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Acute Stress and the Brain Norepinephrine Uptake Mechanism in the Rat

E. D. HENDLEY, G. H. BURROWS, E. S. ROBINSON, K. A. HEIDENREICH AND C. A. BULMAN

Department of Physiology and Biophysics, University of Vermont College of Medicine
Burlington, VT 05401

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HENDLEY, E. D., G. H. BURROWS, E. S. ROBINSON, K. A. HEIDENREICH AND C. A. BULMAN. *Acute stress and the brain norepinephrine uptake mechanism in the rat.* PHARMAC. BIOCHEM. BEHAV. 6(2) 197–202, 1977. – The kinetic constants for norepinephrine uptake in cerebral cortical homogenates were determined in vitro immediately following an acute stress consisting of either forced immobilization, cold-wet exposure, combined cold-plus-restraint, swim stress, or electric footshock in the rat. The kinetic constants, apparent K_m and V_{max}, for uptake of ^{3}H-l-norepinephrine were significantly increased only following 10 min swim at 22°C or following 5 min electric footshock. When severe hypothermia accompanied the stress, the findings suggested that a profound reduction in body temperature was associated with depressed responsiveness of brain noradrenergic mechanisms to stress including decreased uptake kinetic constants. In a series in which the duration of electric footshock was varied from 2 to 30 min, it was noted that the NE uptake kinetic constants were increased at 5 min, but were similar to paired controls at 2, 10 and 30 min following the onset of footshock. It was concluded that various acute stresses did not elicit a generalized response of the cortical NE uptake mechanism to stress in the rat. Furthermore, when uptake kinetic constants did change with stress, the values were often within the range of normal values seen in the rat.

Norepinephrine Uptake Stress Kinetic constants Footshock Hypothermia Restraint stress
Cold stress Swim stress Brain Cerebral cortex

THE application of various aversive treatments, or stresses, in experimental animals has long been known to decrease steady state levels of brain norepinephrine (NE) and to increase its turnover [1, 2, 5, 6, 15, 24]. More recently, Korf *et al.* [13] established that the locus coeruleus was essential for mediating the enhanced NE turnover rate in rat cerebral cortex following electric footshock, and Palkovits *et al.* [17] reported that only the arcuate nucleus among several hypothalamic nuclei in the rat brain was similarly activated in various acute stresses. The present study questioned whether the brain noradrenergic neuronal membrane NE uptake mechanism was also altered by acute stress, in view of the recent findings that the apparent K_m (Michaelis constant) for uptake of ^{3}H-NE was increased following such stressful stimuli as isolation-induced fighting in mice and electroconvulsive shock in rats or mice [7–10, 28]. In the present study short-term (acute) stress paradigms were used in which rats were restrained, or exposed to cold, or subjected to short swim periods at varying water temperatures, or to electric footshock. The rats were killed immediately following each stress procedure, and Michaelis-Menten kinetics were used to determine the apparent K_m and V_{max} for uptake of NE in crude synaptosome-rich homogenates of the rat cerebral cortex.

METHOD

Male adult Sprague-Dawley rats (Canadian Breeding Labs., subsidiary of Charles River Breeding Labs, Inc.) were housed in groups of 6 per cage with food and water ad lib, and alternate 12 hr periods of light and dark. Rats were sacrificed by cervical dislocation between 10 a.m. and 1 p.m. Body weights ranged from 150–300 g but within a given series the mean weights were identical for control and experimental rats.

Restraint stress consisted of immobilizing the rats in a wire mesh restrainer in a prone position at room temperature for 1 hr, then killing immediately following the stress. Control rats were undisturbed cage mates.

Cold exposure was carried out by immersing the rat momentarily in a weak solution of a non-ionic detergent (Triton X-100, 0.05% in tap water) which soaked its fur so that it remained wet throughout the 45 min exposure to cold. The wet rat was placed in a plastic cage without bedding in an empty refrigerator at 2–4° C for 45 min, after which the rat was sacrificed. Controls were undisturbed cage mates.

Combined cold-plus-retraint stress was a combination of the above two procedures. Thus rats were doused with detergent solution and immobilized in the wire mesh restrainer in a prone position and placed in 2–4° C for 45 min then killed. An important feature of this stress was that the rat was not free to curl up, nor to shake excess liquid from its coat, consequently rectal temperature fell precipitously to about 17° C after 45 min cold exposure. Of 13 rats subjected to this procedure, one fatality was observed.

