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Induction of ΔFosB in the Periaqueductal Gray by Stress Promotes Active Coping Responses

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SUMMARY

We analyzed the influence of the transcription factor ΔFosB on learned helplessness, an animal model of affective disorder wherein a subset of mice exposed to inescapable stress (IS) develop a deficit in escape behavior. Repeated IS induces ΔFosB in the ventrolateral periaqueductal gray (vlPAG), and levels of the protein are highly predictive of an individual's subsequent behavorial deficit—with the strongest ΔFosB induction observed in the most resilient animals. Induction of ΔFosB by IS predominates in substance P-positive neurons in the vlPAG, and the substance P gene, a direct target for ΔFosB, is downregulated upon ΔFosB induction. Local overexpression of ΔFosB in the vlPAG using viral-mediated gene transfer dramatically reduces depression-like behaviors and inhibits stress-induced release of substance P. These results indicate that IS-induced accumulation of ΔFosB in the vlPAG desensitizes substance P neurons enriched in this area and opposes behavioral despair by promoting active defense responses.

INTRODUCTION

Members of the Fos family of proteins are induced in the brain upon neuronal activation. By dimerizing with members of the Jun family, Fos proteins participate in the formation of activator protein-1 (AP-1) transcription factor complexes, which bind to DNA at AP-1 sites and modulate the activity of target gene promoters (Morgan and Curran, 1995; Pennypacker, 1998). AP-1-mediated control of transcription has been established as a critical mechanism through which gene expression, and synaptic strength, can be regulated in an experience-dependent manner (Sanyal et al., 2002). Genes encoding Fos and Jun family proteins have been termed immediate early genes, owing to their rapid and transient pattern of induction. However, a subclass of stable Fos-related antigens (FRAs) was later identified in the brain (Chen et al., 1995; Hope et al., 1994). We now know that these chronic FRAs are composed of stabilized isoforms of ΔFosB (Hiroi et al., 1997, 1998), a truncated splice variant of FosB that lacks 101 amino acids from the C-terminal end of the full-length protein (Nakabeppu and Nathans, 1991; Yen et al., 1991). Contrary to FosB and all other Fos family proteins, ΔFosB persists in brain for an extended period of time, and accumulates to high levels in neurons after repeated stimulation by the same stimulus (see McClung et al., 2004; Nestler et al., 2001). These unique biochemical features make ΔFosB an attractive candidate for mediating long-term neuroadaptations in the brain.

Recently, we have shown that exposure to repeated stress causes ΔFosB to accumulate in several brain regions implicated in emotional learning (Perrotti et al., 2004). Here, we further examined the role of ΔFosB in processes of emotional adaptation using models of inescapable stress (IS). Repeated experiences of IS in mice disrupts their ability to later express efficient fight/flight responses. This behavioral plasticity process, referred to as "learned helplessness," is sensitive to antidepressants, and is considered a well validated model of affective disorder in rodents. Neuroanatomical pathways mediating learned helplessness have been partly established, and involve monoaminergic systems (Amat et al., 2005; Besson et al., 1998; Takase et al., 2004, 2005). Therefore, we focused our analysis on three major monoaminergic cell groups: the dorsal raphe nucleus (DR), ventral tegmental area (VTA), and locus coeruleus (LC). Based on the results presented here, we propose that stress-induced expression ΔFosB is part of an adaptive mechanism that promotes resilience to IS by inhibiting selective circuits in the brainstem that mediate the expression of passive defense behaviors.

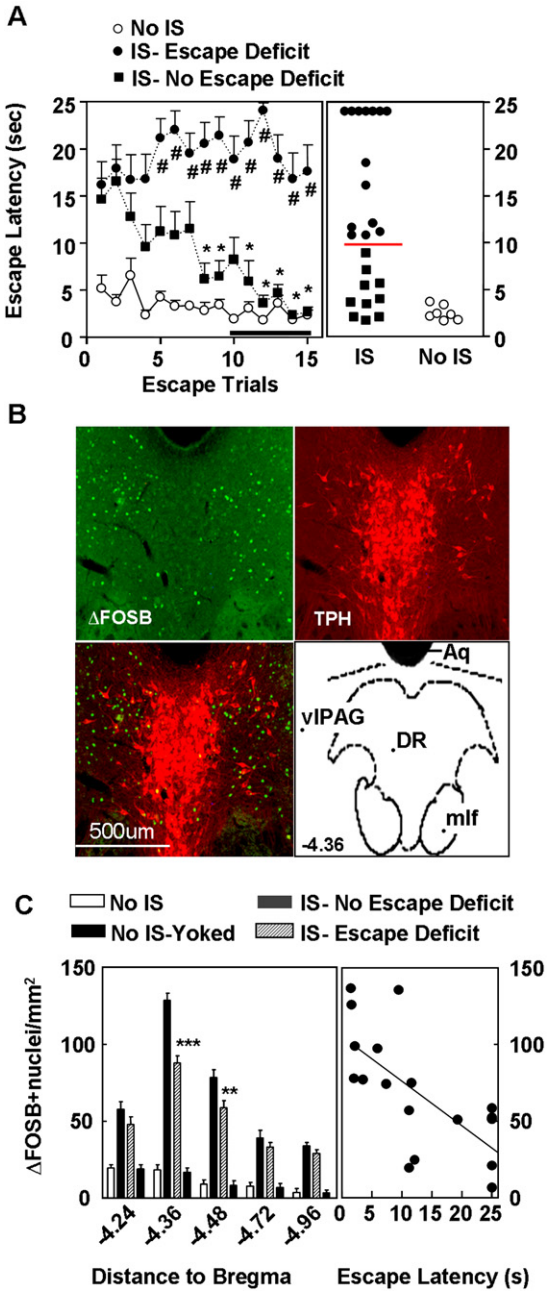

Figure 1. Escape Deficit and ΔFosB Accumulation in the vlPAG after IS

(A) IS-induced escape deficits in mice. (Left) Escape latency across 15 escape trials. (Right) Distribution of the average latency during the last five trials and median split (red line) used to discriminate between Escape deficit versus No escape deficit groups. No-IS group, n = 7, IS group, n = 28, *p < 0.05 versus IS-escape deficit, #p < 0.05 versus no-IS.

(B) ΔFosB accumulation in vlPAG. Top: (Left) Immunolabeling for ΔFosB and (right) tryptophan hydroxylase (TPH). Bottom: (Left) Overlay and (right) coronal view corresponding to AP coordinate −4.36 mm relative to bregma. Aq, aqueduct; DR, dorsal raphe; mlf, medial longitudinal fasciculus.

(C) (Left) ΔFosB accumulates most intensely in the area corresponding to the rostral portion of the DR in IS-no escape deficit group (ANOVA significant interaction between IS and rostrocaudal level, $F_{12,104}$ = 21.33, p < 0.001, ***p < 0.001, **p < 0.01 versus IS-no escape deficit) and correlates negatively with the severity of the behavioral deficit (r = −0.69, p < 0.01, n = 18).

Data are expressed as mean ± SEM.

