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Different Levels of Fos Immunoreactivity After Repeated Handling and Injection Stress in Two Inbred Strains of Mice

ANDREY E. RYABININ, YUAN-MEI WANG AND DEBORAH A. FINN

Department of Behavioral Neuroscience, Oregon Health Sciences University, Portland, OR 97201

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RYABININ, A. E., Y.-M. WANG AND D. A. FINN. *Different levels of Fos immunoreactivity after repeated handling and injection stress in two inbred strains of mice.* PHARMACOL BIOCHEM BEHAV **63**(1) 143–151, 1999.—Expression of Fos and Fos-related antigens was immunohistochemically analyzed in DBA/2J and C57BL/6J inbred mice in response to acute or repeated handling and injection stress. Both strains showed a strong induction of Fos and Fos-related antigens in discrete areas of hypothalamus, amygdala, neocortex, septum, and thalamus 2 h after an acute intraperitoneal injection of normal saline. To habituate animals to this procedure, mice were subjected to repeated handling and injections during 2 weeks preceding the experiment. This procedure led to complete habituation of the immediate early gene response to injection stress in stress-responsive brain areas of C57BL/6J mice, such that no significant difference was found between expression of these proteins in brains of saline-injected animals after repeated stress vs. control animals. In contrast, many brain areas of saline-injected DBA/2J mice still showed elevated Fos and Fos-related antigen expression after repeated injections. These results indicate that identical habituation procedures do not necessarily lead to identical levels of gene expression in brains of inbred strains of mice. In turn, they suggest that genetic components for some behavioral and pharmacological traits identified using inbred strains could be related to different rates of habituation to experimental procedures. © 1999 Elsevier Science Inc.

Fos immunoreactivity Handling Injection stress Inbred mice

STRESS response govern by the hypothalamic–pituitary–adrenal axis (HPA) allows the organism to counteract potential dangers of surrounding environment (57). However, repeated HPA response itself can be harmful for function of many organs (4,35,50). To avoid the potential harm of repeated stress response, the HPA axis can undergo habituation:suppression of activation in response to a familiar stressor (17,46,49).

Mechanisms underlying habituation to stress are unknown. Recent research has shown that while habituation can occur at the level of pituitary and adrenal (25,27,34), a large component of this habituation occurs at the level of central nervous system (CNS) (2,26,42,55). We and others have used expression of immediate early genes (IEGs) to address the mechanisms of habituation to repeated stress (3,42,58,64). IEGs c-*fos*, c-*jun*, *jun*-B encode transcription factors capable of changing expression of the so-called "effector genes" (20,44). In the brain of naive animals expression of most IEGs is very low. However, expression of these genes in neurons is rapidly induced by stimuli that increase neuronal activity, allowing to map neuronal structures involved in processing of these stimuli (43,54).

Exposure of an animal to a stressful situation produces a strong induction of c-*fos* not only in the areas directly mediating the stress response, but also in a large number of brain structures where the role in regulation of the stress response is unclear (5,10,39,51,56,59). Repeated exposure to the same stressor produces habituation of c-*fos* response in these structures (42,58,64). Importantly, this habituation is stressor specific, such that decreased c-*fos* response to a familiar stressor does not prevent induction of this gene by a novel stressor (42,64). Moreover, induction and habituation of c-*fos* is independent of the circulating levels of glucocorticoids, indicating

Requests for reprints should be addressed to A. E. Ryabinin, Department of Behavioral Neuroscience, L470, Oregon Health Sciences University, 3181 SW Sam Jackson Park Rd., Portland, OR 97201.

that stress-induced expression of c-*fos* can be used as a measure of stress- or experience-related activity of CNS neurons independent of the activity of peripheral organs (28,42).

One hypothesis in the laboratory is that genetic differences exist in the rate of habituation of the CNS response to stress. To address this question, we took advantage of mouse genetics and compared induction and habituation of Fos and Fos-related antigens in the brains of two inbred mouse strains. Briefly, an inbred strain is derived from 20 generation of brother–sister mating, resulting in a population of animals that is essentially genetically identical (1,45). Consequently, trait variation within an inbred strain can be attributed to environmental causes, whereas variation between strains can be attributed to genetic causes. Therefore, by measuring expression of IEGs after exposure to a novel or familiar stress (i.e., saline injection) in inbred strains, we could determine whether there were genotypic differences in the habituation to repeated stress.

C57BL/6J (B6) and DBA/2J (D2) are perhaps the two most commonly used inbred strains of mice in behavioral and pharmacological genetics (9,13,14). Moreover, there is a contradiction in that behavioral studies consistently describe D2 mice as more sensitive to stress than B6 mice, whereas neuroendocrine studies show that D2 have a lower corticosterone response to stress than B6 mice (6,15,19,21,40). In the present experiment injection was used as a temporally discrete and simple stressor to which most animals easily habituate. This stressor is commonly used as a control condition in pharmacological experiments with animals, and needs to be thoroughly investigated.

METHOD

Animals and Procedures

Four- to 5-week-old male B6 and D2 mice (30–35 g) were purchased from the Jackson Laboratories (Bar Harbor, ME) and housed four mice per cage. Water and food was provided ad lib. Animals were kept on normal light cycle (12:12 h, lights on at 0600 h). Bedding changes in all cages were performed once per week. All manipulations were performed between 0900 and 1200 h to minimize circadian influences. Cages of mice were assigned to either the naive or repeated stress group. The naive mice remained in their home cages for 3 weeks, and received short handling only during the bedding changes and weighing. Repeatedly stressed animals received the following sequential treatments for a total time of 3 weeks: 1) 7 days of acclimatization to the home cages, 2) 4 days of handling (picking up the animal by its tail, once per day), 3) 3 days of sham injections (needle was penetrated into the peritoneum, but no fluid was injected, once per day), 4) 3 days of intraperitoneal injections (IP) with 10 ml/kg of saline (once per day), 5) 4 days of IP injections of 20 ml/kg saline (resulting in a final volume of 0.4–0.6 ml per mouse, once per day). Our preliminary results suggested that such treatment was necessary to decrease injection-induced c-*fos* expression in B6 mice to a level comparable to that of naive animals.

