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Marked behavioral activation from inhibitory stimulation of locus coeruleus α_1-adrenoceptors by a full agonist

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Abstract

α_1-Adrenoceptors are concentrated in the locus coeruleus (LC) where they appear to regulate various active behaviors but have been difficult to stimulate effectively. The present study examined the behavioral, pharmacological and neural effects of possible stimulation of these receptors with 6-fluoronorepinephrine (6FNE), the only known selective α-agonist that has full efficacy at all brain α-receptors. Infusion of this compound in the mouse LC was found to produce extreme activation of diverse motivated behaviors of exploration, wheel running and operant approach responding in different environments consistent with a global behavioral function of the dorsal noradrenergic system. Infusion of selective antagonists of α_1- (terazosin) or α_2-(atipamezole) receptors or of either the partial α_1-agonist, phenylephrine, or full α2-agonist, dexmedetomidine, indicated that the behavioral effects of 6FNE were due largely due to activation of LC α_1-receptors consistent with the known greater density of α_1-than α_2-adrenoreceptors in the mouse nucleus. Immunohistochemistry of fos in tyrosine hydroxylase-positive LC neurons following IV ventricular infusions indicated that 6FNE markedly depressed whereas terazosin strongly enhanced the apparent functional activity of the nucleus. The changes in fos expression following 6FNE and terazosin were significantly greater than those following dexmedetomidine and atipamezole. It is hypothesized that the α_1-receptors of the mouse LC are strongly activated by 6FNE and serve to potently inhibit its tonic or stress-induced activity which in turn disinhibits prepotent motivated behaviors.

Keywords

α_1-adrenoceptor; locus coeruleus; exploratory behavior; motivated behavior; fos; stress

1. Introduction

Central α1-adrenoceptors have been found to play an essential role in behavioral activation under a variety of experimental conditions. Blockade of these receptors in a number of brain regions produces immobility in novel surroundings whereas stimulation may lead to behavioral activation in familiar environments (Stone et al., 1999; 2001; 2004a). The LC appears to be a

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key region in this system in that it contains a dense concentration of α_1-receptor binding sites (Jones et al., 1985; Stone et al., 2004b) having the above behavioral properties (Stone et al., 2004a,b; Lin et al., 2007). Moreover this nucleus is a site of convergence for systems regulating arousal (Cedarbaum and Aghajanian, 1977; Berridge et al., 1993), motivated behavior (Aston-Jones and Cohen, 2005; Bouret and Sara, 2005), stress (Valentino and VanBockstaele, 2008; Ma et al., 2008; Korf et al., 1973) and pain (Pertovaara, 2006) and can affect a wide range of behavioral and physiological functions.

How α_1-adrenoreceptors of the LC achieve behavioral activation is not presently understood. However, while α_1-receptors have traditionally been thought to mediate postsynaptic excitation (Hermann et al., 2005), several recent studies have shown that they can also depress excitatory synaptic or increase GABAergic neurotransmission in a number brain regions (McElligott and Winder, 2008; Lei et al., 2007). These findings are of interest because a reduced functional activity of the LC is thought to lead to the activation of task-specific behaviors (Aston-Jones and Cohen, 2005; Weiss et al., 1986; Grant and Weiss, 2001), while excessive LC activity has been postulated to cause aversion and the abandonment of rewarding behaviors (Smith and Aston-Jones, 2008; Taylor et al., 1988), and possibly depression (Grant and Weiss, 2001; Simson et al., 1986; Stone, 1982).

It would therefore be of interest to determine how the functional activity of this nucleus is affected by α_1-adrenergic stimulation that produces behavioral activation. Previous work on this problem utilized local infusion of the selective α_1-agonist, phenylephrine (PE), which produces a weak stimulation of exploratory behavior in rats (Stone et al., 2004b). PE, however, is known to be only a partial agonist at brain α_1-receptors (Johnson and Minneman, 1986; Law-Tho et al., 1993). In contrast, we have found in recent preliminary experiments that 6-fluoronorepinephrine (6FNE), which is the only known selective full agonist at all central α-adrenoceptors (Johnson and Minneman, 1986; Brasili et al., 1987), produces extreme hyperactivity in the home cage when infused in the mouse LC.