Swim stress consisted of immersing rats in a plastic pipet cleaning jar filled with tap water at varying temperatures in each series from 15° C–42° C. The level of water was set so

that the rat could not climb out of the water to the rim of the jar and thus the rat swam continuously or treaded water during the full 10 min period, after which it was sacrificed. Control rats were undisturbed cage mates.

Following both cold exposure and swim stress the rectal temperature was measured immediately after decapitation using a laboratory thermometer inserted 10 cm into the colon.

Electric footshock was delivered to the paws of the rat placed in a lucite-walled cage, 20 x 24 x 19 cm high, equipped with a grid floor of electrifiable rods set at 1.5 cm apart. Shocks were of 1 sec duration, 1.6 mA, 7 per min, randomized within each min by intervals of 3–15 sec between shocks. The total shock period was varied in each series from 2–30 min. The rats were sacrificed immediately following the stress. Control rats were undisturbed cage mates.

The kinetic constants for the uptake of ^{3}H-l-norepinephrine (^{3}H-l-NE) were determined as rapidly as possible after sacrificing a pair of rats, one stressed and one control, in alternating order in separate experiments within a given series. The cerebral cortex was rapidly dissected from the whole brain on a chilled surface and stored briefly in chilled 0.25 M sucrose until blotted lightly, weighed, then homogenized gently in 10 volumes of 0.25 M sucrose, using 6 up and down strokes of a Teflon pestle in a smooth glass homogenizer (Kontes Glass, 0.004–0.006 in. clearance). Homogenates were centrifuged in the cold (Dupont/Sorvall Instruments, RC2-B) for 10 min at 1000 g, and the supernatant, a crude synaptosome-rich fraction, was used without further purification for the uptake studies. Aliquots of 100 μl, equivalent to 10 mg of original cerebral cortex, were added in the cold to 2 ml of Krebs phosphate Ringer [26] modified to contain half the usual $CaCl_2$ concentration, disodium ethylene-diaminetetraacetic acid (EDTA, 0.05 mg/ml), ascorbic acid (0.2 mg/ml), glucose (2 mg/ml), Nialamide (10 μM, Pfizer Corp.) to inhibit monoamine oxidase and ^{3}H-l-NE (New England Nuclear Corp., 3.7 Ci/mM, 0.04 μM). Nonradioactive l-norepinephrine hydrochloride (Calbiochem Corp.), dissolved in 1 mM HCl containing EDTA (0.05 mg/ml), added to the media provided a range of 5 or 6 different ^{3}H-l-NE concentrations from 0.04–0.2 or 0.4 μM, while the acid vehicle was kept constant in all tubes at less than 0.5% of the incubation media. Incubations were carried out in duplicate at 37°C, with shaking, in nylon or polypropylene centrifuge tubes (Dupont/Sorvall Instruments) for 5 min, using room air as the gas phase. The uptake process was rapidly terminated by immersion of the tubes simultaneously in an ethanol-ice water bath, then centrifuging for 20 min at 20,000 g in the cold. The surfaces of the pellets were rinsed with 10 ml of cold isotonic NaCl (0.15 M) and centrifuged again for 10 min at 20,000 g. The washings were discarded and the tubes drained over absorbent paper for at least 45 min. Pellets were dissolved in 10 ml of Triton-X 100 in toluene (1:4) containing PPO and POPOP and 0.2 ml distilled water. Tritium was counted in a Packard Tricarb Model 3385 (Packard Instruments Corp.) liquid scintillation spectrometer at 45% efficiency. Since all samples including blanks prepared in a similar manner but without added tissue, as well as samples of 0.2 ml of incubation medium, were found to have the same counting efficiency, monitored by sample channels ratio or by automatic external standardization, no corrections were made for counting efficiency.

Net uptake velocity was calculated as nmoles of NE accumulated in 5 min per gram of original cerebral cortex at 37°C minus that accumulated at 0°C (17% of the accumulations at 37°C). NE accumulated in 5 min under similar conditions was previously found to be almost completely unmetabolized catecholamine [19].

The kinetic constants, apparent K_m and V_{max}, were estimated without weighting from Cleland's [4] Fortran program for non-linear regression (best fit to a hyperbola) using a PDP-8/e computer (Digital Equipment Corp.) and a 4010-1 graphics terminal (Tektronics Corp.) connected to a Sigma 6 (Xerox Corp.) computer.