On the day of test, half of the animals were sacrificed immediately after being taken out of their home cage and were designated as naive control or repeated stress control. The rest of the animals were sacrificed 2 h after an injection of 20 ml/kg saline in the procedure room, and were designated as the acute stress and repeated + acute stress groups (n = 6–10 animals per group). In a separate experiment, animals were sacrificed at shorter time points after the final saline injection to investigate its effects on glucocorticoid levels (n = 3 per time point, per group). Euthanasia was performed by cervical dislocation to avoid potential effects of anesthesia on corticosterone levels and gene expression.

Immunohistochemistry

Immunohistochemistry was performed according to the previously published protocols (52,53) with the slight modifications described below. Dissected brains were postfixed overnight in 2% formaldehyde in isotonic sodium phosphate-buffered saline (PBS) and cryoprotected in 30% sucrose in PBS. Forty-micrometer frozen coronal sections were cut on a microtome from bregma level 1.5 mm to −3.5 mm according to the mouse brain atlas (47), and collected in PBS. Immunohistochemical analysis was performed on every third section. Endogenous peroxidase activity was inhibited by pretreatment with 0.3% hydrogen peroxide. Blocking was performed with 3% goat serum. Fos-specific primary antibody was used in dilution 1:10,000. Antibody recognizing multiple Fos-related antigens (pan-Fra) was used in dilution 1:20,000 (optimal antibody dilution was determined in preliminary experiments). Both antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody specificity was determined by Western blotting. The immunoreaction was detected with Vectastain ABC kit (Vector Laboratory Inc., Burlingame, CA). Enzymatic development was done with the Metal-Enhanced DAB kit (Pierce, Rockford, IL).

Quantitative image analysis was performed using a system consisting of an Olympus microscope BX40 and Sony CCD-IRIS/RGB video camera connected to a Power PC. Digitized video image was analyzed using NIH Image 1.63 software: image was "thresholded" such that a neighboring area with no immunostaining would contain no positive signals. Remaining grains with sizes exceeding 10 pixels were automatically counted. For the majority of brain regions a single section per brain region was analyzed for each animal. For brain areas extending several millimeters (CA1, CA3, CA4 areas of the hippocampus, dentate gyrus, piriform, motor, somatosensory, and insular cortices), positive cells were counted in three separate sections located 1 mm apart. The mean of this value was used as a single data point for further statistical analysis using a three-factorial ANOVA (factors: stress, repetition, strain). Because of the large number of structures analyzed and high probability of type I error, only effects of factors with p less than 0.01 were considered statistically significant. Post hoc analysis was performed using the Fisher partial least-squares difference after a one-factorial ANOVA with a significance level of 99%. The number of data points for some brain areas is lower than the actual number of animals by one or two because sections with these brain areas were lost during handling.

Abbreviations Used for Names of Neuroanatomical Structures

Abbreviations used in this article: Pa—paraventricular nucleus of hypothalamus; PO—preoptic area; AH—anterior hypothalamus; VMH—ventromedial hypothalamus; LH—lateral hypothalamus; CeA—central nucleus of amygdala; BLA—basolateral amygdala; La—lateral amygdala; LSV—lateral septum ventrale; BST—bed nucleus of stria terminalis; AcbC—core of nucleus accumbens; AcbS—shell of nucleus accumbens; DG—dentate gyrus; Cg—cingulate cortex; RS—retrosplenial cortex; M—motor, S—somatosensory, Ins—insular, Pir—piriform cortex; PV—paraventricular nucleus of thalamus; MG—medial geniculate; VLG—ventrolateral geniculate; regions were abbreviated according to (47).

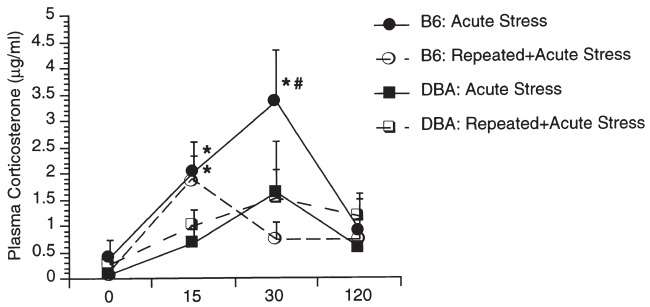

FIG. 1. Plasma corticosterone levels at different time points after the first saline injection (acute stress) and after repeated saline injection (repeated + acute stress) in B6 and D2 mice. Note increased levels of corticosterone in the stress group of B6 mice and a habituation of this response in repeatedly stressed animals. Also note a much smaller corticosterone response in D2 mice and a lack of habituation of this response in these mice. *Indicates significant difference from the time point 0; #Indicates significant difference from same time point of the repeated + acute stress group ($p < 0.05$).

