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Conditioned-Fear Stress Increases Fos Expression in Monoaminergic and GABAergic Neurons of the Locus Coeruleus and Dorsal Raphe Nuclei

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ABSTRACT Many studies have demonstrated that physical or psychological stress can increase Fos expression in brainstem monoaminergic nuclei. Little is known, however, about the extent to which stress increases the expression of Fos in monoaminergic and nonmonoaminergic neurons in the brainstem. We examined the effects of conditioned-fear (CF) stress following mild footshock (FS) as unconditioned stress on Fos expression in the monoaminergic and GABAergic neurons of the ventral tegmental area (VTA), locus coeruleus (LC), and dorsal raphe nucleus (DR) in rats. The CF stress significantly increased the number of Fos-positive (Fos+) cells in both the LC and DR, whereas it did not increase the number in the VTA. Using a double-labeling technique, we combined Fos immunostaining with that for tyrosine hydroxylase (TH), serotonin (5-HT), or GABA for histochemical identification of the CF stress-induced Fos+ neurons. The percentage of TH/Fos double-labeled cells resulting from CF stress was 63% of the Fos+ cells in the LC, whereas 52% of the Fos+ cells contained 5-HT in the DR. We also found that approximately 60% of the CF stress-induced Fos+ cells were GABAergic neurons in these brain regions. These results indicate that CF stress induces intense Fos expression in the noradrenergic LC and serotonergic DR neurons, but not in the dopaminergic VTA neurons. They also indicate that not only monoaminergic neurons but also GABAergic neurons within the LC and DR are activated by the stress. **Synapse 45: 46–51, 2002.**

INTRODUCTION

Fos is a protein product of the immediate early gene c-*fos* that can be induced by a variety of physiological and pharmacological stimuli. The expression of c-*fos* increases in rat brain tissue in response to different stressors, including restraint (Schreiber et al., 1991; Melia et al., 1994), footshock (FS) (Smith et al., 1992), and anxiety (Duncan et al., 1996). Analysis of the effects of a conditioned stressor on regional Fos expression should provide information about the neural substrates of fear at a level of anatomical resolution that cannot be attained with other approaches. So-called conditioned fear (CF), i.e., exposure of rats to an environment in which they had previously received FS, was found to increase Fos expression in widely distributed cortical and subcortical structures (Beck and Fibiger, 1995). Detailed information is still lacking, however, about the relationship between CF and Fos expression in brainstem monoaminergic nuclei.

We have examined the distribution of Fos expression following CF stress in brainstem monoaminergic nuclei in the ventral tegmental area (VTA), a dopaminergic region, the dorsal raphe nucleus (DR), a serotonergic

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TABLE I. Conditioning and testing schedule of the three experimental groups

	Not conditioned–reexposed group	Conditioned–not reexposed group	Conditioned–reexposed group
Habituation	7 × 30 min daily sessions without footshock		
Day 1 (first session)	No footshock	Footshock	Footshock
Day 4 (second session)	No footshock	stayed in home cage	No footshock

Each session was 30 min long and took place in a shockbox with a stainless steel bar floor contained in an isolation box, except for the second session of the conditioned–not reexposed group.

region, and the locus coeruleus (LC), a noradrenergic region. All of these regions are thought to play a role in CF stress-related behaviors (Beck and Fibiger, 1995; Pezzone et al., 1993; Smith et al., 1992). We also examined colocalization of CF-induced Fos with one of three neuronal markers (tyrosine hydroxylase (TH), serotonin (5-HT), and GABA) in two regions that showed a significant increase in Fos induction following CF stress.

MATERIALS AND METHODS

Stressor exposure and behavioral observation

Male Wistar rats (Japan SLC, Hamamatsu, Japan) weighing 180–200 g at the start of testing were used. They were housed in a temperature- and humidity-controlled vivarium under constant light/dark-cycle conditions (lights on 08:00, lights off 20:00) with free access to food and water at all times except during testing. The ethical committee of animal experimentation at Miyazaki Medical College approved the experimental protocols used. To allow adaptation to novelty-induced activation of Fos, the animals were individually placed in the testing apparatus 30 min a day for 7 days preceding the first session. They were randomly divided into three groups: not conditioned–reexposed, conditioned–not reexposed, and conditioned–reexposed (Table I).