The present study was undertaken therefore to investigate the effects of infusion of 6FNE in the LC of unrestrained behaving mice to determine 1) what types of behavior are activated by this agent, 2) which LC adrenoceptor, α_1- or α_2-, mediates its behavioral effects, and 3) what its action is on the functional activity of the LC In these experiments we have examined 3 diverse forms of positively motivated behavior -exploration, wheel running and operant approach behavior - because the LC is believed to regulate motivated behavior in a global rather than specific manner (Aston-Jones and Cohen, 2005). In order to avoid any interference with these behaviors or CNS function, the functional activity of the LC was assessed ex vivo using fos expression.

2. Results

1) Histological verification of cannula placement

Only animals subsequently shown to have cannula tips within 0.5 mm of the LC proper and who had been shown to have positive immobility responses to an initial screening with quinpirole infusion at 2 nmoles (Q2 positive) were used for data analysis in the following experiments. (We have shown previously that over 90% of animals with cannula tips within 0.5 mm of the LC proper show marked immobility responses to Q infusion at this dose (Lin et al., 2008)). The histology for the initial experiment of 35 Q2 positive animals is shown in Fig 1. Similar results were obtained for the remaining experiments of the study. The figure also includes 20 animals with cannulas situated at least 0.75 mm from the LC who were used to further verify the site of action of 6FNE as described below.

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2) Behavioral effects of 6FNE in LC

a) Home cage activity—The effects of unilateral LC infusion of 6FNE, PE, or dexmedetomidine (DMT) on measures of various active behaviors in the home cage are shown are shown in Fig 2A-I. As is readily apparent from these results, 6FNE was capable of producing marked increases in home cage behavioral activation whereas the partial α_1-agonist, PE, and the full α_2-agonist, DMT, given either individually or together, were not.

Analysis of the data for 6FNE revealed that the drug produced significant effects on rearing ($F_{2,37} = 9.88$, $p < 0.001$, Fig 2A), ambulation ($F_{2,37} = 38.56$, $p < 0.0001$, Fig 2B) and total movement ($F_{2,37} = 10.19$, $p < 0.001$, Fig 2C) with the 12 nmoles dose markedly increasing these behaviors (rearing, $F_{1,37} = 10.84$, $p < 0.01$; ambulation, $F_{1,37} = 68.05$, $p < 0.0001$; movements, $F_{1,37}$ 33.22, $p < 0.0001$) and the 2 nmoles dose being ineffective. There was no apparent postural asymmetry or lateralization of behavioral effect to either dose of 6FNE. The 12 nmoles dose was also found to produce a smaller increase in time spent at the food hopper which was of borderline statistical significance (vehicle, 3.23 ± 0.9 min; 6FNE, 6.90 ± 1.89 min, $t_{26} = 2.01$, $p < 0.06$). A subgroup of LC-implanted animals that was not prescreened with Q2 showed a similar activating effect to 6FNE at 12 nmoles (Supplemental data, Fig. S1) indicating that the initial screening with Q did not affect the subsequent response to 6FNE.

Analysis of the responses to PE (Fig 2 G-I) revealed that this agonist produced only a dose-dependent reduction in rearing ($F_{2,32} = 4.77$, $p < 0.05$) and did not significantly alter ambulation or total movement. Similarly, DMT (Fig 2 D-F) was found to produce only dose-dependent, reductions in rearing ($F_{3,32} = 4.74$, $p < 0.01$), ambulation ($F_{3,32} = 3.88$, $p < 0.05$) and total movements ($F_{3,32} = 8.14$, $p < 0.001$). The combination of DMT at 0.04 nmoles and PE at 12 nmoles (Fig 2 G-I) also produced a significant reduction in rearing ($F_{1,38} = 8.48$, $p < 0.01$) but did not significantly alter ambulation or movement.

