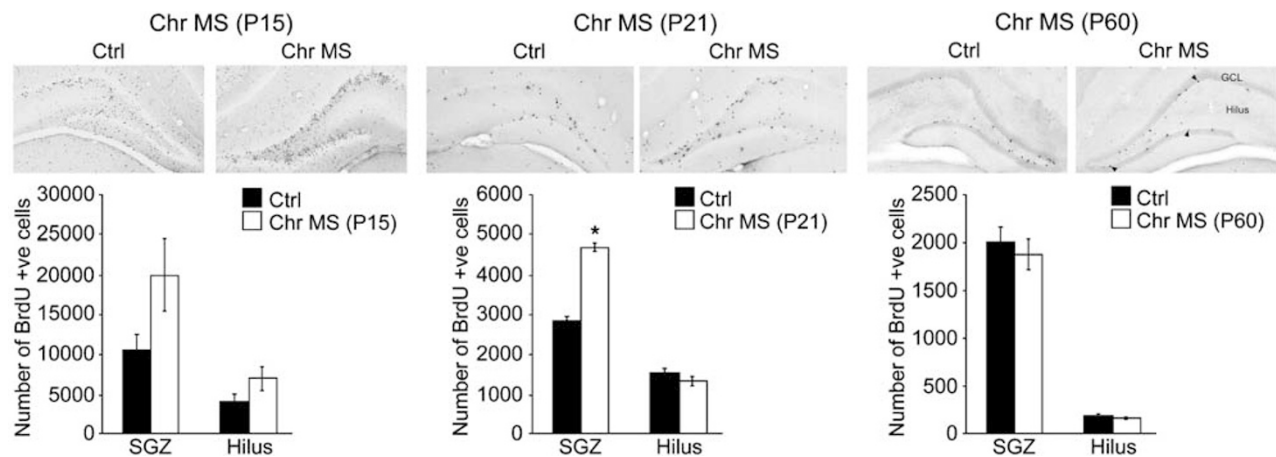


**Figure 8** Influence of Chr MS on the hippocampal regulation of exon-specific BDNF transcripts (I–V) and CREB mRNA by chronic immobilization stress (Chr Stress) in adulthood. Animals were subjected to the Chr MS paradigm from P2 to P14 for 3 h daily, and then to a chronic (10 day) immobilization stress during adulthood (P60 onwards). Exon-specific BDNF mRNA and CREB transcript levels were determined using *in situ* hybridization, and quantitated using densitometric analysis. The levels of the exon-specific BDNF transcripts and CREB mRNA have been shown for the various hippocampal subfields-quantitated- DG, CA1, CA3 and CA4, along with representative autoradiographic images from the 'control', 'Chr MS', 'chronic stress', and 'Chr MS + chronic stress' groups. (a) BDNF exon I, (b) BDNF exon II, (c) BDNF exon III, (d) BDNF exon IV, (e) BDNF exon V, and (f) CREB. The results are presented as percent of control and are the mean  $\pm$  SEM ( $n = 4$ –5/group). \* $p < 0.05$  in comparison to control,  $\delta p < 0.05$  in comparison to Chr MS,  $\#p < 0.05$  in comparison to Chr MS + chronic stress (ANOVA, *post hoc* Bonferroni test). The scales of the y-axis differ across the graphs.