The testing apparatus was a shockbox (29.3 × 26.0 × 28.0 cm) with a stainless-steel bar floor placed in an isolation chamber with a semitransparent window to allow behavior observation. A mild FS (0.3 mA; 60 Hz) was generated with a shock generator using a scrambler (Toyo Sangyo Co., Toyama, Japan) and delivered through grid chamber floors. The timing of the shocks was controlled with a repeated-cycle timer; they lasted 2 sec and were delivered every 15 sec for 20 min. The not conditioned–reexposed rats were placed in the testing apparatus daily for two 30-min sessions without FS on experimental days 1 (first session) and 4 (second session). The conditioned–not reexposed rats were given a first session with FS as an unconditioned fear paradigm: 20-min FS after an initial 10-min orientation period. Thereafter, they remained in their home cages without reexposure to the shockbox until perfusion 3 days later. The conditioned–reexposed rats were subjected to a first FS session, followed 3 days later by a second session of a CF paradigm: 30-min reexposure to the testing apparatus in which the rats had received FS treatment 3 days before. This was a conditioned group for which the unconditioned stimuli were the footshocks and the conditioned stimuli were the contextual cues provided by the testing apparatus.

The animals were undisturbed on days 2 and 3 to permit any direct effects of FS on Fos expression to dissipate. This conditioning procedure produced observable behavioral and autonomic effects that were used to verify the conditioning process. The observations included immobile behavior (a complete lack of movement) and marked bladder and bowel discharge, all in response to the conditioning stimulus. The duration of immobility, defined as the animal being motionless and alert, was measured by direct observation during the first 10-min period of the second session in the testing apparatus for each rat in the not conditioned–reexposed and conditioned–reexposed groups. At the end of each session the rats were returned to their home cages.

Immunohistochemistry

Two hours after the end of the final session the rats were deeply anesthetized with sodium pentobarbital and perfused transcardially, first with heparinized saline, then with 300 ml of cold, freshly prepared 3% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4), and finally with 300 ml of 10% sucrose in 0.1 M phosphate buffer. The brains were removed immediately, immersed in 30% sucrose in 0.1 M phosphate buffer for 2 days at 4°C, then cut coronally into 50-μm sections on a freezing microtome for immunohistochemical examination.

From each animal sections through the VTA (approximately 5.2 mm caudal to the bregma), DR (approximately 7.8 mm caudal to the bregma), and LC (approximately 10.0 mm caudal to the bregma) were selected for histological study (Paxinos and Watson, 1997) (Fig. 2). The sections were collected in a phosphate-buffered saline solution (PBS; pH 7.4) and processed for Fos immunohistochemistry according to the manufacturer's instructions for use with the streptavidin-biotin system (Histofine SAB-PO (R) kit, Nichirei, Tokyo, Japan). After incubation in 10% normal goat serum for 20 min the sections were incubated at 4°C overnight with a Fos antibody, a rabbit polyclonal antibody raised against a peptide corresponding to human c-*fos* amino acid residues 3–16 (diluted 1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). They were then rinsed three times in PBS and incubated at room temperature for 45 min with a secondary biotinylated goat

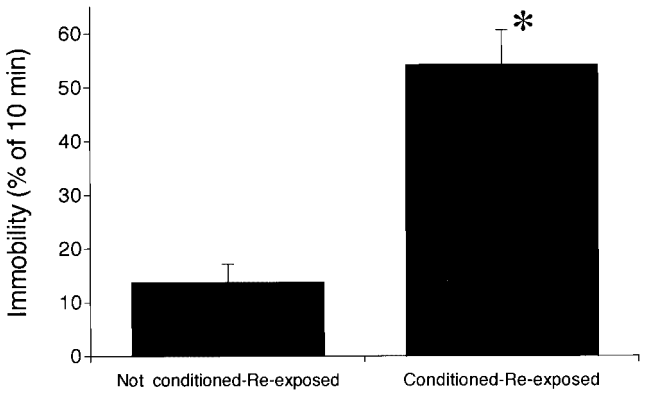

Fig. 1. Immobility in rats in not conditioned–reexposed ($n = 7$) and conditioned–reexposed ($n = 7$) groups during the first 10 min of the second session. Values are mean ± SEM. Asterisk indicates statistically significant difference with not conditioned–reexposed group (*$P < 0.05$, Mann-Whitney U-test).

