

# An Analysis of the Effects of Acute and Chronic Fluoxetine on Extracellular Norepinephrine in the Rat Hippocampus during Stress

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*The locus coeruleus (LC) noradrenergic system is activated by a range of arousing and stressful stimuli. The serotonergic inputs to this structure have been shown to attenuate LC activation under some conditions. The present study examined the effect of fluoxetine, a selective serotonin reuptake inhibitor (SSRI) known to be a clinically effective antidepressant, on basal and stress-induced norepinephrine (NE) release. Basal and stress-induced NE efflux in the rat hippocampus were assessed using in vivo microdialysis techniques. The effect of a 30 minute tailpinch stressor on extracellular concentration of NE was compared in rats treated with fluoxetine either once prior to tailpinch or twice daily for 14 days and, respectively, in unhandled controls and vehicle-treated control animals. A single*

*fluoxetine injection prior to tailpinch did not significantly alter the tailpinch-induced increase of extracellular NE as compared to naive controls. However, there was an enhanced NE response to tailpinch in chronic fluoxetine versus chronic vehicle-treated control rats. Thus, acute blockade of 5-HT uptake by fluoxetine does not affect NE release in response to tailpinch stress. Chronic fluoxetine administration, however, results in a potentiated evoked response of the LC-NE system. One action of chronic fluoxetine, which may relate to therapeutic efficacy, is an increase in responsivity of LC neurons.*

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Alterations in noradrenergic and serotonergic function have long been implicated in depression because clinically effective antidepressants directly interact with these systems and produce observable changes in depressive symptomatology (Schildkraut 1965; Iversen 1975; Owens and Nemeroff 1994). The first generation of antidepressants to be widely prescribed included tricyclic compounds such as imipramine, desipramine, and amitriptyline, and monoamine oxidase inhibitors, such as phenylzine and tranylcypromine. These com-

pounds in general have more potent actions on the biochemistry of central noradrenergic systems (Richelson and Pfenning 1984; Heninger and Charney 1987). The emergence of selective serotonin reuptake inhibitors (SSRIs) as the treatment of choice for depression has shifted the recent emphasis of the monoamine theory of affect toward serotonergic mechanisms rather than noradrenergic dysfunction (Baldwin and Rudge 1995; el Mansari et al. 1995; Stanford 1996; Wong et al. 1995). The present study was designed to test the hypothesis that SSRIs may be effective in part by altering serotonergic modulation of the pontine noradrenergic nucleus, the locus coeruleus (LC). Such a model provides a common mechanistic link between effective antidepressants interacting primarily with central noradrenergic versus serotonergic systems.

Several pieces of data support the idea that serotonergic afferents modulate noradrenergic neuronal activity

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in the LC. Serotonergic immunoreactive fibers provide a dense innervation of the LC area (Palkovits et al. 1974; Leger and Descarries 1978; Pickel et al. 1977). Serotonin (5-HT) attenuates sensory-evoked responses of LC neurons (Segal 1979) and decreases glutamate-induced excitation of these cells when applied locally (Bobker and Williams 1989; Aston-Jones et al. 1991; Charley et al. 1991). The evoked response of LC neurons to repeated sciatic nerve stimulation is enhanced in rats pretreated with the 5-HT synthesis inhibitor p-chloro-phenylalanine (PCPA) and is attenuated in rats treated with the 5-HT precursor 5-HTP (Shiekhatter and Aston-Jones 1993).

Exposure to stress and inability to adequately cope with stress are regarded as important etiological factors associated with the emergence of abnormal affective states (Anisman and Zacharko 1982). It therefore is perhaps significant that stressful stimuli have been shown to elevate indices of activity in both central NE and 5-HT systems. The activity of LC neurons increases in response to a variety of environmental and physiological challenges (Elam et al. 1986; Abercrombie and Jacobs 1987; Morilak et al. 1987; Curtis et al. 1993). The turnover of NE and 5-HT is increased in forebrain regions following exposure to stressful stimuli (Thierry et al. 1968; Korf et al. 1973; Dunn 1988; Corley et al. 1992). *In vivo* microdialysis studies have directly shown stress-induced increases in extracellular concentrations of NE and 5-HT in the hippocampus (Abercrombie et al. 1988; Kalen et al. 1989). Thus, the modulation of LC activity by 5-HT may be important for appropriate behavioral responding to stress.