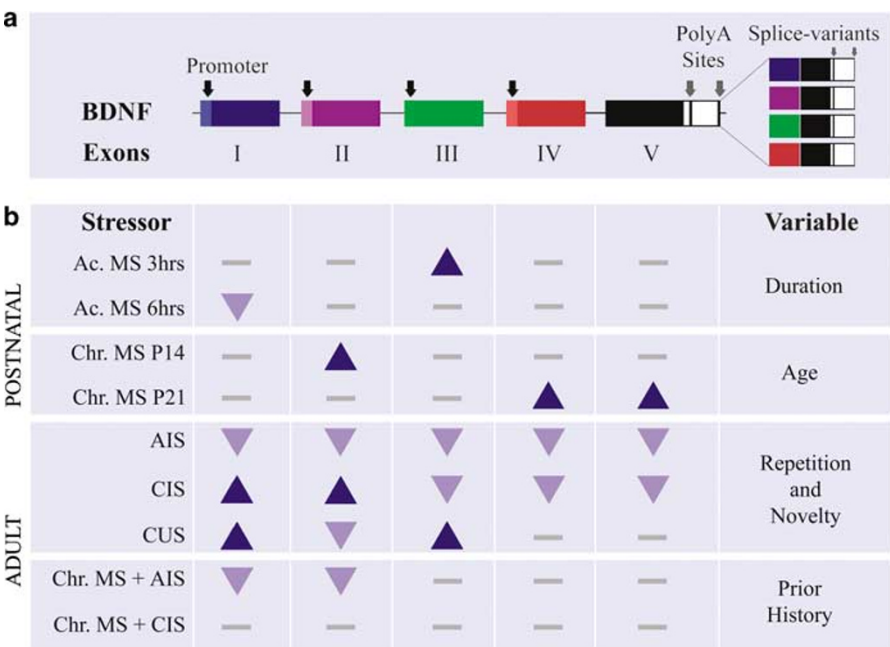
distinct functional outcomes. Although we show that the BDNF exon II and IV promoters are involved in the MS regulation of BDNF expression, there is no information currently available on the effects of PNH on BDNF splice variants. Differential regulation of the BDNF promoters by these paradigms could result in altered translatability, trafficking, and subcellular localization of BDNF, which may underlie the divergent consequences of PNH and MS on hippocampal structure, function and, ensuing behavior.

While our results demonstrate that the early life stress of MS enhances the expression of BDNF and CREB, adult-onset stressors differentially regulate BDNF splice variants in a signature manner (Figure 10), but do not influence CREB mRNA levels. Our results corroborate previous data reporting a decrease in total BDNF (exon V) expression in the DG (Smith *et al*, 1995) following both acute and chronic immobilization stress in adulthood. Notably, we demonstrate that repetitive exposure to immobilization stress has drastically differing consequences on BDNF promoters and the expression of individual BDNF splice variants (Figure 10). Although the decline in BDNF V seen following acute immobilization stress represents a cumulative reduction of all BDNF splice variants (I–IV), the chronic immobilization stress-induced decrease in BDNF V is due to the reduced expression of BDNF III and IV transcripts. This chronic immobilization stress-mediated decrease in total BDNF expression occurs despite a significant upregu-

lation of BDNF I and II mRNAs. Sustained exposure to the same processive stressor, immobilization, recruits distinct BDNF promoters indicative of differences in the activation of signaling cascades and transcription factors by the acute or chronic versions of the same stress. Unlike immobilization stress that resulted in a decrease in total BDNF expression, CUS did not alter total BDNF (V) levels, but did regulate the exon-specific BDNF transcripts (I/III-increase, II-decrease) within distinct hippocampal subfields. It is interesting to note that the stress-mediated downregulation of total BDNF expression is correlated with the ability of the stressor to adversely affect hippocampal cytoarchitecture. Immobilization stress that decreases total BDNF mRNA levels is known to cause dendritic atrophy and debranching within the CA subfields of the hippocampus (Vyas *et al*, 2002), which CUS does not regulate either total BDNF expression or CA subfield cytoarchitecture (Vyas *et al*, 2002). Although BDNF gene regulation appears to be stressor specific, there is also some overlap in the manner in which BDNF is regulated by distinct stressors (Figure 10). Interestingly, a recent report shows that chronic social stress, much like chronic immobilization, may also downregulate total BDNF expression through effects on the BDNF III and IV promoters (Tsankova *et al*, 2006). In addition, short duration immobilization has been reported to upregulate the expression of BDNF splice variants (Marmigere *et al*, 2003). Taken together, this indicates that the



**Figure 9** Influence of Chr MS on the proliferation of dentate granule cell progenitors in the postnatal and adult hippocampus. Chr MS was performed from P2 to P14 for a period of 3 h daily. Control and Chr MS animals were administered the mitotic marker BrdU on P15, P21 and in adulthood (P60) and were killed 2 h after the BrdU injection. Quantitative analysis revealed that the number of BrdU-positive cells within the SGZ/GCL was significantly higher in Chr MS animals at P21, but not in adulthood. At P15, the Chr MS animals exhibited a trend towards an increase in BrdU-positive cell number in both the SGZ/GCL as well as hilus. Shown are representative photomicrographs of BrdU-positive cells from control and Chr MS animals at P15, P21, and at P60. BrdU-positive cells (arrow) were observed in the SGZ, at the border of the hilus and the GCL, and within the hilus. The results are expressed as the mean  $\pm$  SEM ( $n = 4\text{--}6/\text{group}$ ) number of BrdU-positive cells in the SGZ/GCL and hilus. \* $p < 0.05$  indicates significantly different from control (Student's  $t$ -test).