Corticosterone Radioimmunoassay

Corticosterone assay was performed on trunk blood samples. Plasma (5 μl) plasma was diluted with 100 μl sterile water and stored at 4°C until assayed. Samples were immersed in boiling water for 5 min to denature corticosterone-binding globulin. The radioimmunoassay was adapted from a previously reported procedure (33), and utilized antibody from Ventrex (Portland, ME) and [^{125}I]corticosterone from ICN Biomedicals (Costa Mesa, CA). The detectable range of this assay is from 0.1 to 400 μg corticosterone per 100 ml plasma. The specificity of this assay is very high, with only 4% crossreactivity to deoxycorticosterone, 1% crossreactivity to 5b-pregnanedione, and less than 0.6% crossreactivity to other endogenous steroids. Intra- and interassay coefficients of variation were less than 10%. Corticosterone data were analyzed by a three-factorial ANOVA (factors: time, repetition, strain). Post hoc analysis was performed using the Fisher partial least-squares difference after a one-factorial ANOVA with significance level of 95%.

Western Blotting

Western blotting was performed according to previously published protocols (32). Briefly, total neocortex or hippocampus was dissected and placed in protein extract buffer (20 mM HEPES, 0.4 M sodium chloride, 20% glycerol, 5 mM magnesium chloride, 0.5 mM EDTA, 0.1 mM EGTA, 1% Igepal, 1 μg/ml leupeptin, 0.1 mM *p*-aminobenzamidine, 1 μg/ml pepstatin, 0.5 mM PMSF, and 5 mM DTT) at 4°C. Homogenization was performed by three 10-s pulses on the Model 60 Sonic Dismembrator (Fisher Scientific, PA). The lysate was kept on ice for 15 min and then centrifuged 30 min in an Eppendorf microcentrifuge 5415C at 14,000 rpm in a cold room. The supernatant protein extract was collected and stored at −80°C. Protein concentration was measured using the micro-BCA assay kit (Pierce, IL). Eighty micrograms of protein extract was loaded on a 8% SDS-Polyacrylamide gel electrophoresis gel. Western blotting was performed using the Fisher Scientific Semi-Dry Blotting Unit (Fisher Scientific, PA) according to the manufacturer's protocols. Primary antibodies

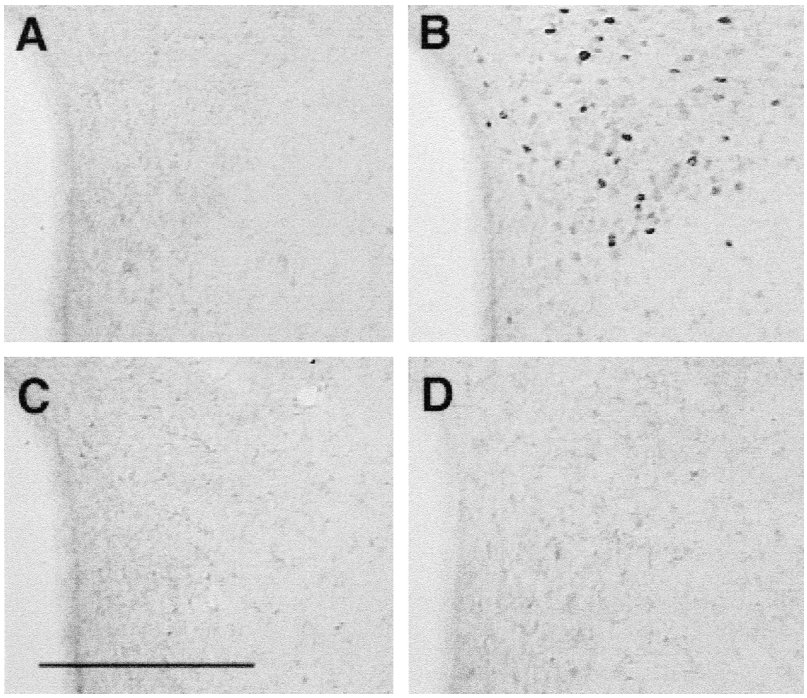

FIG. 2. Fos expression in Pa of B6 mice. Groups: A—naive control, B—acute stress, C—repeated stress control, D—repeated + acute stress. Note induction of c-*fos* in B (dark immunopositive nuclei), and absence of c-*fos* induction in other groups. Induction of Fos in this paradigm is lower than after stronger stressors; therefore, the typical wing-like shape of Pa is not easily visualized. Scale bar corresponds to 150 μm.

were used in dilution 1:3000. Immunodetection was performed using the ECL chemiluminescence kit (Amersham Life Sciences Inc., IL). No statistical analysis of Western blots was performed, as it was used only to check the specificity of the antibody and the presence of similar protein species in the two strains of mice.

RESULTS

To compare the HPA response to a familiar or novel stressor (saline injection) in D2 and B6 mice, we have performed analysis of glucocorticoid levels and Fos and FRA expression in four groups of mice from both strains: naive control, acute stress, repeated stress control, and repeated + acute stress.

Corticosterone Levels

When corticosterone levels were measured in the trunk blood of animals sacrificed for immunohistochemical analysis, they were too low to be statistically evaluated (data not shown). Therefore, a separate experiment was performed where animals were sacrificed at shorter intervals after the injection stress. An acute injection stress led to an increased level of corticosterone 15 and 30 min after the manipulation in B6 mice (Fig. 1). This increase was attenuated in B6 animals repeatedly exposed to handling and injections. In contrast, D2 mice exhibited a smaller increase in corticosterone shortly after the first injection. This response did change after repeated exposure to handling and injections. This observation was reflected in statistical analysis showing a significant effect of time after stress on corticosterone levels, $F(3, 27) = 7.8$, $p < 0.001$, and a significant effect of an interaction between repetition and strain on levels of corticosterone, $F(1, 27) = 4.6$, $p < 0.05$.