antirabbit IgG, again rinsed three times in PBS, and further incubated at room temperature for 15 min with a streptavidin-peroxidase complex. After three rinses in PBS, the reaction products of biotinylated goat antirabbit IgG and streptavidin-conjugated horseradish peroxidase were intensified by pretreatment with 0.125% cobalt chloride, then visualized using 0.01% diaminobenzidine tetrahydrochloride (DAB) and 0.0003% hydrogen peroxide.

For the double-labeling of cells, an antibody against TH (1:20,000; mouse monoclonal, kindly donated by the late Dr. H. Hatanaka at Osaka University, Japan) (Hatanaka and Arimatsu, 1984), 5-HT (1:2,000; rat polyclonal; Eugene Tech International, Ridgefield Park, NJ, USA), or GABA (1:1,000; mouse monoclonal; Sigma, St. Louis, MO, USA) was added during the same stage as the primary Fos antibody was added. Following completion of the Fos processing described above, the tissue was further processed using a procedure similar to that described for Fos except that the avidin-biotin-peroxidase method (Vectastain Elite kit; Vector, Burlingame, CA, USA) was used and cobalt chloride was not included in the visualization process with 0.03% DAB and 0.0009% hydrogen peroxide.

The sections were mounted on gelatin-coated glass slides, air-dried, dehydrated, cover-slipped, and analyzed using light microscopy. The two most heavily labeled sections through each structure of each animal were chosen for cell counting. Cells were counted in two sections per structure in each animal in a standardized manner under 20× magnification using a microscopic 0.15 × 0.15 mm grid. The size of the grid was defined so as not to exceed the horizontal size of the LC, the smallest of the three structures (VTA, DR, and LC). The DR was divided into three parts: dorsal, ventral, and ventrolateral (DRVL) at the mid-level (approximately 7.8 mm caudal to the bregma). The limits of the subnuclei of the DR were defined using the boundaries

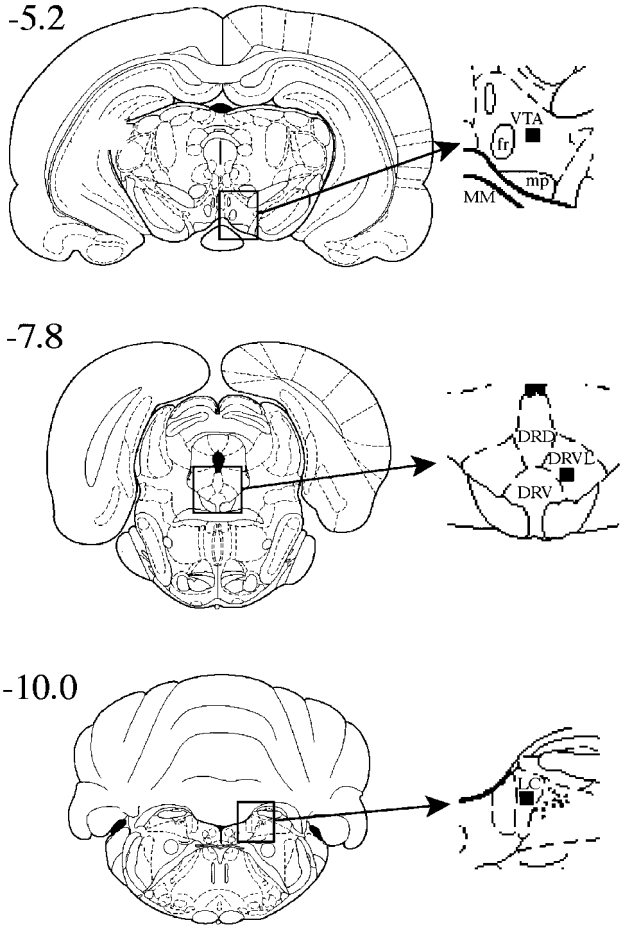

Fig. 2. Schematic representation of anatomical regions in which Fos-immunopositive and/or aminergic cells were evaluated (black squares; drawn from the atlas of Paxinos and Watson, 1997). The open rectangles depict region magnified on right side of each section. Numbers at upper-left side of each section indicate distance (mm) posterior (−) to the bregma. DRD: dorsal part of dorsal raphe nucleus; DRV: ventral part of dorsal raphe nucleus; DRVL: ventrolateral part of dorsal raphe nucleus; fr: fasciculus retroflexus; LC: locus coeruleus; MM: medial part of medial mammillary nucleus; mp: mammillary peduncle; VTA: ventral tegmental area.