In summary, as with other effective antidepressants, although the primary pharmacological action is understood, little is known regarding the ultimate mechanism(s) of action of the SSRIs that serve to confer therapeutic efficacy. One possibility is that affective disorders are the result of a disruption of some appropriate level of balance among central monoaminergic systems (Schatzberg and Schildkraut 1995). As described, a likely site for SSRIs such as fluoxetine to interact with the noradrenergic system of the LC is at the level of 5-HT inputs to the LC cell bodies. The goal of the present study was to determine whether one action of fluoxetine is to alter the responsivity of noradrenergic LC neurons to stress after acute or chronic administration. We examined the ability of acute and chronic fluoxetine administration to alter NE release in an LC terminal field, the dorsal hippocampus, under basal conditions and in response to presentation of an acute stressor.

## METHODS

### Animals

Male Sprague-Dawley rats (Zivic-Miller Laboratories, Pittsburgh, PA) weighing 250 to 300 gm at the start of treatment were maintained on a 12-hour light-dark cy-

cle with food and water available ad libitum. Animals that were not handled prior to the experiment were housed individually in plastic shoebox cages, and animals that received chronic pretreatment injections were housed individually in hanging wire cages. All procedures were performed in accordance with guidelines published in the *NIH Guide for Care and Use of Laboratory Animals* (NIH, 1985), and all protocols were approved by the Rutgers University Institutional Animal Care and Use Committee.

### Drug Treatments and Experimental Design

Fluoxetine (Eli Lilly, Indianapolis, IN) was dissolved in dH<sub>2</sub>O and was administered by IP injection at a dose of 5 mg/kg. This dose was selected because in preliminary studies with chronic fluoxetine we observed that treatment with 10 mg/kg of the drug produced anorexia and unacceptable rates of weight loss in the animals.

In the study of the acute effects of fluoxetine, rats received a single IP injection of drug 75 minutes prior to stress exposure. The control group consisted of naive, unhandled animals. Animals treated with chronic fluoxetine were injected twice daily for 14 days (Byerley et al. 1988; Caccia et al. 1992; Trouvin et al. 1993); control animals received vehicle injections on the same schedule. In these animals, the neurochemical effects of stress were evaluated approximately 18 hours after the last injection.

Stress exposure consisted of a tailpinch stimulus. An artery forcep (Fisher #13-812-4) was applied approximately 5 cm from the tip of the tail and was left in place for 30 minutes.

### Dialysis Probe Calibration

Vertical concentric microdialysis probes with a 2 mm active region of dialysis membrane were used (for details see Abercrombie et al. 1988). To characterize the *in vitro* recovery rate, probes were calibrated prior to implantation by being placed in a beaker containing artificial cerebrospinal fluid (aCSF: 147 mM NaCl, 2.5 mM KCl, 1.3 mM CaCl<sub>2</sub>, 0.9 mM MgCl<sub>2</sub>; pH 7.4) to which NE standard (0.5 μM) was added. However, because the diffusion properties of neurochemicals in brain tissue are likely different *in vivo* from *in vitro*, dialysate values were not corrected for the recovery of the probe. Rather, determining the recovery of the probe allowed us to discard probes that were not within the normal range of recovery (10–14%). The aCSF was continuously perfused through the probe at a rate of 1.5 μl/minute by a microliter infusion pump (Harvard Apparatus, South Natick, MA).