RESULTS

Heterogeneous Escape Deficits Induced by IS in Mice

Mice with no experience of IS (no-IS group, n = 7) displayed consistently short escape latencies and an absence of escape failures across 15 successive escape trials (average escape latency: 3 ± 0 s, escape failures: 0% ± 0%). In the IS group (n = 28), repeated administration of IS resulted in an overall increase in escape latencies (14 ± 1 s, p < 0.001) and an increased proportion of escape failures (44% ± 6%, p < 0.01). Individual responses were highly heterogeneous within the IS group, especially during the last trials of the test session, as indicated by the striking spread in the average escape latencies for the last five sessions (Figure 1A, right panel). A median split of the average latency for the last five trials defines two subgroups of animals that display dramatically divergent patterns of responses within the IS group. One subgroup (IS-escape deficit) showed an escape deficit that was sustained across the 15 trials, whereas the other subgroup (IS-no escape deficit) recovered escape latencies identical to those of the no-IS group by the end of the test session (Figure 1A, left panel; two-way ANOVA, significant trial x subgroup interaction $F_{28,448}$ = 2.48, p < 0.001).

IS Induces ΔFosB Accumulation in Monoaminergic Regions

We found a robust increase in the number of FosB immunoreactive cell nuclei in the DR and LC 24 hr after repeated IS, but no change in the VTA (Figure 1B and Figure S2 in the Supplemental Data, available with this article online). Since the anti-FosB antibody used here targets an epitope common to both ΔFosB and full-length FosB, and thus does not allow us to differentiate between the two isoforms, we demonstrated in an additional experiment that FosB immunoreactivity at this time point reflects the accumulation of ΔFosB and not FosB (see Figure S1). Thus, no immunolabeling was observed in the same brain sections when an anti-FosB (C terminus) (Perrotti et al., 2005) antibody was used, which recognizes an epitope present only in the full-length FosB protein. The ability of the anti-FosB (C terminus) antibody to effectively detect full-length FosB was confirmed by detection of the protein in mice sacrificed 3 hr after an acute exposure to inescapable foot shocks (Figure S1), when expression of the full-length isoform is peaking. Similarly, ΔFosB accumulation did not coincide with induction of c-Fos, which, like full-length FosB, is a short-lived Fos family member (Figure S1).

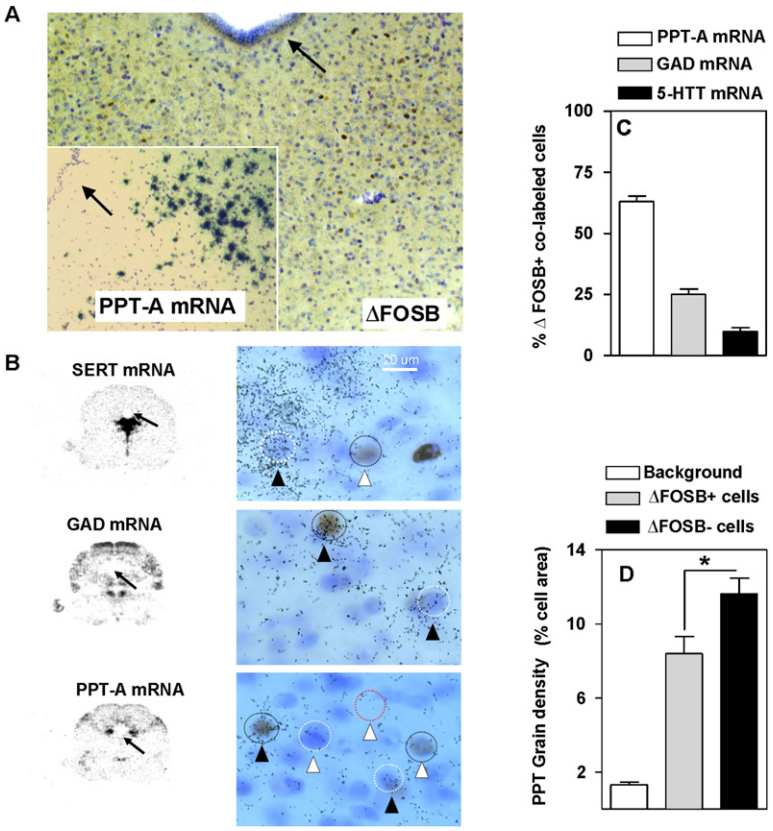

Figure 2. ΔFosB Accumulates in Substance P-Positive Neurons after IS

(A) Relative distribution of ΔFosB-positive cells (visualized with DAB) and *PPT-A* mRNA (insert, silver grains) in the vlPAG at 10× magnification. The black arrow indicates the position of the aqueduct on each view.

(B) (Left) Autoradiographs illustrating the relative distribution of SERT mRNA, GAD-67 mRNA, and *PPT-A* mRNA at the same rostrocaudal level. The thin black arrows indicate the position of the aqueduct on each view. (Right) Examples of views (100×) used to quantify mRNA grain density over ΔFosB-positive cells (black circles), ΔFosB-negative cells (white circles), and background (red circle). Arrowheads indicate cells which are either positive (black) or negative (white) for a given mRNA signal.

(C) Proportion of ΔFosB-positive cells colabeled with each type of probe in the vPAG; note the major colocalization of ΔFosB-positive cells with *PPT-A* mRNA.

(D) Density of *PPT-A* silver grains is decreased in ΔFosB-positive cells compared with ΔFosB-negative cells; *$p < 0.05$.

Data are expressed as mean ± SEM.

ΔFosB Levels in the Ventrolateral Periaqueductal Gray Correlate Negatively with the Severity of the Behavioral Deficit

The majority of FosB-like immunoreactivity induced in the DR region was observed in the lateral margins of this nucleus and comprised an area corresponding to the ventrolateral periaqueductal gray (vlPAG). Comparison of ΔFosB levels in this area across the spectrum of escape latencies (Figure 1A) indicates that mice from the IS-no escape deficit group displayed significantly higher levels of ΔFosB in the vlPAG compared with the animals from the IS-escape deficit group (Figure 1C). A similar pattern was seen for ΔFosB induction in the LC: ΔFosB levels in IS-no escape deficit group were significantly greater (26 ± 3 cells/mm^2) than those of the IS-escape deficit group (17 ± 3; $p < 0.05$).

Since the strongest induction of ΔFosB was observed in the vlPAG, as opposed to the LC (Figure S1A versus Figure S1B), we performed a more detailed characterization of the cell populations expressing ΔFosB in this region. Using tryptophan hydroxylase (TPH) as a marker of serotonin neurons, we found that the highest degree of ΔFosB accumulation occurs specifically at a rostrocaudal level corresponding to the rostral portion of the DR (between −4.24 mm and −4.48 mm relative to bregma). At this level, the number of cells immunoreactive for ΔFosB correlates inversely with the severity of the behavioral deficit following IS ($r = -0.69$, $p < 0.01$, $n = 18$), with a lower level of ΔFosB accumulation being predictive of a stronger escape deficit after IS (Figure 1C).

We performed a similar analysis in mice exposed to social defeat, another model of IS. In mice perfused 24 hr after the last defeat episode, we also found an overall increase in the number of ΔFosB-positive nuclei in the vlPAG (ANOVA main effect, $F_{2,47} = 3.34$, $p < 0.05$). Interestingly, as we had observed in the learned helplessness paradigm, this effect was significant only in the subgroup of resilient animals (Figure S3).