Fos Immunohistochemistry

As expected, basal levels of Fos-immunopositive cells in the brains of naive animals from both strains were low. Consistent with previous findings, a single exposure to the stressor produced a dramatic increase in Fos expression in hypothalamic, amygdalar, and several other stress related- and sensory areas (Figs. 2, 3, and Table 1) (5,10,52). Fos-positive cells were counted in 25 areas (Table 1). Multivariate ANOVA confirmed the significant effect of stress on Fos expression in all 25 brain areas. Although statistically significant, this induction was lower than observed after more severe stressors. For example, the number of Fos-positive cells was three to four times higher in Pa in response to injection of 4 g/kg of alcohol (data not shown). For most brain regions there was also a significant effect of repetition (with exception of La and nucleus accumbens) and a significant effect of interaction between stress and repetition (with the exception of CeA, La, AcbC, AcbS, CA4, and PV) on Fos expression.

Importantly, there were clear differences between strains in the effect of repeated stress on Fos expression following injection stress. Thus, in most stress-related regions Fos levels were strongly elevated over the basal expression in the repeated + acute stress group of D2, but not B6 mice. Increased level of Fos expression in D2 vs. B6 mice was confirmed by the significant main effect of strain on Fos expression in LH, CA1, RS, and M, and a significant effect of interaction between stress and strain in LH, AcbS, CA1, RS, M, and PV. There was also a significant interaction between repetition

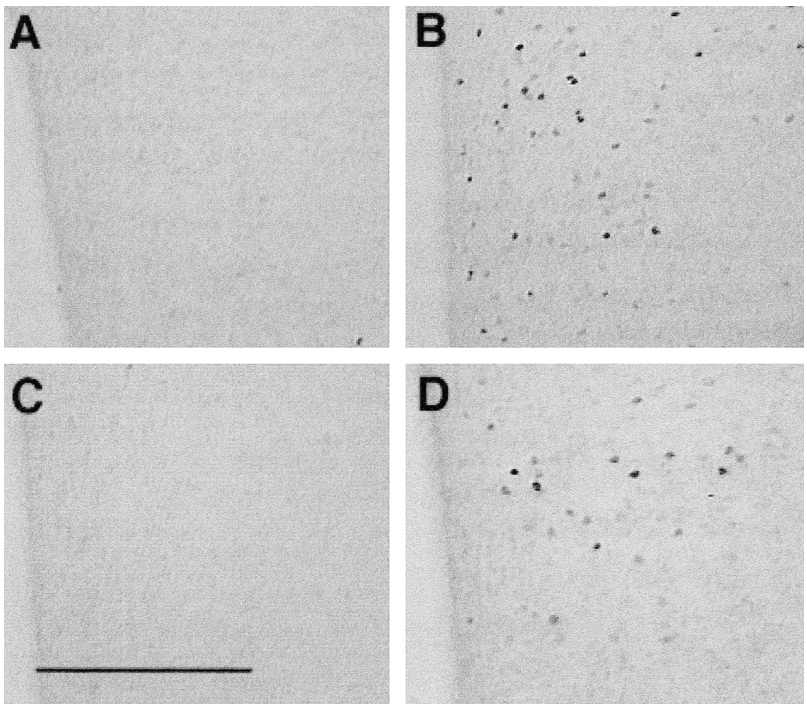

FIG. 3. Fos expression in Pa of D2 mice. Groups: A—naive control, B—acute stress, C—repeated stress control, D—repeated + acute stress. Note induction of Fos in B and D (dark immunopositive nuclei), and absence of induction of Fos in other groups. Scale bar corresponds to 150 μm.