### Surgical Procedures

Animals were anesthetized with chloral hydrate (400 mg/kg IP) and placed in a stereotaxic apparatus with

the skull flat. A small burr hole was made in the skull centered 3.8 mm posterior and 2.0 mm lateral to bregma. The probe was slowly lowered 3.8 mm from the dura into the dorsal hippocampus and secured with skull screws and dental acrylic. The inlet of the probe was connected to a fluid swivel (Instech Laboratories Inc., Plymouth Meeting, PA), and the rat was placed into a cylindrical Plexiglass container and allowed to recover overnight. Dialysate samples were collected every 15 minutes until a stable baseline efflux (defined as three samples that did not vary by more than 10%) was observed. In most cases, at the end of the experiment rats received an IP injection of the  $\alpha_2$ -agonist clonidine (100  $\mu\text{g}/\text{kg}$  IP) to verify the identity of the NE peak. At the end of the experiment rats were anesthetized and perfused transcardially with 10% buffered formalin. The brains were removed and stored in formalin. Subsequently 60- $\mu\text{m}$  frozen sections were cut on a microtome, mounted on gelatinized slides, and stained with cresyl violet to histologically verify placement of the dialysis probe. Data were discarded if the placement was observed to be outside of the hilar region of the dentate gyrus.

### NE Quantification

The amount of NE in the dialysates was determined with high-pressure liquid chromatography coupled to electrochemical detection (HPLC-EC, see Abercrombie and Finlay 1991 for full description). Briefly, dialysate samples were collected at 15-minute intervals and 20  $\mu\text{l}$  were injected directly onto the HPLC, which consisted of an ESA 580 solvent delivery system, a Waters U6K injector, and a Velosep RP-18 column (100  $\times$  3.2 mm, 3  $\mu\text{m}$ ; Brownlee Labs, Foster City, CA). The mobile phase consisted of 60 mM sodium phosphate buffer (pH 4.2) with 100  $\mu\text{M}$  EDTA, 1.5 mM sodium octyl-sulfate, and 3.5% (v/v) methanol. The flow rate through the system was 700  $\mu\text{l}/\text{minute}$ . The detection system used was an ESA 5100A electrochemical detector with three electrodes in series. The conditioning electrode was set at +270 mV. The applied potential of the second electrode was set at -250 mV, and the compounds of interest were quantified at the third electrode, which was set at +270 mV. Peak heights were measured and compared to the peak height of  $10^{-8}$  M standard calibrated daily. The sensitivity of this assay is 0.5 to 1.0 pg of NE.

### Data Analysis

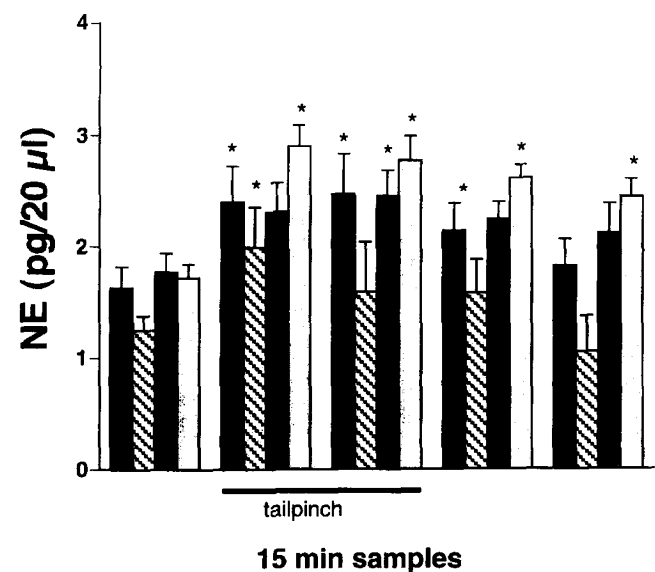
The data are expressed as the mean  $\pm$  SEM. The effect of exposure to tailpinch on hippocampal NE efflux was analyzed using one-way ANOVA with repeated measures over time ( $p < .05$ ) coupled to Dunn's post-hoc test for comparison of treatment values to a baseline mean. The absolute amount of NE measured in dialy-

sates (pg/20  $\mu\text{l}$  sample) was used as the dependent variable to assess within-group effects; the value for the mean of the three final baseline samples and the values for four samples collected during and immediately after the tailpinch were analyzed. Between-group analyses of treatment differences were conducted using two-way ANOVA with repeated measures over time ( $p < .05$ ) coupled to Dunnett's post hoc test for comparison of group means relative to a control mean. To eliminate any potential contribution of baseline value differences to the overall treatment effects, the dependent variable employed in the between-group analyses was absolute change in NE ( $\Delta$  pg) for the four samples collected during and immediately after the tailpinch relative to the baseline NE value. The level of significance for all post-hoc analyses was  $p < .01$ .