ΔFosB Is Induced Primarily in Substance P Neurons in the vlPAG

We next investigated the cell type in the vlPAG in which IS induced ΔFosB. By use of combined immunohistochemistry (for ΔFosB) and in situ hybridization (for substance P mRNA), we found that a majority (63%) of ΔFosB-positive cells also express substance P (Figures 2A–2C). In contrast, much lower levels of colocalization were observed between ΔFosB and serotonin (~10%, using serotonin transporter [SERT] mRNA as a marker) or GABA (~25%, using glutamic acid decarboxylase [GAD-67] as a marker). All ΔFosB expression was neuronal, since complete colocalization was observed with the neuronal marker NeuN and no colocalization was observed with glial fibrillary acidic protein (GFAP) (not shown). The pattern of ΔFosB induction in nonserotonergic neurons located at the periphery of the DR is similar to the pattern

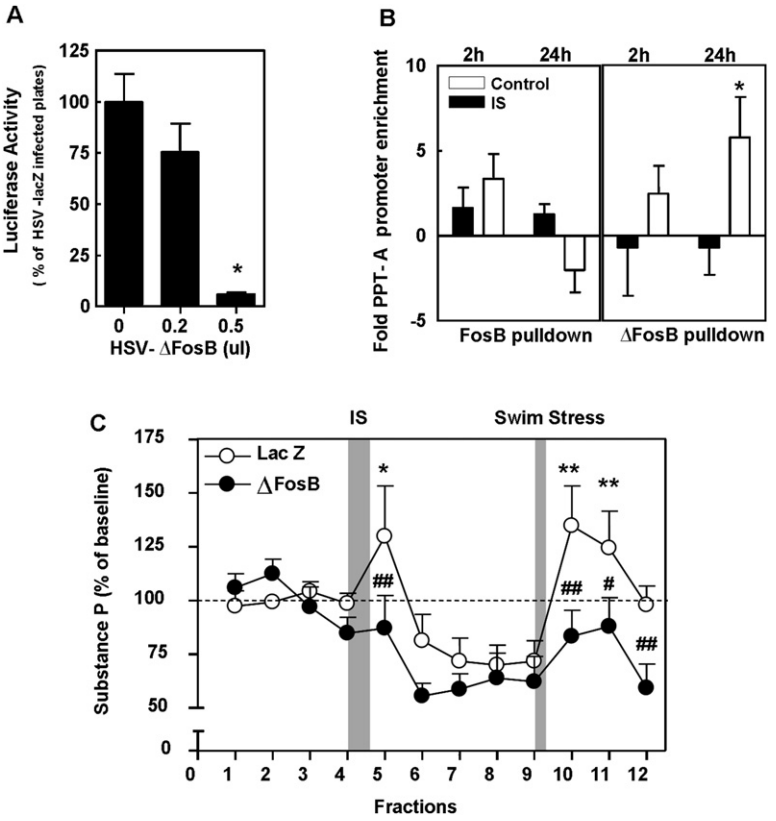

Figure 3. ΔFosB Regulates *PPT-A* Gene Expression and Stress-Induced Substance P Release

(A) Viral overexpression of ΔFosB dose-dependently inhibits *PPT-A* gene promoter activity in luciferase reporter assays; *p < 0.05.

(B) ChIP from vlPAG demonstrates the direct binding of ΔFosB (but not full-length FosB) to the *PPT-A* gene promoter in vivo, 24 hr after IS administration; *p < 0.05.

(C) In vivo microdialysis in freely moving rats. Exposure to IS or forced swimming stress increases substance P release in the NAc, an effect blocked by previous bilateral infusion of HSV-ΔFosB in the vlPAG. ANOVA effect of time ($F_{11,165}$ = 6.74, p < 0.0001) and interaction between virus and time ($F_{11,165}$ = 2.07, p = 0.02), *p < 0.05 and **p < 0.01 from baseline, #p < 0.05 and ##p < 0.01 from LacZ (n = 8–9 rats per group).

Data are expressed as mean ± SEM.

observed in the LC. In this latter region most ΔFosB-positive cells were seen just lateral to the boundary of LC noradrenergic neurons, and very few ΔFosB-positive cells colocalized with expression of the catecholaminergic marker tyrosine hydroxylase (Figure S1).

ΔFosB Decreases Substance P Gene Expression In Vitro and In Vivo

We tested whether IS-induced ΔFosB regulates substance P gene expression in three different ways. First we analyzed levels of preprotachykinin A (*PPT-A*) mRNA, the substance P precursor, over ΔFosB-positive and ΔFosB-negative cells by use of a combined immunohistochemistry-in situ hybridization method (Figure 2B). The average density of substance P mRNA silver grains was significantly lower over individual ΔFosB-positive cells compared with ΔFosB-negative cells (ANOVA effect of cell type, $F_{2,228}$ = 27.8, p < 0.001) (Figure 2D). This result led us to hypothesize that ΔFosB accumulation may contribute to repress *PPT-A* gene transcription. We tested this hypothesis in vitro by a using a promoter assay in PC12 cells in which a luciferase reporter is placed under the control of a 1.3 kb fragment of the *PPT-A* 5′ gene promoter. We found that overexpression of ΔFosB, using a herpes simplex virus (HSV) viral vector, causes a dose-dependent repression of the *PPT-A* promoter compared with use of a control HSV-LacZ vector (ANOVA dose effect, $F_{2,5}$ = 5.55, p = 0.053) (Figure 3A).

We next sought evidence as to whether this regulation represents a direct effect of ΔFosB on the *PPT-A* gene. To test this hypothesis, we performed chromatin immunoprecipitation (ChIP) assays from vlPAG tissue in mice killed 1 or 24 hr after administration of IS (n = 6 per group). We observed a significant enrichment of the *PPT-A* gene promoter (the region which contains its AP-1 sequences) after immunoprecipitation of chromatin complexes for ΔFosB (ANOVA main effect of stress, $F_{1,20}$ = 4.99 p < 0.05), but not for full-length FosB (ANOVA no effect of stress, $F_{1,20}$ = 0.367, nonsignificant) (Figure 3C). This effect reached significance only 24 hr after the administration of IS (p < 0.05). This result indicates that ΔFosB accumulating in substance P-positive neurons in the vlPAG after IS binds directly to the promoter region of the *PPT-A* gene in vivo.

ΔFosB in the vlPAG Modulates Stress-Induced Substance P Release in the Nucleus Accumbens

To test whether the regulation of substance P gene expression in vlPAG neurons by ΔFosB may contribute to functional alterations of forebrain substance P neurotransmission, we overexpressed ΔFosB (or an inactive LacZ construct) locally within the vlPAG using HSV vectors and looked for changes in substance P release in a known projection area of these neurons, the nucleus accumbens (NAc). To mimic the endogenous pattern of expression of ΔFosB, viral infusions were aimed at the lateral margins of

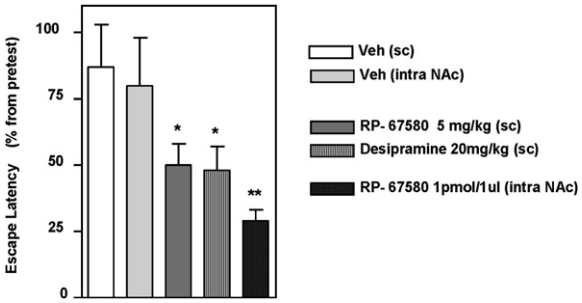

Figure 4. NK1 Receptor Blockade in the NAc Has an Antidepressant-like Effect

The selective NK1 receptor antagonist RP67580, administered either systemically or directly into the NAc, significantly reduced escape latencies (ANOVA main drug effect, $F_{4,54} = 3.13$, $p < 0.05$), an effect shared by the subchronic administration of the antidepressant desipramine in this test. *$p < 0.05$ and **$p < 0.01$ from respective control conditions. Intracerebral injection, n = 7 mice per group; systemic drug injection, n = 17–18 group. Data are expressed as mean ± SEM.