TABLE 1
EFFECTS OF STRESS AND HABITUATION ON c-fos EXPRESSION IN B6 AND D2 MICE

	B6				D2				
Structure	Naive Control	Acute Stress	Repeated Stress Control	Repeated + Acute Stress	Naive Control	Acute Stress	Repeated Stress Control	Repeated + Acute Stress	Significant Effects
Hypothal									
Pa	6.5 ± 3.5	72 ± 11	3.8 ± 1.7	9 ± 3.8	4.2 ± 1	59 ± 4	6.8 ± 1.4	38 ± 5	A,B,AB,BC
PO	2.1 ± 1.5	56 ± 8	2.9 ± 2.5	5.8 ± 3.1	1.3 ± 1.3	70 ± 20	1.4 ± 0.8	26 ± 11	A,B,AB
AH	4 ± 1.3	43 ± 6	8.2 ± 2.9	7 ± 1.7	4 ± 1.9	43 ± 6	3.4 ± 1.5	31 ± 7	A,B,AB
VMH	6.3 ± 3.6	76 ± 10	3.1 ± 1.1	11 ± 3	3.2 ± 1.4	85 ± 14	5.8 ± 1.7	35 ± 6	A,B,AB
LH	3.6 ± 0.9	46 ± 7	4.8 ± 1.6	5.1 ± 1.7	13 ± 5	67 ± 13	7.8 ± 2.2	42 ± 10	A,B,AB,C,AC
Amygdala									
CeA	6.4 ± 2.7	56 ± 12	16 ± 7	7.5 ± 2.9	2.7 ± 0.8	34 ± 7	2.9 ± 1.3	44 ± 13	A,ABC
BLA	1.5 ± 0.5	36 ± 11	11 ± 8	3.8 ± 1.7	1 ± 0.4	21 ± 5	1.9 ± 0.8	15 ± 3	A,AB
La	0.1 ± 0.1	1.6 ± 0.9	0.6 ± 0.3	0.9 ± 0.6	0	2.3 ± 1.2	0	3.1 ± 1.3	A
Septum and struatum									
LSV	12 ± 7	141 ± 38	7.3 ± 2.8	6.5 ± 1.7	1.7 ± 1.2	123 ± 27	2 ± 0.7	29 ± 3	A,B,AB
BST	6.6 ± 4.3	87 ± 14	11 ± 7	6 ± 1.5	0.7 ± 0.4	97 ± 19	1.1 ± 0.5	32 ± 9	A,B,AB
AcbC	1.4 ± 1.2	22 ± 4	0.5 ± 0.2	1.1 ± 0.7	0.5 ± 0.3	30 ± 13	0.6 ± 0.4	25 ± 11	A
AcbS	2.3 ± 2	47 ± 4	2.4 ± 0.9	4.9 ± 2.1	0.7 ± 0.7	78 ± 25	1 ± 0.4	50 ± 22	A,AC
Hippocamp									
DG	3 ± 0.8	16 ± 2	3.6 ± 1.3	3 ± 0.9	4.1 ± 1	16 ± 4	4.5 ± 0.6	9.1 ± 2.7	A,B,AB
CA1	0.5 ± 0.4	15 ± 1	0.9 ± 0.5	1 ± 0.4	0.6 ± 0.3	24 ± 4	0.3 ± 0.1	6.9 ± 1	A,B,AB,C,AC
CA3	0.4 ± 0.1	8.7 ± 1.7	0.4 ± 0.3	0.4 ± 0.2	0.2 ± 0.1	16 ± 7	0.3 ± 0.1	4.5 ± 1	A,B,AB
CA4	0	0.7 ± 0.5	0.1 ± 0	0.1 ± 0.1	0	1.3 ± 0.7	0	0.6 ± 0.3	A
Neocortex									
Cg	5.3 ± 4.6	55 ± 7	2.1 ± 1.1	1.9 ± 0.8	0.1 ± 0.1	127 ± 57	0.1 ± 0.1	40 ± 14	A,B,AB
RS	1.8 ± 1.4	29 ± 8	2 ± 1.3	2.6 ± 1.1	0.2 ± 0.2	87 ± 26	0	27 ± 9	A,B,AB,C,AC
M	3.3 ± 2.6	37 ± 3	2.9 ± 1.8	5 ± 2	0.9 ± 0.2	65 ± 16	0.4 ± 0.2	22 ± 5	A,B,AB,C,AC
S	3 ± 2.7	23 ± 4	2.8 ± 1.5	1.2 ± 0.4	0.4 ± 0.2	25 ± 3	1.1 ± 0.7	16 ± 6	A,B,AB
Ins	5.9 ± 4.4	52 ± 12	8.4 ± 5.8	5.8 ± 2.3	1.3 ± 0.4	62 ± 18	1 ± 0.3	28 ± 10	A,B,AB
Pir	8.9 ± 5.9	53 ± 7	14 ± 9	8.1 ± 1.6	0.9 ± 0.3	66 ± 22	3.2 ± 1	32 ± 10	A,B,AB
Thalamus									
PV	64 ± 14	131 ± 22	45 ± 11	58 ± 18	17 ± 4	150 ± 30	23 ± 6	118 ± 13	A,AC
MG	1.4 ± 1.3	32 ± 6	0.5 ± 0.4	2.9 ± 1.5	0.8 ± 0.4	28 ± 7	1.1 ± 0.8	14 ± 3	A,B,AB
VLG	1.5 ± 1.3	39 ± 10	1.2 ± 0.8	3.5 ± 1.5	1.3 ± 1.1	39 ± 11	3 ± 2.7	22 ± 6	A,B,AB

Values are means (±SEM) of immunopositive cells/brain region/brain slide.
Significant effects are indicated according to a three-way ANOVA with factors: A—stress, B—repetition, C—strain, $p < 0.01$ (doubled letters indicated significant effects of interaction between factors).

and strain in Pa, and a significant interaction between stress, repetition, and strain in CeA.

Post hoc analysis confirmed that Fos expression in the repeated + acute stress group was significantly higher in D2 vs. B6 mice in Pa, AH, LH, VMH, CeA, AcbC, AcbS, CA1, and S. In contrast, there was no brain region in which Fos expression in the repeated + acute stress group was significantly higher in B6 than in D2 mice. Significantly higher expression of Fos in D2 vs. B6 mice in the acute stress group was found in CA1, Cg, RS, and M. Thus, most of these brain areas were different from those showing significantly higher Fos expression in D2 vs. B6 in the repeated + acute stress group. The difference in Fos expression between strains was not due to differences in basal levels (no statistically significant differences were found between strains in naive control or repeated stress control groups in any brain regions). There were no statistically significant differences between repeated + acute stress and naive control or repeated stress control groups of B6 mice, indicating complete habituation of animals of the repeated + acute stress group. The lack of habituation in D2 animals is confirmed by the significantly higher Fos expression in the repeated + acute stress group than in the naive control in Pa, PO, AH, LH, VMH, CeA, La, AcbC, AcbS, CA1, S, Ins, PV, and MG, and than repeated stress control group in Pa, AH, LH, VMH, CeA, La, AcbC, AcbS, CA1, M, S, Ins, PV, and MG of D2 mice (indicating lack of habituation).