Special care was taken to treat control and stress tissues concomitantly and identically with respect to time, temperature of incubation and centrifugation conditions, thus ensuring a legitimate statistical comparison between pairs of control and stressed animals (paired *t*-test). The kinetic constants are derived values at best, and would not be comparable except under identical conditions of measurement.

RESULTS

Exposure of rats, while wet, to 2–4°C for 45 min (cold stress) failed to alter the kinetic constants for ^{3}H-l-NE uptake (Table 1). These rats had significantly reduced rectal temperature (30°C). Immobilization of rats for one hr at room temperature (restraint stress) was also ineffective in altering the kinetic constants for ^{3}H-l-NE uptake (Table 1).

During combined cold-plus-restraint, rats were immobilized in the restrainer, then placed in the refrigerator (2–4°C) for 45 min. Following this stress their snouts, paws, tails and genitalia were bright red in appearance, and they were grossly inactive prior to sacrificing at the end of the stress period. They failed to right themselves when released from the restrainer and gave no resistance to handling and sacrificing. Their rectal temperatures fell markedly to an average of 17°C (Table 1) as a result of being deprived of two important means of conserving body heat, namely shaking off excess moisture and curling up to lower total body surface area. More importantly, peripheral vasodilation in the skin of these rats was evident, undoubtedly contributing significantly to the profound heat loss and indicating a failure of the noradrenergically-mediated response to heat loss. In these rats the apparent K_m fell to an average of 17% below paired control values and this decrease was of borderline significance (Table 1). There was no significant alteration in the V_{max}.

In rats subjected to swim stress (10 min swim in tap water of varying temperatures) the results obtained varied with the temperature of the water (Table 2). When rats swam in water at body temperature (38°C), rectal temperature increased slightly but consistently ($p < 0.01$) by 0.5°C, presumably as a result of inability to dissipate heat generated by the muscular exertion of swimming. There were no significant changes in the kinetic constants apparent K_m and V_{max} in these rats. They swam vigorously at the beginning of the 10 min period but after several minutes, spent much of the time in treading water. They did not appear to search continuously for escape routes as did rats that swam in cold water.

Rats that swam in relatively hot water (42°C) were severely hyperthermic after 10 min (41.5°C, Table 2). They appeared to be somewhat inactive at the end of the swim period although they were aggressive when handled. There were no significant differences in the NE uptake kinetic

TABLE 1

KINETIC CONSTANTS FOR UPTAKE OF ^{3}H-l-NOREPINEPHRINE IN HOMOGENATES OF RAT CEREBRAL CORTEX: EFFECTS OF COLD, RESTRAINT STRESS

Rectal Temp °C		Apparent K_m μM		V_{max} nmol/g/5 min	
Control	Stress	Control	Stress	Control	Stress
		Cold stress: 45 min at 2-4°C, wet fur			
37.6 ± 0.2	30.0 ± 0.1	0.19 ± 0.01	0.20 ± 0.01	0.76 ± 0.06	0.75 ± 0.05
(16)	(16)	(16)	(16)	(16)	(16)
$p<0.001$		$p>0.05$		$p>0.05$	
		Restraint stress: 1 hr at room temp			
—	—	0.22 ± 0.01	0.22 ± 0.02	0.74 ± 0.10	0.77 ± 0.04
		(6)	(6)	(6)	(6)
		$p>0.05$		$p>0.05$	
		Combined Cold-Plus-Restraint for 45 min			
37.6 ± 0.1	16.7 ± 0.7	0.18 ± 0.01	0.15 ± 0.01	0.63 ± 0.04	0.61 ± 0.04
(12)	(12)	(12)	(12)	(12)	(12)
$p<0.001$		$0.1>p>0.05$		$p>0.05$	

Values are means ± SEM, number of rats in parentheses.
p values from paired t-test.