the serotonergic cell population (Figure 6). We measured substance P release, both under baseline conditions and following stress exposure, using in vivo microdialysis in freely moving animals. Baseline extracellular concentrations of substance P in the NAc were not significantly altered by ΔFosB overexpression (13.9 ± 3.0 fmol/dialysate for the LacZ group and 13.5 ± 1.7 fmol/dialysate for the ΔFosB group). In contrast, the substance P response to stress and its return to baseline were significantly affected by ΔFosB: ANOVA effect of time, $F_{11,165} = 6.74$, $p < 0.0001$; and interaction between virus and time, $F_{11,165} = 2.07$, $p = 0.02$) (Figure 3C). Foot shocks and swim stress each significantly increased substance P efflux in the NAc of LacZ-injected animals by 30% and 120%, respectively. Overexpression of ΔFosB in the vlPAG significantly attenuated the foot shock-induced and swim stress-induced enhancement of substance P release in this brain region (Figure 3C).

NK1 Receptor Blockade in the NAc Has an Antidepressant-like Effect

Our results so far indicated that exposure to IS leads, in resilient animals, to a strong induction of ΔFosB in the vlPAG, and apparently to a desensitized substance P input to the NAc. To further understand the functional significance of this decreased substance P input, we blocked substance P receptors in the NAc by administering RP67589, a selective NK1 receptor antagonist, prior to the test session. We found that injection of RP67580, either systemically or directly into the NAc, significantly reduced escape latencies (ANOVA main drug effect, $F_{4,54} = 3.13$, $p < 0.05$) (Figure 4). We observed a similar profile of activity in this test after subchronic administration of the traditional antidepressant desipramine (Figure 4). Together, these results indicate that decreased substance P signaling in the NAc exerts an antidepressant-like effect and promotes active escape responses in the learned helplessness paradigm.

Overexpression of ΔFosB in the vlPAG Opposes Behavioral Despair

To further test the hypothesis that ΔFosB induction in the vlPAG modulates defense responses to IS, we manipulated levels of ΔFosB or other AP-1-related transcription factors in this brain region using HSV vectors, and we evaluated behavioral responses after exposure to IS. Mice that received intra-vlPAG injections with a control HSV vector expressing LacZ displayed a significant increase in escape latency and an increased proportion of escape failures following IS in the learned helplessness test (Figures 5A and 5B), a result in line with our previous observation in noninjected mice (Figure 1). These findings indicate that stereotaxic surgery and viral infection per se do not affect IS-induced escape deficits. In contrast, mice injected with HSV-ΔFosB showed less severe, and nonsignificant, behavioral deficits after IS. This result is consistent with the correlation observed previously and provides direct evidence that induction of this transcription factor in the vlPAG increases resilience to IS. Interestingly, a similar effect was obtained after overexpression of the truncated protein ΔJunD. In contrast, viral-mediated overexpression of full-length FosB caused the opposite effect and potentiated the severity of the IS-induced escape deficit (Figures 5A and 5B). Importantly, none of these viral vectors altered escape behaviors in the absence of IS administration, nor did they have any effects on general locomotion, motor coordination, and sensitivity to foot shocks (Figures 5C–5E). Moreover, a similar antidepressant-like effect of HSV-ΔFosB in the vlPAG was observed in a second, well-validated model that is also sensitive to antidepressant treatments, i.e., the forced swimming test. As illustrated in Figure 6D, ΔFosB bilaterally injected in the vlPAG reduced floating behavior ($p < 0.01$) and increased time spent struggling ($p < 0.05$) and swimming ($p < 0.05$) compared with control conditions.

DISCUSSION

We have reported recently that exposure to IS causes ΔFosB to accumulate in several brain regions implicated in the expression of emotional responses (Perrotti et al., 2004). The goal of the present study was to examine the functional consequences of ΔFosB induction by IS.

Learned Helplessness: Neuroplasticity of Defense Systems

Repeated exposure to IS in mice disrupts their ability to later express active fight/flight responses in escapable situations (Chourbaji et al., 2005). This experience-dependent shift from active defense to a passive behavioral repertoire (i.e., learned helplessness) occurs in some, but not all, animals exposed to IS (Figure 1A) and can be corrected by short-term treatment with a broad spectrum of antidepressants (Caldarone et al., 2000; Chourbaji et al., 2005)

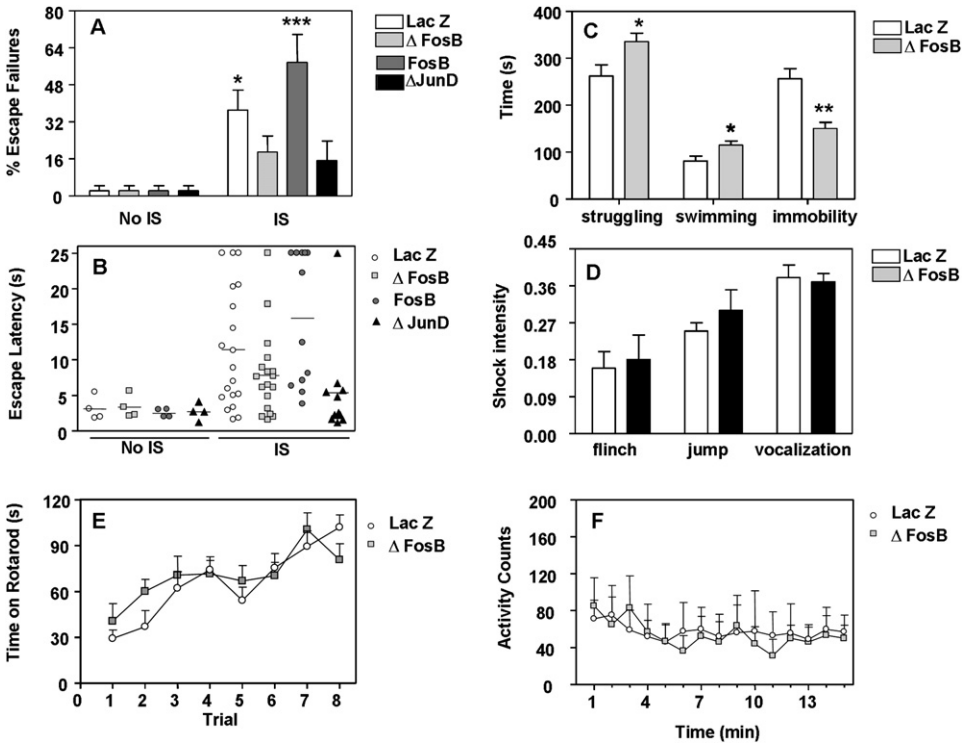

Figure 5. Viral Overexpression of ΔFosB in the vlPAG Opposes the Development of an IS-Induced Escape Deficit and Immobility in the Forced Swimming Test