of Paxinos and Watson (1997). The grid was placed in the center of the VTA, LC, or ventromedial part of the DRVL (Fig. 2). The average number of Fos+ cells, of cells immunopositive for each cellular marker and of double-labeled cells in each structure of the brain was computed.

Statistical analysis

To evaluate the behavioral data statistically, the nonparametric Mann-Whitney U-test was used to assess the difference in immobility between the control and CF groups. One-way ANOVA was used to test for group effects on the Fos-positive cell counts within each structure. A finding of significant group effects, $P < 0.05$, was followed by Newman-Keuls testing to assess the group differences.

TABLE II. Means and SEMs of Fos-positive cell numbers/structure (0.15 × 0.15 mm²) in the three experimental groups

Structure	Not conditioned–reexposed group (n = 7)	Conditioned–not reexposed group (n = 6)	Conditioned–reexposed group (n = 7)
VTA	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.2
DR	0.9 ± 0.3*	1.3 ± 0.4*	9.0 ± 0.8
LC	1.1 ± 0.1*	1.5 ± 0.5*	10.3 ± 0.8

Following a significant group effect with one-way ANOVA regarding each structure, groups were compared with Newman-Keuls tests.
*P < .01 compared to the corresponding value of the conditioned–reexposed group.

TABLE III. Quantification of the double-labeling experiment in the conditioned–reexposed group (cell numbers/0.15 × 0.15 mm²; mean ± SEM; n = 6–7)

DR		LC	
Cellular character		Cellular character	
5-HT+	22.3 ± 1.0	TH+	30.4 ± 1.7
Fos+	7.9 ± 1.0	Fos+	10.7 ± 2.2
5-HT+/Fos+	4.1 ± 0.7	TH+/Fos+	6.7 ± 1.4
GABA+	25.3 ± 1.8	GABA+	28.3 ± 0.9
Fos+	8.4 ± 1.3	Fos+	10.9 ± 2.1
GABA+/Fos+	4.9 ± 0.6	GABA+/Fos+	6.6 ± 1.4

RESULTS

Behavior

During reexposure to the testing apparatus, the rats previously exposed to FS stress (the conditioned–reexposed group) remained relatively immobile (Fig. 1).

Immunohistochemistry

Following the CF stress, the number of Fos-positive cells significantly increased in the DR ($F_{2,16}$ = 79.07, P < 0.01; Newman-Keuls test, P < 0.01) and LC ($F_{2,16}$ = 73.34, P < 0.01; Newman-Keuls test, P < 0.01), but not in the VTA ($F_{2,16}$ = 0.15, n.s.) (Table II).

The percentage of TH/Fos double-labeled cells resulting from CF stress was 63% of all Fos+ cells in the LC, whereas 52% of all Fos+ cells contained 5-HT in the DR. We also found that approximately 60% of the stress-induced Fos+ cells were GABAergic neurons in these two brain regions (Table III, Fig. 3).

DISCUSSION

These results indicate that CF stress may induce intense Fos expression in the serotonergic DR and noradrenergic LC neurons, but not in the dopaminergic VTA neurons, and also that GABAergic neurons in the DR and LC are commonly activated by the stress.