To determine if the above activating effects of 6FNE were mediated by the LC as opposed to a neighboring pons region, animals with cannulas at least 0.75 mm distant from the LC proper were examined as were the effects of a prior unilateral lesion of the LC with 6-OHDA (Fig 3). Mice with cannulas more than 0.75 mm distant failed to show a significant stimulatory effect of 12 nmoles of 6FNE on any behavior. Similarly, animals given unilateral LC lesions via Q2-positive cannulas showed significant attenuations of the rearing ($t_8 = 2.40$, $p < 0.05$), ambulation ($t_8 = 4.61$, $p < 0.002$) and movement responses ($t_8 = 2.71$, $p < 0.05$) to a subsequent infusion of 6FNE (12) compared to sham-lesioned controls.

b) Wheel running behavior—The effects of LC 6FNE infusion on wheel running behavior are shown in Fig. 4. As can be seen, the drug produced a large dose-dependent increase in this behavior that was evident for most of the 2 h post-infusion period in the 12 nmoles group. The latter dose produced a total running time of 46.4 ± 12.4 min spread over a 2 hr period compared to 10.2 ± 2.7 for vehicle-infusion. For analysis, the running times of days with vehicle, 2 and 12 nmole 6FNE were compared in a 3 × 4 (6FNE × interval) ANOVA for repeated measures on the interval variable. This revealed a significant dose-dependent effect of 6FNE ($F_{2,15} = 5.55$, $p < 0.05$) with the mean of the 12 nmole 6FNE day being significantly greater than that of the vehicle day ($F(1,15) = 10.62$, $p < 0.05$). The interaction between day and interval was not significant.

c) Operant approach behavior—The effects of 6FNE on operant approach behavior are shown in Fig. 5. In this experiment, vehicle and 6FNE (12 nmoles) infusions were tested for their ability to restore approach behavior in trained animals that were deficient in this behavior as a result of infusion of the α_1-antagonist, TER. A 2 × 2 (TER × 6FNE) ANOVA revealed a significant main effect of TER ($F_{1,18} = 7{,}58$, $p < 0.05$) and a significant TER × 6FNE interaction ($F_{1,18} = 25.65$, $p < 0.0001$). As can be seen from the figure, infusion of TER by itself at 2

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nmoles produced a virtual abolition of approach of the water dipper with the mean latency increasing some 15-fold to close to the 2 min limit ($F_{1,18} = 38.52$, $p < 0.0001$). Coinfusion of 6FNE at 12 nmoles produced a complete rescue of approach behavior ($F_{1,18} = 23.20$, $p < 0.005$) with the mean latency returning to, and no longer significantly different from the vehicle level. 6FNE given alone did not significantly affect the approach response.

2) Role of α_1- and α_2-adrenoceptors in behavioral effects of 6FNE

The effects of coinfusion of antagonists of α_1- (TER) and α_2-receptors (ATI) on the behavioral activation effect of 6FNE in the home cage are shown in Fig 6. The data for the rearing, ambulation and movement responses were analyzed by separate $2 \times 2 \times 3$ (6FNE $\times$ Antagonist $\times$ Dose) ANOVAs. These indicated a significant main effect of Antagonist on rearing ($F_{1,56} = 11.43$, $p < 0.005$), ambulation ($F_{1,56} = 6.44$, $p < 0.05$) and movement responses ($F_{1,56} = 28.54$, $p < 0.0001$) which was due to greater inhibitions of these behaviors by TER than ATI for both the 6FNE and vehicle-treated groups combined. Post-hoc comparisons of the effects of each antagonist versus vehicle in the 6FNE condition revealed that TER dose-dependently reduced rearing responses ($F_{1,56} = 7.57$, $p < 0.01$) and movements ($F_{1,56} = 21.35$, $p < 0.0001$) while ATI failed to affect any of the behaviors of the drug-infused animals. There was a significant interaction between antagonist and dose for total movements ($F_{2,56} = 8.43$, $p < 0.001$) which resulted from a greater inhibition by TER of this behavior of the vehicle than 6FNE-treated animals

3. Effect of 6FNE infusions on LC functional activity – double label studies

This experiment examined the effects of 4th ventricle infusions of 6FNE or vehicle in the presence or absence of TER or ATI, on fos expression in TH-positive LC neurons by double label immunohistochemistry. Also included in this experiment were animals receiving DMT alone. It was necessary to use intraventricular rather than LC infusions as preliminary experiments indicated that cannula implantations near the nucleus led to the appearance of numerous fos-positive, TH-negative glial-like cells (i.e, cells having small fos-stained nuclei). The use of the ventricular route, which avoided this problem, was justified in that 6FNE, TER and ATI given via this route produced similar behavioral effects as when given near the LC (Supplemental data, Fig. S2) and these were significantly reduced by prior bilateral 6OHDA lesions of the LC (Supplemental data, Fig. S3).