IS administration increased the percentage of escape failures (A) and average escape latency (B) in mice that received intra-vlPAG injections of the control HSV-LacZ vector (n = 19), similar to results seen in noninjected animals (see Figure 1). In contrast, HSV-ΔFosB (n = 18), as well as HSV-ΔJunD (n = 11), reduced the development of such escape deficits, while HSV-FosB (n = 12) had the opposite effect. ANOVA main effect of IS on escape latency, $F_{3,68} = 12.88$, $p < 0.001$, and percentage of escape failures, $F_{3,68} = 12.36$, $p < 0.001$, *$p < 0.05$, ***$p < 0.001$ versus animals from the no-IS condition. None of these viral vectors altered escape performances in the absence of IS administration. Overexpression of ΔFosB also decreased immobility in the rat forced swimming test (C) ($p < 0.01$) while increasing time spent struggling ($p < 0.05$) and swimming ($p < 0.05$); n = 8–9 per group. On the other hand, HSV-ΔFosB did not alter sensitivity to foot shocks (D), general locomotion (E), motor coordination (F), or social interactions (not shown). Data are expressed as mean ± SEM.

such as the tricyclic antidepressant desipramine used in the present study (Figure 4). In the absence of treatment, extinction of learned helplessness occurs after a short delay in undisturbed animals (Chourbaji et al., 2005; Maier, 2001). However, exposure to contextual reminders stabilizes escape deficits for several weeks after interruption of the stress (Maier, 2001), an observation suggesting that IS causes stable neuroplastic changes in neuronal networks mediating defensive responses.

ΔFosB Accumulates in the DR and LC, but Not in the VTA, after Repeated IS

We show here that ΔFosB accumulates in the DR and LC, but not in the VTA, after repeated administration of IS. A previous study reported an identical pattern of induction of c-Fos upon re-exposure to an environment previously associated with IS (Ishida et al., 2002). Taken together, these results suggest a preferential involvement of serotonergic and noradrenergic versus dopaminergic cell groups in the associative processes supporting the development of learned helplessness.

ΔFosB Induction in the DR Area Predicts Individual Degrees of Resilience to IS

It is important to point out that, in our study, increased levels of ΔFosB were observed in mice perfused 24 hr after the last administration of repeated IS, and immediately after a brief "escapable" test session. In these conditions, we presumed that the FosB accumulation is caused by the repeated IS administered 24 and 48 hr beforehand, and not by the brief test session performed 15 min preceding perfusion (which would not allow sufficient time for new protein synthesis). This was confirmed by the observation that mice from the no-IS-yoked group, which received no repeated IS during the training session but the same amount of foot shocks as the IS animals during the test session, showed virtually no ΔFosB accumulation (Figure 1C). We also demonstrated that the FosB/ΔFosB signal 24 hr after IS reflects the accumulation of the stable, truncated isoform ΔFosB, and not full-length FosB (Figure S1). These results are in good agreement with the patterns of expression of ΔFosB that we have reported earlier in other brain regions in response to stressors

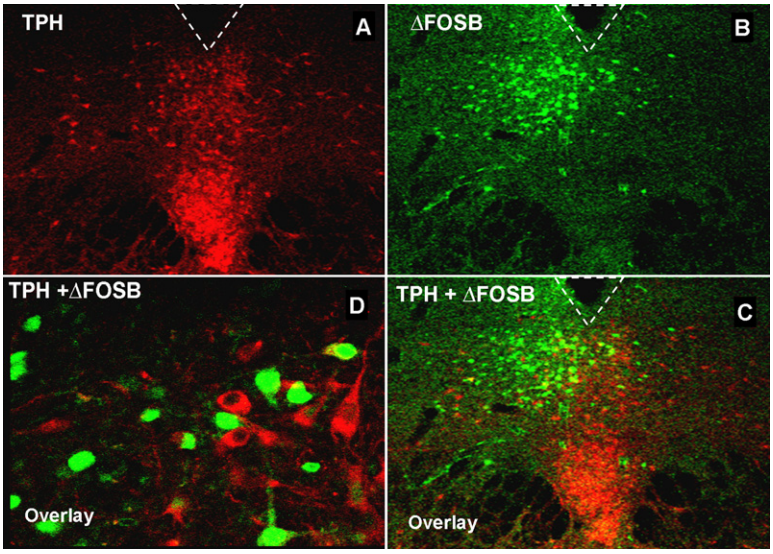

Figure 6. Viral Overexpression of ΔFosB in the vlPAG

Example of HSV-ΔFosB infection in the vlPAG. The injections were aimed at the lateral margins of the DR in order to mimic the endogenous pattern of ΔFosB induction after IS (see Figure 1B and Figure S1). (A) Serotonergic neurons were immunolabeled with an antibody against tryptophan hydroxylase, and (B) the viral overexpression was revealed with an antibody against ΔFosB. The overlays at low (C) and high (D) magnification confirm the location of the infection at the lateral margins of the serotonergic cell groups (D). Dashed triangles indicate the position of the aqueduct on each picture.

and many other types of stimuli (McClung et al., 2004; Perrotti et al., 2004, 2005).

In the region where the highest degree of induction is observed (the vlPAG, corresponding to the rostral portion of the DR), the number of cells immunopositive for ΔFosB was inversely proportional to the severity of the escape deficit after IS (Figure 1C). In other words, the level of ΔFosB induction by IS in this brain region predicts the individual degree of resilience to IS. To test whether this pattern of ΔFosB expression is produced specifically by the learned helplessness paradigm (or whether it may result from exposure to other types of repeated IS), we evaluated ΔFosB levels in the vlPAG of mice subjected to another IS paradigm which elicits learned effects that are sensitive to antidepressants. Using a repeated social defeat paradigm, which also triggers long-lasting alterations of defense responses (Berton et al., 2006), we observed a similar relationship between the level of ΔFosB in the vlPAG and the individual degrees of resilience (see Figure S1). This observation suggests that ΔFosB induction may constitute a signature neuroadaptation in defense circuits that is uniquely linked to the development of resilience to IS. The origin of the behavioral and physiological interindividual variations observed here is unknown. Since our experimental population is composed of inbred mice, these variations cannot be attributed to genetic heterogeneity, but may reflect epigenetic, stochastic, or environmental influences (Maier and Watkins, 2005).

ΔFosB Induction Occurs in Passive Coping-Related Circuits in the vlPAG

Closer examination of the pattern of induction of ΔFosB in the DR region indicates that immunopositive cell nuclei are clustered in the lateral margins of the rostral DR, within the vlPAG (Abrams et al., 2004). This distinctive pattern of induction is particularly interesting since the vlPAG has been characterized previously as an important neural substrate for passive coping responses (Bandler and Shipley, 1994). IS, which induces primarily passive behavioral responses, engages the vlPAG (Bandler and Shipley, 1994). Reciprocally, activation of the vlPAG (using electrical stimulation or infusion of excitatory amino acids) evokes passive behavioral coping reactions of quiescence or immobility and hyporeactivity to the environment (Bandler and Shipley, 1994; Johnson et al., 2004), i.e., a behavioral profile similar to the one observed in helpless mice during the test session. Based on these observations, we hypothesized that IS-induced accumulation of ΔFosB, and subsequent alterations in AP-1 transcription, may act to desensitize passive coping-related circuits in the vlPAG.