Fos and FRA Western Blot Analysis

To investigate whether the IEG response in B6 and D2 mice consisted of similar FRAs, we performed Western blot analysis of proteins from neocortices and hippocampi of these mice. Blots were analyzed with either the Fos-specific antibody used in the immunohistochemical studies described above, or with an antibody recognizing most of the FRAs (pan-Fra) (Fig. 4). As expected, the Fos-specific antibody recognized a single band of approximately 58 kDa in the protein extracts from both strains of mice corresponding to Fos. In contrast, the pan-Fra antibody recognized several protein bands. One of them corresponded in size to the Fos protein.

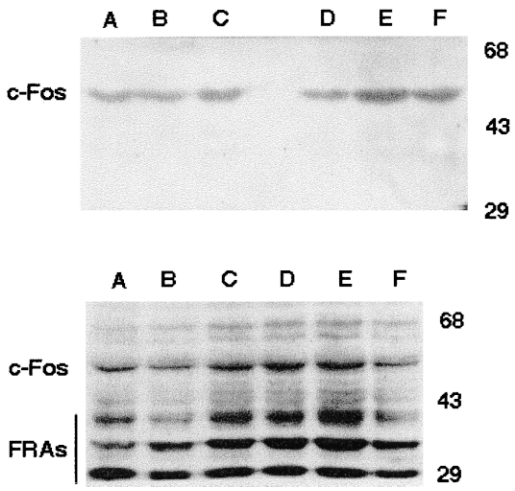

FIG. 4. Western blot analysis of Fos and FRA expression in neocortex of individual B6 and D2 mice. Blots were incubated either with a Fos-specific antibody (upper panel), or with a pan-Fra antibody (lower panel). Lanes: A—naive control group of D2 mice; B—acute stress group of D2 mice; C—repeated + acute stress group of D2 mice; D—naive control group of B6 mice, E—acute stress group of B6 mice; F—repeated + acute stress group of B6 mice. Numbers correspond to molecular weights of standards from Gibco BRL (Rockville, MD). Note single band on the blot incubated with the Fos antibody, and several bands on the blot incubated with the pan-Fra antibody (with stronger recognition of other FRAs than Fos). For unknown reasons, the band corresponding in size to slightly lower than 43 kDa showed a variable band intensity in replicate blots, which was unrelated to the group or strain of the animal.

Comparable or stronger bands of 29–43 kDa also were seen corresponding to additional FRAs. Similar results were observed with protein extracts from hippocampi of B6 and D2 mice (data not shown). We found that Western analysis in our hands was less quantitatively accurate (i.e., gave higher variability) than immunohistochemistry. Therefore, no quantitative analysis was performed.

FRA Immunohistochemistry

Because the pan-Fra antibody has broader immunoreactivity, FRA immunohistochemistry had a expected higher background than Fos immunohistochemistry. Similar to Fos, there was a significant effect of stress on FRA expression in all 25 analyzed brain regions (Table 2). A significant effect of repetition was seen in all stress-reactive areas except amygdala. The effect of interaction between stress and repetition on FRA expression was significant in all analyzed areas except amygdala and PV, indicating that exposure to repeated stress does not alter basal FRA expression in the majority of stress-reactive areas, but appears to change responsivity of FRA to the familiar stressor. Strain significantly influenced FRA expression in LH, DG, and CA1. There was a significant interaction between strain and stress in Pa, AH, LH, CA1, and RS. These strain differences, as with Fos, are due to higher FRA expression in D2 vs. B6 in acute stress and repeated + acute stress groups, rather than in the control groups.

Post hoc analysis confirmed that FRA expression was higher in many regions of the repeated + acute stress D2 mice vs. similarly treated B6 mice. There were significant differences between these mice in AH, LH, VMH, DG, M, S, Ins, and Pir. FRA expression in the acute stress group was significantly higher in D2 than in B6 mice in Pa, LH, LSV, BST, CA1, and RS. There was no statistically significant difference in FRA expression between the repeated + acute stress and naive control or repeated stress control groups of B6 mice. In contrast, FRA expression in D2 mice was significantly higher in the repeated + acute stress group vs. naive control in AH, LH, VMH, BLA, DG, M, S, Ins, Pir, and PV, and vs. repeated stress control in AH, LH, VMH, M, S, Ins, Pir, and PV.

DISCUSSION

Our studies confirm previous investigations showing that exposure of animals to a novel experimental situation, such as simple intraperitoneal injection, results in increased expression of Fos protein and FRAs in the brain (12,23,24,53,59,61). This increase is widespread, and affects many brain regions, making it difficult to identify the neural circuitry of the stress response. According to previous studies, this stress-related induction of IEG is experience specific and reflects the CNS response to the particular behavioral or experimental situation (42,64). Recently, it has been shown that there are two main circuits leading to the activation of the HPA axis, processive (originating in the associative areas of the brain) and systemic (originating in the brain stem). Involvement of one or both circuits depends on the type of stressor that activates the HPA axis (8,29,38,58). Injection of normal saline, although not a strong stressor, should involve both circuits. Involvement of the systemic circuit is confirmed in the present study by the increased expression of these IEGs after saline injection in CeA, PO, and BST, areas in which Fos expression is rarely observed after processive stress (58).