**Figure 10** Stressors regulate multiple BDNF splice variants in a signature manner. The rat BDNF gene through the alternate splicing of four distinct 5' exons (I–IV), with unique promoters, to a common 3' exon (V), and by the use of two separate polyadenylation sites, can generate eight unique BDNF transcripts (a). Distinct stressors differentially regulate exon-specific BDNF splice variants in the postnatal and adult hippocampus (b). The postnatal stress of MS regulates specific BDNF splice variants in a duration and age-dependent manner. Adult-onset stress exhibits heterogeneity in the regulation of BDNF splice variants that is dependent on the nature, repetition and novelty of the stress. The signature manner in which adult-onset stressors regulate the expression of the BDNF gene is completely altered based upon prior history of exposure to Chr MS in early life. Ac MS—3- or 6-h duration; Chr MS—killed at P14 or P21; acute/chronic immobilization stress (AIS or CIS); CUS. Arrowheads reflect an up- or downregulation, and (—) reflects no change as compared to controls.

stress regulation of BDNF appears to be dependent on the stressor, its intensity, duration, frequency, and number of exposures (Figure 10). Although the physiological consequences of BDNF splice variants are at present unknown, they may contribute to the versatile effects of BDNF, which can serve as both an anterograde and retrograde factor,

influencing neuronal survival, cytoarchitecture, and function. Stressor-dependent differences in the regulation of the BDNF splice variants may have an important consequence in shaping the hippocampal subfield-specific vulnerability to diverse stressors. Studying the stress regulation of BDNF splice variants may prove to be an immensely valuable

starting point in identifying both the individual and overlapping stressor-recruited pathways that underlie the stressor-specific functional outcomes on the hippocampus.

In addition, the signature manner in which adult-onset stressors regulate the expression of the BDNF gene can be completely altered based upon the early life experiences of the animal (Figure 10). MS is known to influence the development of stress neurocircuitry thus directly impacting adult stress responses (Kalinichev *et al*, 2002; Ladd *et al*, 2000). Our results demonstrate that the adult-onset immobilization stress-mediated regulation of BDNF splice variants, as well as the decline in total BDNF mRNA levels, is lost in animals that have received MS in early life. However, the exceptions to this are the acute immobilization stress-induced decline in BDNF I and II mRNA in the DG and the chronic immobilization stress-mediated increase in BDNF II in the CA1, which is preserved irrespective of the prior history of the animal. It is almost as though the early life stress of MS acts to predominantly generate a 'cross-tolerance' to the adult-onset stress regulation of BDNF transcripts. This view is further supported by previous evidence that adult-onset stress does not result in a decline in neurogenesis in animals with a history of MS in early life (Mirescu *et al*, 2004). One can evoke a possible role for epigenetic mechanisms that mark specific BDNF promoters through histone modifications and altered methylation following Chr MS, which may then alter the availability of these BDNF promoters to regulation by stress in adulthood. Indeed, such epigenetic modifications have been reported for the glucocorticoid receptor gene following MS (Meaney and Szyf, 2005; Weaver *et al*, 2004). The idea that BDNF promoters may undergo epigenetic modifications is supported by evidence that such a mechanism is involved in generating a dynamic control over the developmental expression of the BDNF splice variants (Dennis and Levitt, 2005). Future investigations need to test whether MS does epigenetically modify individual BDNF promoters thus influencing their availability for regulation by stress in adult life. Multiple BDNF promoters, and the ability of experience to epigenetically modify their control, would provide a flexible mechanism via which a unique individual pattern of BDNF gene regulation by stress can be generated.

Understanding the mechanisms that contribute to the generation of individual differences in vulnerability to stress-related psychopathology is of critical importance. Our results suggest that early life adverse experience may program lasting alterations to hippocampal circuitry through a perturbation of the normal developmental profiles of CREB and BDNF transcripts, as well as hippocampal progenitor proliferation. Given the central role of the hippocampus in mood circuitry, a consequence of such long lasting alterations in hippocampal stress responses may be the establishment of individual variability in vulnerability to stress-related psychopathology.

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