ΔFosB in the vlPAG Mediates Its Antidepressant-like Effects by Acting as an Endogenous Antagonist of AP-1 Sites

Considerable evidence indicates that ΔFosB can act as either a transcriptional repressor or activator both in vitro and in vivo (Chen et al., 1997; Dobrazanski et al., 1991; McClung and Nestler, 2003; Nakabeppu and Nathans, 1991; Wisdon and Verma, 1993; Yen et al., 1991). The similar antidepressant-like activity of both ΔFosB and ΔJunD overexpression in the vlPAG (Figure 5) suggests that ΔFosB acts in this region after IS as an endogenous antagonist of AP-1 transcription. Indeed, ΔJunD, which lacks an N-terminal transactivation domain, is a truncated variant of JunD, the major in vivo partner for ΔFosB (Chen et al., 1995; Hiroi et al., 1998). In Jun family members, truncation of the highly conserved N-terminal transactivation domain always creates dominant-negative antagonists of AP-1-mediated transcription (Brown et al., 1996; Hirai et al., 1989; Lloyd et al., 1991; Peakman et al., 2003; Struhl, 1988). This conclusion is also reinforced by the observation of an opposite (prodepressant) behavioral

influence exerted by full-length FosB, a transcription factor unambiguously characterized as an AP-1 activator (Schuermann et al., 1991; Wisdon and Verma, 1993; Yen et al., 1991). It will be important in future studies to identify the factors that determine whether ΔFosB represses or activates a given target gene.

Substance P Is a Direct Target of ΔFosB in the vlPAG

Given the clustering of ΔFosB-expressing cells at the periphery of the DR in the vlPAG, we hypothesized that these cells may express the neuropeptide substance P, which is known to be expressed in this area with a similar pattern (Barbaresi, 1998; Harlan et al., 1989; Hurd et al., 1999; Warden and Young, 1988). Our in situ hybridization results confirmed that the ΔFosB immunopositive cell population is composed primarily of substance P-expressing neurons. It is not clear which type of neurons coexpress substance P in this region of the mouse brain. Although substance P has been shown to be highly colocalized with serotonin in the human DR, and to a lesser extent in the rat's, this does not seem to be the case in the mouse, at least at this rostrocaudal level (Chan-Palay et al., 1978). Colabeling for GAD-67 mRNA suggests that GABAergic neurons represent only a low proportion of the ΔFosB-expressing population, although we cannot exclude the possibility that some GABAergic neurons in this region may express other isoforms of GAD (Day et al., 2004).

The *PPT-A* gene encoding substance P is reported to contain AP-1-like sites within its 5′ promoter region (Fiskerstrand et al., 2000; Paterson et al., 1995). Therefore, we tested the possibility that IS induction of ΔFosB in substance P-positive neurons might feed back and regulate expression of substance P in these cells. Our ChIP data indicate that the *PPT-A* gene is in fact a direct target of ΔFosB in these neurons: increased binding of ΔFosB, but not full-length FosB, occurs at the *PPT-A* promoter after IS. This binding activity is delayed and reaches significance at 24 hr after the administration of IS. The *PPT-A* promoter region evaluated for FosB/ΔFosB binding spans the nucleotides −420 to −256 upstream of the transcription start site. Binding of c-Fos containing AP-1 complexes to this regulatoty region, which contains two tandem AP-1 elements, has been shown to stimulate *PPT-A* transcription (Paterson et al., 1995). This is consistent with our interpretation that ΔFosB, acting as a transcriptional repressor, inhibits *PPT-A* expression, while full-length FosB activates it.

ΔFosB Limits the Activation of Substance P Neurotransmission during IS: An Adaptive, Negative-Feedback Mechanism

Substance P neurons within the vlPAG are known to send their principal ascending projections to the NAc and the amygdala (Li et al., 1990a, 1990b), a subset of brain regions implicated in defense responses wherein the primary receptor for substance P (NK1) is enriched. As shown in the present study, exposure to experimental stresses triggers the release of substance P in the NAc, whereas blockade of NK1 receptors in this brain region has antidepressant properties, consistent with the substantial literature for a role of substance P and NK1 in depression models (Bilkei-Gorzo et al., 2002; Boyce et al., 2001; Ebner et al., 2004; Rupniak et al., 2000; Smith et al., 1999). Our in vitro and in vivo results indicate that the accumulation of ΔFosB in the vlPAG after IS represses substance P gene expression in this brain region and desensitizes stress-induced substance P release in the NAc (Figure 2 and Figure 3). These data support a model in which repeated activation of substance P neurons in the vlPAG would lead to an AP-1-mediated desensitization of the vlPAG-NAc substance P pathway. This desensitization, the state of which can be mimicked by NK1 antagonists infused into the forebrain (Ebner and Singewald, 2006), would occur only in a subset of animals, and contribute to the inhibition of passive coping-related circuits and the maintenance of an active behavioral repertoire. In the rest of the population, in which ΔFosB levels do not accumulate sufficiently to repress substance P signaling, a quiescent behavioral repertoire would gradually develop upon re-exposure to the stressor. In addition to these effects in distal projection areas, a possible regulation of neuronal networks within the PAG itself, by substance P expressed and released locally, might be relevant to the development of learned helplessness (Barbaresi, 1998; Jansen et al., 1998; Johnson et al., 2004). Indeed, substance P neurons from the vlPAG project to, and possibly regulate the activity of, the nearby serotonin neurons in the DR through a glutamate-dependent mechanism (Valentino and Commons, 2005). Accordingly, the infusion of substance P or NK1 agonists within the DR/vlPAG modulates the firing of serotonin neurons (Valentino and Commons, 2005) and inhibits escape responses induced by IS (Johnson et al., 2004; Mongeau et al., 1998).

Our present results provide a fundamentally novel and testable model for the mechanisms of resilience. Our future studies will test the hypothesis that antidepressant treatments may enhance resilience by stimulating these same adaptive processes which occur spontaneously in some, but not all, of the individuals in a population exposed to chronic stress.

EXPERIMENTAL PROCEDURES

Animals

Eight-week-old C57 male mice (Jackson Labs, Bar Harbor, ME) were used in all but the microdialysis experiments. Animals were housed four to five per cage in a colony maintained at constant temperature (23°C) with a 12:12 hr light/dark cycle (lights on from 7:00 a.m. to 7:00 p.m.) and ad libitum food and water. All procedures were approved by the Institutional Animal Care and Use Committee of UT Southwestern.

For microdialysis experiments male Sprague-Dawley rats weighing 250–350 g (Charles River, Sulzfeld, Germany) were used. Prior to use, the animals were housed in groups of four to six under controlled laboratory conditions (12:12 hr light/dark cycle with lights on at 7:00 a.m., 21°C ± 1°C, 60% humidity, and ad libitum food and water).

Behavioral Procedures

All behavioral procedures in mice, save for the measures of motor coordination and social interaction, were performed using the active avoidance system of Med Associates (Georgia, VT). This system is a shuttle box divided into two compartments separated by an automated door. A grid floor delivers scrambled shocks to either side of the box. Movements of mice are detected using photocells.

IS, or Training Sessions

On days 1 and 2, mice receiving inescapable shocks (IS group) were confined in one compartment of the shuttle box and received 120 instances of IS through the grid floor (0.45 mA, 5 s duration). IS was distributed randomly over 1 hr, with an average intershock interval of 30 s. Animals from the no-IS and no-IS-yoked groups were placed in the same apparatus for 1 hr, but were not administered any IS.