TABLE 2

KINETIC CONSTANTS FOR UPTAKE OF ^{3}H-l-NOREPINEPHRINE IN HOMOGENATES OF RAT CEREBRAL CORTEX: SWIM AT VARYING TEMPERATURES

Rectal Temp °C		Apparent K_m μM		V_{max} nmol/g/5 min	
Control	Stress	Control	Stress	Control	Stress
		10 min Swim at 15°C			
37.6 ± 0.2	23.1 ± 0.2	0.24 ± 0.02	0.24 ± 0.03	0.86 ± 0.07	0.94 ± 0.15
(8)	(8)	(8)	(8)	(8)	(8)
$p<0.001$		$p>0.05$		$p>0.05$	
		10 min Swim at 22°C			
37.5 ± 0.2	27.4 ± 0.3	0.20 ± 0.02	0.30 ± 0.04	0.60 ± 0.06	0.81 ± 0.09
(9)	(9)	(9)	(9)	(9)	(9)
$p<0.001$		$p<0.05$		$p<0.05$	
		10 min Swim at 38°C			
38.0 ± 0.1	38.5 ± 0.1	0.18 ± 0.01	0.19 ± 0.02	0.53 ± 0.02	0.61 ± 0.07
(12)	(12)	(12)	(12)	(12)	(12)
$p<0.01$		$p>0.05$		$p>0.05$	
		10 min Swim at 42° C			
37.8 ± 0.1	41.5 ± 0.1	0.24 ± 0.01	0.23 ± 0.01	0.79 ± 0.04	0.75 ± 0.02
(20)	(20)	(20)	(20)	(20)	(20)
$p<0.001$		$p>0.05$		$p>0.05$	

Values are means ± SEM, number of rats in parentheses.
p values from paired t-test.

constants in these rats when compared with nonstressed controls.

When rats swam for 10 min in water at 22°C, rectal temperature fell to 27°C (Table 2). These rats swam vigorously and continually searched for exits to escape, such as by diving to the bottom of the jar or attempting to reach for the rim of the jar. The apparent K_m in this series increased significantly by 50% above nonstressed controls, and V_{max} by 35%.

In rats that swam for 10 min at 15°C, rectal temperature fell to an average of 23°C (Table 2). These rats swam vigorously and searched for exits for escape early in the swim but soon gave up these efforts and attempted to keep as much of their bodies out of the water as they could. They could accomplish this by kicking the hindlimbs in place and avoiding swimming movements that immersed the whole body. At the end of 10 min these rats were exhausted and relatively unresponsive to handling or to

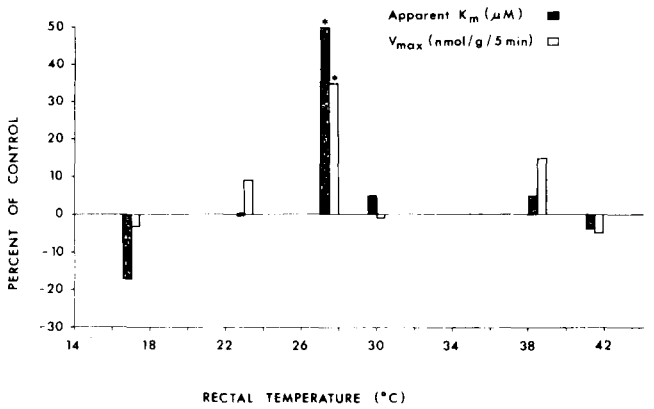

FIG. 1. Effects of alterations in body temperature on percent change from paired controls in the kinetic constants for ^{3}H-l-norepinephrine uptake in cerebral cortical homogenates. Data are taken from series in Tables 1 and 2, in which rectal temperature was measured at the end of the stress procedure. *At 27°C both apparent K_m and V_{max} were significantly increased (Table 2). At 17°C the fall in apparent K_m was of borderline significance (Table 1). All other differences from paired controls were not statistically significant.

righting themselves. Their kinetic constants for ^{3}H-l-NE uptake were not significantly different from nonstressed paired control rats (Table 2).

In order to evaluate the effects of temperature change per se on NE uptake kinetic constants, we plotted the percent change in kinetic constants against mean rectal temperature regardless of the applied stress, using the series in Tables 1 and 2 in which rectal temperature had been recorded (Fig. 1). We felt justified in ignoring the effects of physical exercise in swimming on the basis of the findings of Stone [23] that the neurochemical changes during cold swim stress were due to hypothermia and not to the muscular exertion involved. The data in Fig. 1 suggested that the uptake kinetic constants were not altered by hyperthermia (rectal temperature 38.5° or 41.5°C) or by hypothermia to a level of 30°C. When rectal temperature averaged 27°C we observed significant increases in apparent K_m and V_{max}, however, as hypothermia became even more severe the kinetic constants progressively declined from a significant increase above controls to a decrease in the apparent K_m(albeit of borderline statistical significance), and to no significant change in the V_{max}. The effects on the kinetic constants observed at rectal temperatures of 23° and 17° were associated with depressed behavioral reactivity although no objective behavioral measurements were made in these studies.