Our studies also confirmed that habituation to an experimental situation tends to blunt the brain IEG response to this familiar stressor (42,64). Consistent with these reports, preexposure to stress did not change basal levels of Fos expression in the majority of stress-related areas, but changed responsivity of Fos to the familiar stressor. However, the aim of this study was to determine whether two commonly used inbred strains of mice will equally habituate their IEG response to injection stress. Although B6 and D2 mice and their progeny strains are widely used to study genetic predisposition of pharmacological, cognitive, and emotional traits, it was not clear whether these mice have equal rates of habituation to stress (9,13,14). Moreover, data on the reactivity of D2 and B6 mice to acute stress were also contradictory. Early studies described B6 as "calm" and having "low emotionality" (14,15,62). However, the majority of studies including the present one, which analyzed glucocorticoid levels, have found higher stress-induced corticosterone levels in B6 vs. D2 mice (6,19,21). One potential explanation for this discrepancy could be that despite the higher levels of "emotionality" of D2 mice (which agree with higher levels of Fos and FRA in several areas of the brain of D2 mice in the acute stress group in this study), there is a decreased sensitivity of the HPA axis to stress. In contrast to this hypothesis, there was a higher level of FRA expression in the Pa of D2 mice vs. B6 mice in the acute stress group, and no statistically significant difference in Fos expression. Thus, there remain two potential explanations for the difference between these strains in their acute response to stress. First, that B6 and D2 mice differ in their response to acute stress in peripheral regions of the HPA axis. This is in agreement with higher rates of liver corticosterone metabolism in D2 mice (60) and higher pituitary ACTH content in B6 mice (19,21,37). Second, that stress-induced FRA expression

TABLE 2

EFFECTS OF STRESS AND HABITUATION ON FRA EXPRESSION IN B6 AND D2 MICE

	B6				D2				
Structure	Naive Control	Acute Stress	Repeated Stress Control	Repeated + Acute Stress	Naive Control	Acute Stress	Repeated Stress Control	Repeated + Acute Stress	Significant Effects
Hypothal									
Pa	4.9 ± 2.2	53 ± 10	5.1 ± 2.8	8.1 ± 5.4	2.5 ± 0.7	87 ± 16	2.2 ± 0.6	23 ± 5	A,B,AB,AC
PO	1.6 ± 0.5	52 ± 15	1.8 ± 0.8	3.3 ± 1.4	4.3 ± 3	80 ± 17	1.9 ± 0.4	17 ± 4	A,B,AB
AH	11 ± 7	53 ± 8	4.4 ± 1.6	5.1 ± 1.6	4.1 ± 1.4	58 ± 5.2	2.1 ± 1.1	38 ± 6	A,B,AB,AC
VMH	5 ± 2.2	64 ± 7	5.5 ± 2.3	7.1 ± 2.3	3.9 ± 1	63 ± 9	5.4 ± 1	31 ± 6	A,B,AB
LH	4.1 ± 1.3	41 ± 7	1.8 ± 0.6	3 ± 0.7	5.6 ± 1.3	73 ± 8.6	3 ± 1.3	38 ± 3.6	A,B,AB,C,AC
Amygdala									
CeA	8.6 ± 2.3	94 ± 33	20 ± 14	15 ± 8	4 ± 0.6	86 ± 28	12 ± 8	61 ± 22	A
BLA	0.5 ± 0.2	32 ± 12	1.1 ± 0.6	2.8 ± 0.7	1 ± 0.3	26 ± 4	2 ± 0.8	23 ± 7	A
La	0.1 ± 0.1	4 ± 1.6	0.4 ± 0.3	2.8 ± 2	0.3 ± 0.3	3.2 ± 1.5	0.1 ± 0.1	1.1 ± 0.6	A
Septum and struatum									
LSV	18 ± 6	75 ± 11	19 ± 4	18 ± 4	4.2 ± 1.3	132 ± 39	4.5 ± 1	36 ± 13	A,B,AB
BST	21 ± 5.8	107 ± 13	38 ± 8	21 ± 8	3.5 ± 0.7	244 ± 110	15 ± 8	43 ± 15	A,B,AB
AcbC	2.9 ± 1.4	43 ± 4	9.6 ± 4.1	6.5 ± 3.2	1.8 ± 0.6	48 ± 10	1.9 ± 0.7	15 ± 2	A,B,AB
AcbS	9.4 ± 4.4	87 ± 11	16 ± 5	12 ± 4	2.1 ± 0.5	92 ± 27	4.8 ± 1.3	25.7 ± 6	A,B,AB
Hippocamp									
DG	5.5 ± 0.8	28 ± 4	4 ± 0.9	3.9 ± 0.5	4.3 ± 0.5	35 ± 3	13 ± 3	17 ± 5	A,B,AB,C
CA1	2.8 ± 0.6	16 ± 2	0.7 ± 0.3	1.8 ± 0.7	1.9 ± 0.6	29 ± 6	1.6 ± 0.5	6.5 ± 1.2	A,B,AB,C,AC
CA3	2 ± 0.7	15 ± 2	1 ± 0.4	2 ± 1	3.3 ± 0.8	21 ± 5	1.9 ± 0.9	6.5 ± 1.4	A,B,AB
CA4	0.2 ± 0.1	1.2 ± 0.9	0	0.2 ± 0.2	0.2 ± 0.1	0.7 ± 0.3	0	0.6 ± 0.3	
Neocortex									
Cg	3.1 ± 1	63 ± 6	4.2 ± 1.5	3 ± 0.5	1.7 ± 0.4	88 ± 29	2.6 ± 0.5	33 ± 10	A,B,AB
RS	4 ± 2.6	44 ± 7	4.9 ± 2.8	1.1 ± 0.6	0.3 ± 0.2	63 ± 8	1.1 ± 0.4	15 ± 5	A,B,AB,AC
M	5 ± 1.5	57 ± 7	4.5 ± 0.7	3.3 ± 0.7	1.3 ± 0.2	53 ± 4	1.8 ± 0.4	17 ± 3	A,B,AB
S	5.2 ± 1.6	37 ± 6	3.8 ± 1.3	2 ± 0.5	0.8 ± 0.3	33 ± 4	1.3 ± 0.4	14 ± 2	A,B,AB
Ins	2.4 ± 1	31 ± 3	3.3 ± 2.4	1.8 ± 0.6	0.7 ± 0.2	35 ± 10	1.2 ± 0.4	14 ± 3	A,B,AB
Pir	3.5 ± 1.6	45 ± 5	6.2 ± 3.3	3.4 ± 1	0.7 ± 0.2	44 ± 7	1.1 ± 0.2	18 ± 4	A,B,AB
Thalamus									
PV	44 ± 22	125 ± 11	18 ± 6	50 ± 20	10 ± 2	137 ± 26	13 ± 4	107 ± 18	A,B
MG	1.1 ± 0.5	29 ± 9	0.6 ± 0.3	1.8 ± 1	0.8 ± 0.3	38 ± 12	1.6 ± 1.2	14 ± 4.4	A,B,AB
VLG	0.8 ± 0.5	26 ± 8	0.6 ± 0.3	1.5 ± 1.1	0.9 ± 0.3	22 ± 8	1.8 ± 1.2	8.3 ± 2.2	A,B,AB