Contrary to the view that dopamine neurons in the VTA are involved in CF stress (Beck and Fibiger, 1995; Campeau et al., 1997), we observed limited Fos-induction in the dopaminergic nucleus. This disagreement might be due, at least in part, to the differences between the methods used, such as the specificity of the conditioned stimuli; we relied on contextual fear conditioning whereas other studies conditioned fear to specific stimuli (visual and/or auditory conditioned stimulations). Although the rats in the CF group displayed reliable freezing immobility, the preceding FS intensity (0.3 mA) used in our experiment was relatively mild in comparison with that used by other investigators (Pezzone et al., 1993; Smith et al., 1992). Furthermore, an FS session has often been delivered on each of 2 consecutive days or more in previous studies (Beck and Fibiger, 1995; Campeau et al., 1997; Pezzone et al., 1993; Smith et al., 1992), while we delivered only one FS session on day 1 for conditioning prior to the reexposure session on day 4. Moreover, there were differences between the strains of rats (Wistar vs. Sprague-Dawley) and between the antibodies we used and the ones the other studies used.

The reciprocal connection of the LC to the amygdala (Cedarbaum and Aghajanian, 1978; Jones and Moore, 1977) may play a role in the behavioral and physiological responses to stress (Butler et al., 1990; Koegler-Muly et al., 1993). The DR also projects to the amygdala (Imai et al., 1986; Ma et al., 1991), where 5-HT can increase neural activity (Compaan et al., 1996) and produce anxious behavior (Gonzalez et al., 1996). Given that activity in the amygdala is necessary for fear conditioning (Davis, 1994), increased activity in this area should enhance fear conditioning. In addition, behaviors that are considered indices of anxiety can be directly modulated by DR manipulation (Higgins et al., 1988, 1992; Hindley et al., 1985). Furthermore, the projection of the DR to the LC (Cedarbaum and Aghajanian, 1978; Pasquier et al., 1977) may also be involved in the behavioral and physiological responses to the CF stress.

Although it is unknown whether Fos induction specially reflects synaptic activity in catecholamine neurons, the gene for the rate-limiting enzyme in catecholamine synthesis, TH, contains an AP-1 binding site where Fos may bind and regulate its transcription (Cambi et al., 1989; Lewis et al., 1987). Additionally, Fos expression in central catecholaminergic neurons closely parallels the increase in TH protein levels (Fritschy et al., 1991), strongly suggesting that Fos regulates TH transcription in central catecholaminergic neurons. CF stress increases Fos expression in the LC (Beck and Fibiger, 1995; Pezzone et al., 1993; Smith et al., 1992), as we confirmed in our study. A previous study demonstrated that immobilization stress increased the mRNA levels of tryptophan hydroxylase (TPH), the rate-limiting enzyme in 5-HT synthesis, in the DR and median raphe nuclei of rats (Chamas et al., 1999). Taken together with the previous findings, our