The effects of footshock on the kinetic constants for ^{3}H-l-NE uptake varied with the duration of the shock procedure (Table 3). Rats shocked for only 2 min exhibited no significant changes in the kinetic constants. Following 5 min footshock there was a significant increase in apparent K_m (33% above controls) as well as in V_{max} (28% above controls). After footshock for 10 min or 30 min there were no detectable differences in kinetic constants from paired control values. Behaviorally the rats appeared to detest the footshock procedure more than any of the other stresses in this study. During 2, 5 or 10 min footshock periods the rats made continual efforts to escape, including digging attempts with their paws in the corners of the shock apparatus, and making continual futile searches and jumps for escape routes. Rats shocked for 2 min were without exception extremely irritable when removed from the apparatus and usually attempted to bite the investigator. After 5 or 10 min footshock most of the rats exhibited similar irritable behavior, although others were seen to cower in a corner. During 30 min footshock the rats made continual attempts to search for exits for the first 15 min, after which time they appeared to cease resisting the inevitability of the shock procedure. At the end of the 30 min period they were docile when removed from the apparatus.

DISCUSSION

In the present study the lability of the neuronal membrane NE uptake mechanism in the rat cerebral cortex was tested using various acute stresses that have been associated in the literature with enhanced adrenocortical activation [12, 14, 16, 17, 27] and with sympathoadrenomedullary stimulation [18]. In this limited series we were not able to detect a generalized response of the neuronal membrane NE uptake mechanism to stress in the rat, although a more thorough investigation, such as varying the intensities and/or the durations of each stress, might have revealed a common generalized response to stress once a presumed threshold had been reached. Keim and Sigg [12] concluded from a study of 3 stress paradigms in the rat that the time course and direction of the various neurochemical changes observed were not consistent with the concept of a generalized neurochemical response to stress. Among the acute stresses tested in the present study, only 10 min swim at 22°C, or electric footshock for 5 min, increased apparent K_m and V_{max} significantly. It must be emphasized, however, that for the most part, the significantly elevated kinetic constants in these two series were well within the normal range of values observed among control rats in other series within this study. The variability of control data observed from one series to another was not attributable to differences in mean age or weight of the rats between series, nor to the season in which each series was run. It should be reemphasized here that within a given series, ages and mean body weights were identical for control and stressed rats, as were also the in vitro conditions for measuring uptake in pairs of control and stressed rats. Nevertheless, the fact that stressed rats recorded kinetic constants within the physiological range compels us to draw only tentative conclusions from this study.

It was also noted that the magnitude of the change in kinetic constants observed in the rat was only 25–50% above paired controls, and considerably lower than the change in apparent K_m following one fight (68% above controls) or one electroconvulsive shock (110% above controls) in the mouse [28]. However, considering that uptake has been estimated to account for removal of about 70–80% of the released NE [11], a change in kinetic constants of only 25–50%, if real, should nevertheless be consequential in altering the availability of NE for stimulation of postsynaptic sites.

Assuming that Michaelis-Menten kinetics for enzyme systems may be also applied to carrier-mediated transports such as the NE uptake mechanism [29], then the increased apparent K_m may reasonably be interpreted as indicating a decreased affinity of the NE terminal membrane carrier for uptake of NE, and the increased V_{max} as an increase in the number of NE transport sites on the terminal membrane as a result of these stress procedures.