Values are means (±SEM) of immunopositive cells/brain region/brain slice.
Significant effects are indicated according to a three-way ANOVA with factors: A—stress, B—repetition, C—strain.
$p < 0.01$ (doubled letters indicate significant effects of interaction between factors)

could be primarily occurring in inhibitory neurons, such that higher levels of FRA expression would actually reflect a greater inhibition of Pa response. Recently, it has been demonstrated that a subpopulation of inhibitory neurons projecting to Pa express Fos in response to stress (16,36,58). We are currently involved in studies to address this second possibility.

A recent study in rat strains has shown that strain differences in responsivity of the HPA axis to acute stress is paralleled by differences between strains in their abilities to habituate to repeated stress (18). Our results agree and extend these finding by demonstrating that differences in the ability of certain rodent strains to adapt to a familiar stressor are accompanied by differences in the CNS response to this stressor. Indeed, the major finding of this study is that identical habituation procedures can lead to different levels of habituation of IEG expression in stress-reactive areas of brains of B6 and D2 mice. Thus, significant differences in Fos and FRA expression between the repeated + acute stress and control animals were found in areas of hypothalamus, amygdala, hippocampus, neocortex, hippocampus, and thalamus of D2 mice, but not of B6 mice.

The specific composition of the IEG response to a stimulus have been shown to differ, depending on whether the treatment is acute or repeated (31,32). Thus, in contrast to acute treatments, repeated treatments can induce the so-called chronic Fra, a FosB-related product (11). In our study we did not observe increased levels of FosB-related products on Western blots of proteins isolated from neocortex and hippocampus. This may be due to the mild stressor, that was used in the present study, in comparison with previous investigations. Although our immunohistochemical studies found less statistically significant differences for FRAs than for Fos (probably due to higher background staining with the pan-Fra antibody), for most of the brain regions in the present study habituation of IEG expression was similar when analyzed by an antibody recognizing either all FRAs or only Fos. Therefore, the differences in expression of FRAs between B6 and D2 mice after habituation to injection are due to changes in the re-

sponsivity of FRAs to the stressor, and not due to changes in the expression of components of the FRA complex.

Our studies suggest that stress-related Fos and FRA induction in B6 and D2 mice can reach habituation at a different time point of the habituation procedure. The mechanism leading to this difference between strains is not clear. Recently, it has been shown that repeated restraint stress led to increased expression of CRF receptor 1 mRNA in the cortex of B6, but not D2 mice (22). However, it is not clear whether this difference contributes to the differences in habituation between these two strains, or whether the differences in habituation lead to changes in CRF-R1 expression in one strain, but not the other.

Another important consideration is whether the inability of the IEG response to habituate to the saline injection stress in D2 mice is due to different rates of habituation or to initial differences in the IEG response to acute stress between the D2 and B6 strains. Indeed, several brain regions showed higher levels of Fos and FRA expression after acute stress in D2 vs. B6 mice. However, most of these areas were different from those showing significantly higher Fos and FRA expression after the familiar stressor. This result does not support a strong role of differences in stress sensitivity between B6 and D2 mice, and is in favor of different rates of habituation to stress in these two strains of mice. In fact, D2 mice have been previously shown to perform significantly worse than B6 mice in several learning paradigms (14,30,48,63). However, an important implication from the present results is that B6 and D2 mice tested in these more complex cognitive tasks may have been at different levels of habituation to the experimental manipulations. Strain differences in the level of stress and habituation could, in turn, affect the ability of these mice to perform in these tasks (41). On the other hand, our experiments did not address the generality of observed phenomena for other types of stressors. Therefore, it is not clear whether the same differences in rates of habituation will be observed between these mice when they would be habituated to different behavioral manipulations, other vehicle solutions, or different patterns of saline administration. Indeed, different stimuli may elicit opposite strain-dependent patterns of stress response (7), which could be dependent on interactions of many factors, including differences in pain sensitivity, learning ability, exploratory activity, and others.

In addition to implications for studies evaluating the learning abilities of inbred mouse strains, the present results suggest that strain differences in IEG responsivity to a stressor or to habituation may affect pharmacological experiments with these strains of mice and their progeny strains. Thus, many pharmacological experiments use a saline injection as a control. If the experimental paradigm involves habituation to the injection procedure, differences in habituation at the time of testing could influence a drug's pharmacological effect. Future studies using these strains would benefit from examining genetic predisposition to pharmacological together with stress-related traits.

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