## RESULTS

### Controls

The concentration of NE in dialysates from nonhandled control rats increased by 56% from  $1.6 \pm 0.2$  pg/20  $\mu\text{l}$  to  $2.5 \pm 0.4$  pg/20  $\mu\text{l}$  in response to tailpinch [ $F(4,16) = 9.71$ ,  $p < .001$ ; Figure 1]. In chronic vehicle control rats the stress-induced increase in NE of 39%, from  $1.8 \pm 0.2$  pg/20  $\mu\text{l}$  to  $2.5 \pm 0.2$  pg/20  $\mu\text{l}$ , was significant for the second stress sample, although there was no significant overall effect in this group [ $F(4,16) = 2.1$ ,  $p = .13$ ; Figure 1]. However, between-group analysis revealed no signifi-



**Figure 1.** Effect of 30-minute tailpinch stress on hippocampal NE efflux in the experimental groups. *Solid bars*, controls; *cross-hatched bars*, acute flu; *gray bars*, chronic vehicle; *open bars*, chronic flu. For all groups  $n = 5-6$ . Baseline values are the mean of three samples collected prior to tailpinch. Dunn's post hoc analysis: \* $p < .01$  versus baseline.

cant difference between naive controls and chronic vehicle controls in the absolute change in NE output in response to tailpinch [ $F(1,8) = 0.27, p = .62$ ]. In addition, there was no significant interaction of group and time [ $F(3,24) = 0.5, p = 0.69$ ]. Posthoc analysis revealed no significant differences.

### Acute Fluoxetine

Extracellular NE concentration did not differ significantly before and after fluoxetine administration;  $1.3 \pm 0.2$  pg/20  $\mu$ l versus  $1.2 \pm 0.2$  pg/20  $\mu$ l [data not shown,  $F(4,1) = 0.25, p = .64$ ]. Application of the tailpinch stimulus for 30 minutes increased NE efflux from  $1.2 \pm 0.2$  pg/20  $\mu$ l to  $2.0 \pm 0.2$  pg/20  $\mu$ l, an increase of 67% [ $F(4,16) = 3.7, p = .03$ ; Figure 1]. There was no significant effect of group between acute fluoxetine and naive controls [ $F(1,8) = 1.33, p = .27$ ], nor was there a significant interaction between the acute fluoxetine group and naive controls [ $F(3,24) = 0.84, p = .48$ ; Figure 2]. Posthoc analysis likewise revealed no significant differences.

### Chronic Fluoxetine

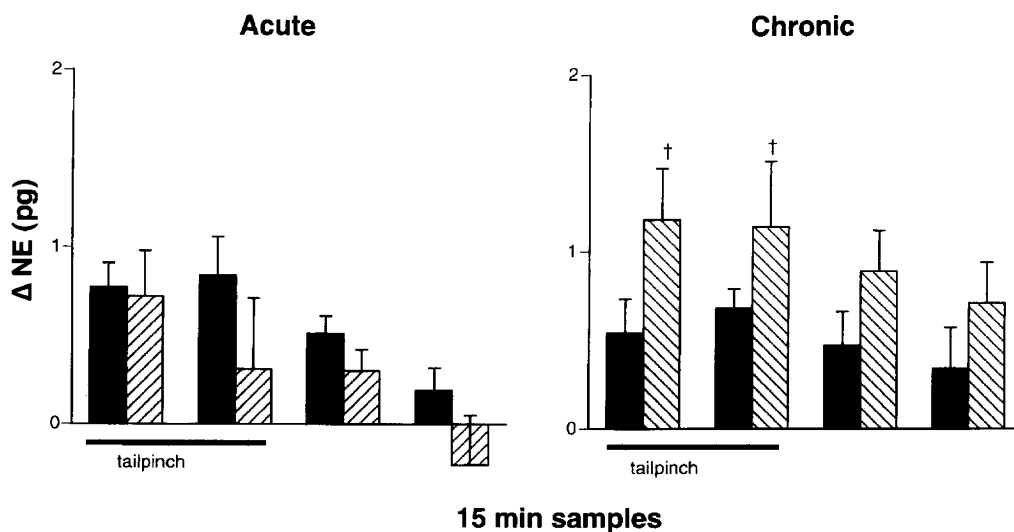
Tailpinch significantly increased extracellular NE by 69%, from  $1.7 \pm 0.1$  pg/20  $\mu$ l to  $2.9 \pm 0.4$  pg/20  $\mu$ l [ $F(4,20) = 9.84, p < .0001$ ]. NE was significantly elevated for the entire hour following the onset of the tailpinch stimulus in the chronic fluoxetine group, whereas a significant elevation in NE occurred only