Escape Trials, or Testing Sessions

Mice were reintroduced into the shuttle box for 15 consecutive escape trials. During each trial, a continuous shock was delivered and mice were given the opportunity to escape it by entering the adjacent, unelectrified compartment. Following a successful escape, the door was automatically shut and the escape latency recorded. When mice did not escape within 25 s, the trial was terminated and recorded as a failure. An additional no-IS-yoked group was used, in which mice were not given the opportunity to escape shocks during the test session, but were individually coupled to the mice from the IS group, and thus received the exact same amount of shocks.

In experiments including pharmacological treatments, mice were pretested on day 3, after vehicle administration, to establish baseline escape latency and balance experimental groups. They were tested again on day 6 after repeated vehicle or drug administration.

Locomotor Activity

Independent groups of mice were used to determine effects of viral infusions on locomotor activity. On day 4 and 5 after surgery, animals were habituated to the shuttle box for 1 hr, and on day 6 locomotor activity was measured for 15 min.

Sensitivity to Foot Shocks

Independent groups of mice were used to determine the threshold sensitivity to foot shocks. Mice were placed in the shuttle box and after 2 min of habituation, foot shocks were administered every 30 s, starting at 0.05 mA with a 0.05 mA increment between each shock. The first appearance of a flinch, a jump, and an audible vocalization were recorded.

Rotarod Test of Motor Coordination

For the rotarod test (IITC Life Science, Woodland Hills, CA), each mouse was placed on a rotating rod accelerating from 0 to 45 rpm in 60 s. The speed, the latency to fall, and the distance traveled on the rod were measured. The test was repeated for a total of 8 runs, each 15 min apart.

Social Defeat and Avoidance

Social defeat and avoidance testing were performed according to previously published protocols (Berton et al., 2006; Tsankova et al., 2006). Experimental mice were submitted to 10 consecutive days of social defeat and were evaluated for social approach/avoidance on day 11. Time spent in the interaction zone during the first (no social target) and second (target present) 2.5 min interaction trials was evaluated. "Truly defeated mice" were defined as the mice with an interaction ratio <100%, whereas resilient mice had interaction ratios >100% (V.K., M. Han, D. Graham, O.B., W. Renthal, Q. Laplant, A. Graham, S. Russo, M. Lutter, and D. Lagace, unpublished data). For immunohistochemistry, mice were perfused immediately following the termination of the 5 min test session, 24 hr after the last defeat experience.

Stereotaxic Surgery

Mice were anesthetized using ketamine/xylazine solution, and stereotaxic surgery was performed under standard conditions (Hommel et al., 2003). For bilateral viral infusions in the vPAG area, Hamilton syringe needles (at a 22° angle) were targeted to 0.56 mm caudal to interaural line, +0.80 mm lateral to midline, and 2.80 mm ventral from dura. A total of 0.5 μl of purified HSV virus was delivered on each side over a 4 min period, followed by 6 min of rest. Mice were then left undisturbed for 3 days (Barrot et al., 2002) and started the first training session on day 4 post surgery. For verification of injection sites, brains were processed as described above and slices corresponding to the region of interest were inspected under a microscope. Animals were rejected when injection sites were found on slices outside of the −4.24 mm to −4.72 mm interval respective to bregma, or outside of the 1 mm^2 region described above. Less than 15% of all animals were rejected due to inaccurate injection sites.

To administer drugs into the NAc of awake, freely moving mice, bilateral guide cannulas (26G, 4.5 mm long, 1.5 mm apart, Plastics One, Roanoke, VA) were chronically implanted in this brain region. (+1.2 mm anteroposterior [AP], −4.5 mm dorsoventral [DV]) and secured with Cerebond (myNeuroLab, St. Louis, MO). Mice were singly housed and left to recover for 10 days post surgery. On the day of the intracerebral infusion, bilateral injectors (33G, projecting 0.5 mm below the tip of the guide, Plastics One, Roanoke, VA) were inserted into the guides for the duration of the infusion.

For microdialysis experiments, rats were anesthetized with sodium pentobarbital/ ketamine. They first received bilateral microinjections of HSV virus (1 μl on each side over a 6 min period, followed by 2 min of rest) into the vlPAG (coordinates: −7.6 mm AP, +0.5 lateral (L), 5.8 mm below the surface of the skull). Immediately after vlPAG infusions, a U-shaped microdialysis probe (1.0 mm membrane, Hemophan, molecular cutoff of 18 kDa) was implanted into each animal's NAc (1.6 mm rostral to bregma, 1.2 mm lateral to the midline, 7.8 mm below the surface of the skull). After surgery, rats were housed individually and left to recover for 3 days, during which they received daily handling. For histological verification of the localization of viral injections and microdialysis probe placement, brains were sectioned and 30 μm coronal sections were stained with cresyl violet.

Microdialysis with Simultaneous Behavioral Analysis

Microdialysis experiments were conducted as described (Ebner et al., 2004), 3 days after surgery, in rats previously habituated to the procedure room for 24 hr.

Briefly, the probe was connected to a microsyringe pump and superfused with artificial cerebrospinal fluid (see Ebner et al., 2004 for details). Samples were collected every 30 min and stored at −80°C until assay. The first four samples were collected as baseline release for substance P. At the beginning of the fifth dialysis interval, animals were transferred for 20 min into a Plexiglas chamber equipped with a grid floor delivering electric shocks (0.7 mA, ten inescapable shocks, duration of 5 s each, 1 shock every 120 s). After the last shock, rats were returned to their home cages and four consecutive 30 min dialysates were collected. Rats were then transferred from their home cages to a swim tank (35 × 35 × 42 cm; filled with water at 20°C up to a 30 cm height) for 10 min with ongoing dialysis. During the forced swimming procedure, behavior was videotaped and scored a posteriori by a trained observer. The behavior of the animals was assigned to one of the three following behavioral categories: (1) struggling, (2) swimming, and (3) floating. After 10 min the animals were returned to their home cages and microdialysis was continued for 80 min. The concentration of substance P was measured in microdialysates using a highly sensitive radioimmunoassay (detection limit 0.1 fmol/sample). For detailed descriptions see Ebner et al. (2004).

Drug Treatments

Desipramine (20 mg/kg, Sigma) and RP67580 (5 mg/kg, Tocris) were given subcutaneously in 10 ml/kg of 1% DMSO. The injections were performed once daily for four consecutive days, with the last injection 30 min prior to testing in the learned helplessness paradigm. For intracerebral administration, RP67580 to a concentration of 1 μM was infused in a volume of 1 μl over 5 min, with 0.1% DMSO as a vehicle. The injector was left in place for 2 extra minutes to allow diffusion of the drug, and the mice were immediately tested in the learned helplessness paradigm.

Immunohistochemistry and Combined Immunohistochemistry-In Situ Hybridization

Immunohistochemistry

Perfusion (conducted immediately after the 15 min test session) and staining were performed according to standard procedures (Peakman et al., 2003). For FosB detection we used a 1:500 dilution of a rabbit polyclonal antiserum (SC7203, SantaCruz Biotechnology, Santa Cruz, CA), and TPH immunolabeling was performed using a 1:1000 dilution of a sheep polyclonal antiserum (AB1541, Chemicon, Temecula, CA).

Combined Immunohistochemistry-In Situ Hybridization

Sections were prepared and stained with the FosB antibody as described above but with the following modifications (Peakman et al., 2003). Imunolabeling was conducted in RNase-free conditions and revealed using the avidin-biotin complex method and DAB. Sections were hybridized overnight at 60°C in buffer containing ^{35}S-labeled RNA probes at 2.0 × 10^6 counts per minute per slide. Autoradiography and emulsion procedures were conducted as described elsewhere (Peakman et al., 2003).