Swim stress for 10 min at varying temperatures revealed

that the response of the NE uptake mechanism varied with the temperature of the water bath. From signs of the rats' continual attempts to escape, it was clear that the stress became more aversive to the rats as water temperature fell below normal body temperature. After 22°C swim rectal temperature fell to 27°C and apparent K_m and V_{max} were significantly elevated. After 15°C swim the kinetic constants were no different from unstressed controls. We propose that the severe hypothermia in the latter series (rectal temperature 23°C), which is known to be associated with decreased brain NE synthesis and utilization rates [22,23] also prevented the increase in NE uptake kinetic constants that we observed during less severe hypothermia (Fig. 1). This conclusion is further borne out by the series on cold-plus-restraint stress (Table 1, Fig. 1) where rectal temperature fell to an average of 17°C, apparent K_m seemed to decrease rather than increase, and rats gave evidence of failure of the noradrenergically-mediated heat conservation mechanism to operate effectively (marked peripheral vasodilation during severe cold exposure). Stone [22,23] proposed that cold stress differed from other forms of stress in that brain NE metabolism was decreased rather than increased as in other stresses. He also associated a decreased brain NE utilization rate with marked behavioral withdrawal and torpor in severely hypothermic rats, as supported also by our findings.

One can assume from the decrease in apparent K_m with severest hypothermia (Fig. 1) that an increase in intraneuronal metabolism of NE and a decrease in extraneuronal breakdown of brain NE would prevail under such conditions. In accordance with this prediction, Stolk *et al.* [21] reported that deaminated metabolites of brain NE had increased in the rat after 20 min swim at 15°C while the O-methylated metabolite, normetanephrine, was decreased.

The sequence of changes in various brain noradrenergic mechanisms following acute footshock in the rat can now be discerned from several studies, although certain key measurements that would give a more complete picture are still lacking. As early as 5 min after onset of footshock, brainstem NE was significantly depleted [15], turnover of brainstem NE newly synthesized from dopamine was increased [20], and brain NE uptake kinetic constants in the cerebral cortex were significantly elevated (Table 3). After 10 min footshock the kinetic constants returned to control levels (Table 3). After 15 min footshock there was an increase in rate of utilization of newly synthesized NE in the brainstem while at the same time there was no detectable change in the accumulation of intracisternally administered ^{3}H-l-NE into the brainstem [25]. Following 30 min footshock NE turnover in cerebral cortex was elevated, but only if the locus coeruleus was also intact [13]. At the same time (30 min), NE uptake kinetic constants were no different from control levels in the cerebral cortex (Table 3). Following 45 min tailshock, a related stress paradigm, endogenous NE levels in cerebral cortex and hypothalamus were markedly reduced, plasma corticosterone was elevated, tyrosine hydroxylase activity in the forebrain was unchanged, and no change in ^{3}H-NE uptake was discernible in cerebral cortical slices [27]. From this composite picture it appears that the earliest neurochemical changes were detectable not only in the brainstem, where the noradrenergic cell bodies are located, but also in the cerebral cortex, where noradrenergic nerve terminals exhibited increased apparent K_m and V_{max} for NE uptake. With increasing duration of footshock stress, NE utilization rate was elevated at a time when rate of NE synthesis could not keep pace with turnover, resulting in marked depletions of endogenous levels of NE, as originally proposed by Bliss *et al.* [3]. Interestingly, with more prolonged duration of footshock (3 hours; [24]), or chronic footshock (15 days; [27]), brain NE uptake mechanisms again appeared to be altered in response to stress.

TABLE 3

KINETIC CONSTANTS FOR UPTAKE ^{3}H-l-NOREPINEPHRINE IN HOMOGENATES OF CEREBRAL CORTEX: ELECTRIC FOOTSHOCK* FOR VARYING TIME PERIODS

Apparent K_m (μM)		V_{max} (nmol/g/5 min)	
Control	Stress	Control	Stress
2 min Footshock			
0.23 ± 0.02 (13)	0.24 ± 0.05 (13)	0.80 ± 0.10 (13)	0.67 ± 0.09 (13)
$p>0.05$		$p>0.05$	
5 min Footshock			
0.15 ± 0.01 (9)	0.20 ± 0.02 (9)	0.54 ± 0.05 (9)	0.69 ± 0.05 (9)
$p<0.025$		$p<0.05$	
10 min Footshock			
0.18 ± 0.01 (11)	0.16 ± 0.01 (11)	0.70 ± 0.04 (11)	0.65 ± 0.06 (11)
$p>0.05$		$p>0.05$	
30 min Footshock			
0.23 ± 0.01 (16)	0.22 ± 0.01 (16)	0.85 ± 0.04 (16)	0.82 ± 0.04 (16)
$p>0.05$		$p>0.05$	

Values are means ± SEM, number of rats in parentheses.
p values from paired *t*-test.
*Shocks were of 1 sec duration, 1.6 mAmp, 7/min.

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