during the second stress sample in the control group (Figure 1). Comparison of the absolute change in NE output in response to tailpinch between the chronic fluoxetine group and the chronic vehicle group revealed no significant effect of group [ $F(1,9) = 2.73, p = .13$ ]. There was no significant interaction between these groups [ $F(3,26) = 1.00, p = .42$ ]. Post-hoc comparisons, however, revealed significantly greater NE output during the two stress samples in the chronic fluoxetine group (Figure 2).

## DISCUSSION

Acute administration of fluoxetine did not significantly alter NE release evoked by tailpinch. Chronic fluoxetine administration, in contrast, produced an enhancement of the NE response to the tailpinch stimulus along with a significant prolongation of the time course of this response. Neither acute nor chronic fluoxetine altered the basal level of NE efflux in the hippocampus. These results on NE release in awake animals are similar to previous work by Valentino and colleagues (1990) examining LC unit activity in anesthetized rats. These authors showed that the evoked sensory response of LC neurons was somewhat enhanced in rats treated chronically with the SSRI sertraline. However, these authors also observed a decrease in the evoked LC response with acute sertraline.

Based on published data regarding the ability of serotonin to attenuate LC responsiveness to excitatory



**Figure 2.** Left: effect of acute fluoxetine treatment ( $n = 5$ ) on hippocampal NE output during and after tailpinch relative to unhandled controls ( $n = 5$ ). Solid bars, vehicle; cross-hatched bars, fluoxetine. Right: effect of chronic fluoxetine treatment (cross-hatched bars,  $n = 6$ ) on hippocampal NE output during and after tailpinch relative to vehicle-treated controls (solid bars,  $n = 5$ ).  $\Delta$  NE (pg) is the absolute increase in NE obtained in the samples relative to the baseline mean (see text). The tailpinch stimulus was applied for 30 minutes. Dunnett's post hoc analysis:  $p < .01$  versus control.

stimuli (Segal 1979; Bobker and Williams 1989; Valentino et al 1990; Aston-Jones et al. 1991; Charley et al. 1991), we hypothesized that acute fluoxetine administration would attenuate the tailpinch-induced release of NE. Acute fluoxetine previously has been shown to increase extracellular concentration of 5-HT *in vivo* (Perry and Fuller 1992; Sabol et al. 1992; Rutter and Auerbach 1993; Rutter et al. 1994), and a number of studies have shown that 5-HT within the LC is able to attenuate evoked responses of LC neurons to noxious sensory stimuli or to glutamate (see Introduction). Although we observed a trend toward a diminished NE response to tailpinch after acute fluoxetine, this effect did not reach statistical significance. As described, the predicted decrease in an evoked sensory response of LC neurons was obtained in the study of Valentino et al. (1990). The failure to observe a significant decrease in evoked NE release in the present study may be due to the differences in temporal resolution of single-unit recording of LC activity versus microdialysis monitoring of NE efflux.