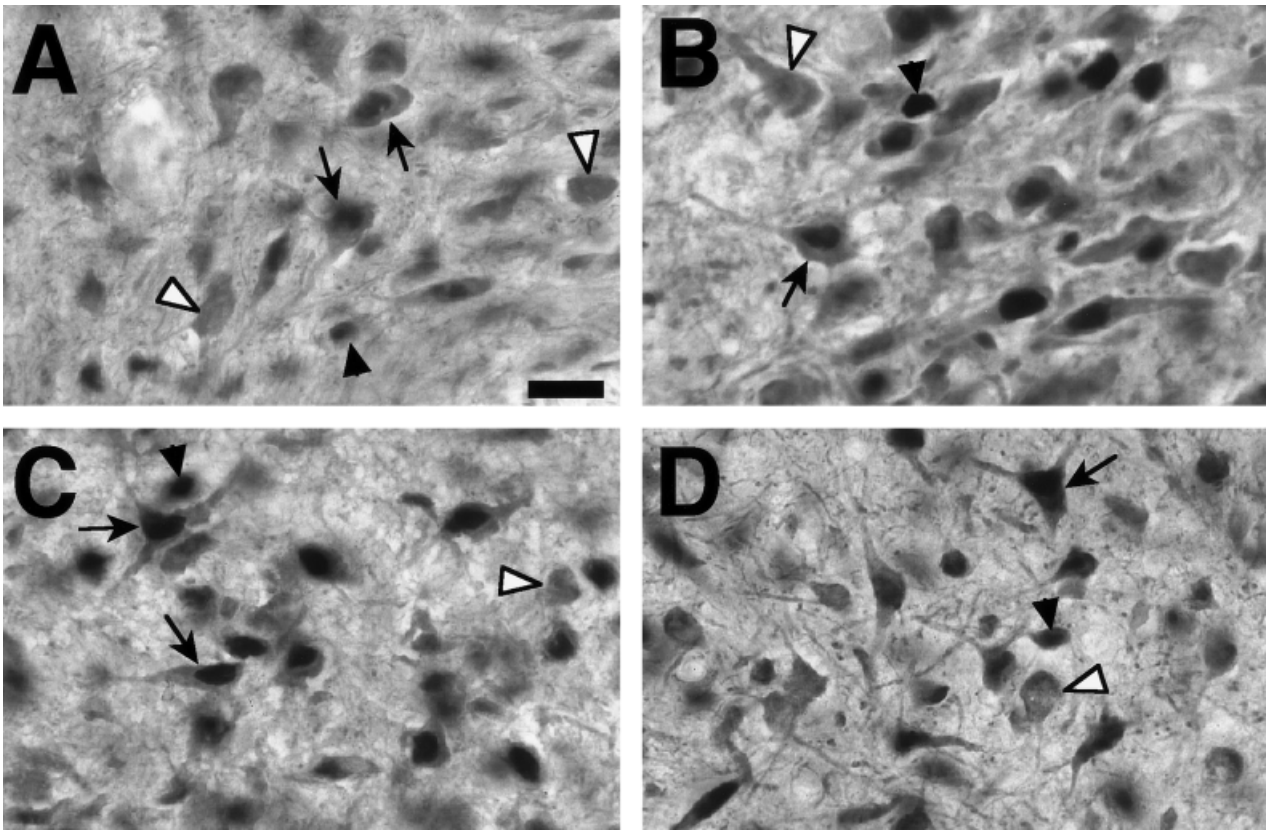

Fig. 3. Photomicrographs of brain sections double-labeled for Fos (black: cobalt-enhanced DAB) and 5-HT (**A**), TH (**B**), or GABA (**C,D**) (brown: DAB) in the DR (**A,C**) and LC (**B,D**) of rat in conditioned–reexposed group. Black arrowheads indicate examples of single-labeled Fos-immunopositive cells and white arrowheads indicate single-labeled TH-, 5-HT-, or GABA-immunopositive cells. Arrows indicate double-labeled cells. Scale bar = 20 μm.

finding of CF-induced Fos expression in noradrenergic LC neurons and serotonergic DR neurons may be related to the alteration of TH and TPH protein/mRNA levels, respectively.

It has been generally suspected that the LC is rich in GABAergic neurophil, but possibly lacks GABAergic neurons. This suspicion is based on the results of glutamic acid decarboxylase (GAD) immunohistochemistry (Ford et al., 1995; Maloney et al., 1999). We used GABA immunohistochemistry with intra-aortic perfusion and fixation to prevent serious deterioration of immunoreactivity. This technical difference may account for the discrepancy between our results and the previous results (Ishida et al., 2001). We found that 63% and 52% of all Fos+ cells in the LC and DR were TH+ and 5-HT+ cells, respectively, and that approximately 60% of all Fos+ cells in the LC and DR were GABAergic neurons in these two regions in the CF group. These inconsistent results can be explained as follows. Because there is colocalization of 5-HT and GABA neurons in the DR (Belin et al., 1983; Harandi et al., 1987; Stamp and Semba, 1995) and of TH and GABA neurons in the LC (Iijima, 1993), some of the monoaminergic Fos+ cells in the serotonergic and noradrenergic nuclei could be GABAergic cells.

Fos-expressing GABAergic cells in the DR may, at least in part, correspond to neurons that were originally considered inhibitory interneurons, which connect reciprocally with the neighboring serotonergic neurons (Aghajanian et al., 1978). Fos-expressing GABAergic cells in the LC were codistributed with the noradrenergic cells; therefore, a portion of the GABAergic cells acted as local inhibitory neurons. The local GABAergic neurons in the DR and LC may be important when noradrenergic and serotonergic neurons are excited during stress. The gene that codes for GAD, the GABA-synthesizing enzyme, is also regulated by Fos (Hughes and Dragunow, 1995). Regulation of gene transcription by Fos proteins may thus contribute to the DR- and/or LC-dependent behavioral effects of CF stress.

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