Riboprobes

Antisense ^{35}S-labeled riboprobes were transcribed in the presence of ^{35}S-UTP and purified by using RNA Quickspin columns (Roche, Basel, Switzerland). *PPT-A* riboprobe template was a 468 bp fragment corresponding to nucleotides 25–532 of the rat PPT mRNA (Krause et al., 1987). The template was linearized using HinDIII and transcribed using T7 RNA polymerase. GAD-67 riboprobe was transcribed using T7 RNA polymerase from a 360 bp template (nucleotides 1324–1683 of the feline GAD-67 cDNA, 94% homologous to the murine sequence) linearized with BamHI (Jones et al., 1994). To produce a ribobrobe against the SERT, a 470 bp fragment of the murine 5-HTT cDNA corresponding to the 3′UTR of the short spliced variant (nucleotides 143–613 of exon 14; see Bengel et al., 1997) was subcloned into a TOPO cloning vector (Invitrogen, Carlsbad, CA). For in vitro transcription of antisense probe, the vector was linearized using XhoI and transcribed using SP6 RNA polymerase.

Cell Countings

After double-labeling for FosB and trypyophan hydroxylase, FosB-positive nuclei were counted from coronal views of the DR (1.1 mm^2 area extending 1.1 mm ventral to the tip of the aqueduct and 0.55 mm lateral to the midline). All pictures were captured the same day under fixed microscope and camera settings. Cell counts from captured images were averaged for each animal across three consecutive slides at six independent rostrocaudal levels ranging from −4.24 mm to −4.96 mm relative to bregma. Rostrocaudal levels were determined using the pattern of TPH labeling as a landmark (see Figure S1). We also referred to a published anatomic atlas of TPH immunoreactivity (Abrams et al., 2004).

Silver Grain Countings

Silver grains were quantified after in situ hybridization using a Leeds microscope equipped with an x-y-z stage connected to a personal computer running Bioquant Nova (Bioquant Image Analysis, Nashville, TN). Pictures were captured from random sites inside of the area of interest using a 100× magnification. All the pictures were captured the same day under fixed microscope and camera settings.

Quantification of Double-Labeling

The relative degree of colocalization of FosB with SERT mRNA, GAD-67, or *PPT-A* mRNA was evaluated. Cresyl violet (FosB-negative) or DAB (FosB-positive) -stained cells were surrounded by a 20 μm diameter circle. Silver grains inside of each defined circle were digitized and their cumulated area was determined using the Bioquant software. The same procedure was used to measure grain density at random background sites. A cell was considered positive for a given mRNA signal when the density of silver grains was more than three times over the average background density for a given riboprobe. A total of 150 cells from three slides in three animals from the IS condition were counted for each riboprobe. The same approach was used to evaluate the effect of ΔFosB expression on the levels of *PPT-A* mRNA. Grain density was expressed as a ratio of grain area to cellular area. A total of 60 FosB-positive and 150 FosB-negative cells were analyzed from three slices in three animals from the IS condition.

ChIP Assays

Punch dissections of vlPAG from mice submitted to the IS or control condition were taken 1 and 24 hr after the end of the second training session. Tissues were crosslinked, washed, and pooled between three to four mice. ChIP was performed based on a protocol from Upstate Biotechnology ChIP kit and published methods (Tsankova et al., 2004). Aliquots of fixed, sheared chromatin were incubated overnight, first with mouse anti-FosB antibody (C terminus antibody; Center for Biomedical Inventions, UT Southwestern), which recognizes full-length FosB only, followed by rabbit anti-FosB antibody (SC048 Santa Cruz, CA), which recognizes the middle region of both full-length FosB and ΔFosB. Nonimmune IgG was used as a control. The binding of full-length FosB and ΔFosB to the *PPT-A* promoter was determined by measuring the amount of the *PPT-A* promoter pulled down in chromatin immunoprecipitates by use of real-time PCR (ABI Prism 7700, Applied Biosystems, Foster City, CA) using the following oligonucleotide pairs. For the *PPT-A* promoter the primers 5′-GCGGAAGTTATTTGGCTGTC-3′ and 5′-ACAATCTGACGCCCTCC TC-3′ amplified a 164 bp product spanning the portion −420 to −256, a region known to contain an AP-1-AP-1-E Box sequence necessary for transcriptional regulation of *PPT-A* by AP-1 complexes (Paterson et al., 1995). The oligonucleotides used for the synaptophysin control promoter were TCATCTGGTAGAACTGAGCGGTC-3′ and 5′-GAGGCTGTGGGTTTTAGAGGAA-3′.

Viral Vectors

Construction and packaging of viral vectors has been described previously (Barrot et al., 2002). Transgenes (cDNAs for Lacz, FosB, ΔFosB, and ΔJunD149) were inserted into the HSV amplicon HSV-PrPUC and packaged into the virus using the helper 5 dl1.2, as described previously (see Neve et al., 2005). The average titer of viral stocks was 2.0 × 10^7 infectious units/ml. Clone #63024, obtained from the American Type Culture Collection (ATCC, Rockville, MD) and comprising a 1.67 kb insert encoding mouse JunD, was used to produce ΔJunD (or ΔJunD149) by PCR. ΔJunD is a truncated JunD insert that begins at amino acid 149, immediately after the homologous His that terminates the transactivation domain in all Jun family members. A FLAG epitope tag, a suitable Kozak sequence, and an initiating Met residue were also incorporated into the forward PCR primer. The resulting 617 bp PCR product was gel purified and subcloned into the HSV PrpUC at the EcoR1 site. This ΔJunD is distinct from the previously described endogenous short variant of JunD, ΔJunD48, which only lacks 48 amino acids (144 bp) at the N-terminal extension (Okazaki et al., 1998) and displays only subtle functional differences when compared with the full-length JunD protein (Yazgan and Pfarr, 2001, 2002). In contrast, ΔJunD149 used here has virtually no transactivational activity and, instead, functions as a dominant-negative of AP-1-mediated transcription (Hirai et al., 1989; McClung and Nestler, 2003; Peakman et al., 2003; Struhl, 1988).

Luciferase Assays

Luciferase assays were performed as described previously (Peakman et al., 2003) using a *PPT-A*-LUC reporter plasmid (courtesy of J.P. Quinn, University of Edinburgh, UK). A fragment of the *PPT-A* promoter spanning nucleotides –865 to +447 was subcloned into a pGL3-basic vector (Promega, Madison, WI) expressing firefly luciferase (Fiskerstrand et al., 2000). PC12 cells were cotransfected with the *PPT-A*-LUC reporter plasmid and the Renilla luciferase reporter pRL-TK to control for transfection efficiency. After 24 hr, cells were infected with HSV-LacZ or HSV-ΔFosB and harvested after 48 hr. Aliquots normalized for protein concentrations were assayed for reporter gene activity using the Dual-Luciferase Reporter Assay System from Promega.

Statistics

Dual comparisons were performed using Student's t tests except for microdialysis results which were analyzed using nonparametric Mann-Whitney U-tests. For multiple group comparisons and repeated measures, we conducted one- or two-way ANOVAs followed by Fisher post hoc tests.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/55/2/289/DC1/.

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