We found that the group of animals exposed to fluoxetine for 14 days exhibited a *greater* increase in NE release in response to tailpinch. Although testing in this group occurred 18 to 20 hours after the final dose of fluoxetine, drug washout is unlikely to be a factor in this result because the elimination half-life of fluoxetine is 8 to 13 hours and the metabolite, norfluoxetine, has a half-life of 15 to 16 hours and also is an active 5-HT reuptake blocker (Caccia et al. 1990; Gardier et al. 1993). Rather, this result presumably reflects the occurrence of cellular adaptation to chronically elevated extracellular concentration of 5-HT (Bel and Artigas 1993; Rutter et al. 1994; Kreiss and Lucki 1995). Previous work has demonstrated an involvement of the 5-HT<sub>1A/1B</sub> receptor subtypes in the serotonergic inhibition of glutamatergic responses in LC (Bobker and Williams 1989; Charley et al. 1991). These latter data are consistent with the distribution of 5-HT receptor subtypes (Pazos and Palacios 1985; Weissman-Nanopoulos et al. 1985). Li et al. (1993) observed that long-term (21-day) treatment with fluoxetine, but not desipramine, decreased 5-HT<sub>1A</sub> receptor function in the hypothalamus. Likewise, Neumaier et al. (1996) demonstrated decreased 5-HT<sub>1B</sub> mRNA in the dorsal raphe following 21 days of fluoxetine treatment. It remains unknown whether such receptor changes occur in other brain regions, including the LC. If indeed downregulation of 5-HT<sub>1A/1B</sub> receptor function occurs in the LC with chronic fluoxetine treatment, the antidepressant activity of fluoxetine may be expressed, in part, as an increase in the evoked responsivity of LC neurons.

The present data suggest that the antidepressant efficacy of SSRIs such as fluoxetine may involve a time-dependent ability of the drug to enhance the activation of LC neurons by environmental stimuli. This suggestion implies that hyporesponsivity of the LC system

may be a factor in the neural basis of depression. Unfortunately, neither the basic nor the clinical literature provides clear answers regarding possible alterations in central noradrenergic function that might occur in depressed individuals. Chronic stress has been implicated in the etiology of affective disorders (Redmond and Huang 1979; Charney et al. 1993; Abercrombie and Zigmond 1995) and therefore has been used as a paradigm for depression in animal models. In rats, exposure to severe or chronic uncontrollable stress can lead to a performance deficit on subsequent exposure to a similar or different stressor (Seligman and Maier 1967; Weiss and Simson 1985), and SSRIs have proven effective in reversing stress-induced behavioral deficits (Sherman and Petty 1980). Chronic stress has been shown to result in increased tissue concentration of NE (Irwin et al. 1986; Adell et al. 1988), increased activity of tyrosine hydroxylase in LC neurons (Thoenen 1970; Zigmond et al. 1974; Stone et al. 1978; Richard et al. 1988), and enhanced NE turnover in response to an acute stress (Thierry et al. 1968; Kvetnansky et al. 1983; Nisenbaum et al. 1991; Petty et al. 1994). To the extent that the noradrenergic changes reported in these animal studies do indeed reflect the biochemical alterations that exist in clinical depression, it would appear that *hyper-* rather than *hypore-*sponsivity of the LC system may underly some of the symptoms of this disorder. On the other hand, a recent report shows that animals selectively bred for a predisposition to stress-induced behavioral depression display *decreases* in a number of indices of central noradrenergic function (Scott et al. 1996). Data from *in vivo* neurochemical studies using an animal model of depression to study antidepressant action(s) should be informative.

Finally, it is recognized that clinical depression is not a unitary disorder and that multiple subtypes with different etiologies likely exist. In this regard, it is interesting to note the recent data of Schildkraut and coworkers showing that patients with *low* urinary concentration of the NE metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) responded more favorably to treatment with fluoxetine than did patients with high MHPG concentrations (Schatzberg and Schildkraut 1995). An ability of fluoxetine to restore adequate levels of responsivity to environmental stimuli in the LC system may contribute to this clinical profile of the drug (present study; Valentino et al. 1990). The results we obtained reflect the diverse actions of fluoxetine on central monoaminergic systems.

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