Representative single-label and merged fos/TH-stained sections from the vehicle, 6FNE and TER groups are shown in Fig 7A-I. It is evident that, in addition to staining the nuclei of LC neurons, the fos antiserum also unexpectedly stained the cytoplasm of neurons in the adjacent mesencephalic trigeminal nucleus (Fig 7B). However, judging from the single and merged images, this nonspecific staining, which disappeared when the antibody was omitted, did not occur in the LC or compromise the nuclear fos staining in LC neurons, and was therefore disregarded.

The proportions of double labeled LC cells after the various drug treatments are shown in Table 1. (As the total number of fos-positive cells was virtually identical to that of double-labeled cells, these data were not analyzed and are not shown). Because the proportion of the vehicle control group was low, there was a skewing of the scores toward low values which tended to obscure detection of reductions in labeling. For the statistical analysis, therefore, the proportions were first $\log_{10}$-transformed before being analyzed with a 2×3 (6FNE $\times$ Antagonist) ANOVA. The latter showed highly significant main effects for both 6FNE ($F_{1,26} = 46.27$, $p < 0.0001$) and Antagonist ($F_{2,26} = 22.66$, $p < 0.0001$) with no significant interaction between the two. Compared to the vehicle-alone group, the animals treated with 6FNE-alone showed a highly significant decrease in the log proportion of double labeled neurons ($F_{1,26} = 18.29$, $p < 0.005$) whereas those treated with TER-alone showed a highly

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significant increase ($F_{1,26} = 23.56$, $p < 0.001$). Animals given ATI-alone did not differ significantly from the vehicle-alone group ($F_{1,26} = 5.65$, $p > 0.1$). Furthermore TER coinfusion significantly reduced the effect of 6FNE ($F_{1,26} = 21.96$, $p < 0.001$) whereas ATI coinfusion failed to do so ($F_{1,26} = 6.74$, NS). While DMT-alone also tended to reduce the proportion of double-labeled neurons at both doses tested (0.056 and 0.1 nmoles) this effect failed to reach statistical significance (one way ANOVA comprising the vehicle-alone and the two DMT-alone groups, $F_{2,16} = 1.83$, NS). None of the above drugs had any effect on the total number of TH positive neurons.

Correlational analysis applied to all treatments of Table 1 indicated that the log proportion of double-labeled LC cells was inversely related to home cage movement at 0-30 min post infusion ($r = -.80$, $p < 0.05$; Fig 8). Similar inverse correlations were obtained with rearing (-.73, $p < 0.05$) and ambulation (-.64, NS)

Because unilateral LC 6FNE infusions in the previous behavioral experiments did not appear to produce gross postural or behavioral asymmetry it was of interest to determine if the unilateral injections were affecting both LC's. For this purpose the proportion of double-labeled neurons in the LC contralateral to a unilateral infusion of vehicle, 6FNE (12) or TER (2) was examined. The results indicated that the latter two drugs exerted actions on the contralateral LC that were similar to their effects in the above intraventricular experiment (Fig. S4 Supplemental data).

3. Discussion

The present results show that 6FNE infused in the vicinity of the LC produces extreme hyperactivity in the home cage. The drug produced a marked dose-dependent increase in home cage rearing, ambulation and movement that persisted for about 1 h after a dose of 12 nmoles. Total rearing was increased some 2.6 fold, ambulation, 4.2 fold and gross movements, 1.8 fold by the latter dose. A lower dose (2 nmoles) was ineffective.

While this finding is suggestive of a direct action of 6FNE on LC receptors, the small size of the nucleus in the mouse brain in comparison with the cannula and volume of infusion, however, has raised questions as to whether this nucleus is, in fact, the site of action of the above drug. However, a number of findings of the present study support an LC site. First, most animals used in the present study showed a positive immobile response to quinpirole infusion which had been shown in a previous study to produce immobility in over 90% of mice when infused within 0.5 mm of the LC proper (Lin et al., 2008). Second, home cage behavioral stimulation was not seen with cannulas located more than 0.75 mm distant from the nucleus but was seen with cannulas located within 0.5 mm. This agreed with a previous finding that stimulation of exploratory behavior produced by another α_1-agonist, phenylephrine, in the rat LC increased as a function of proximity to the LC (Stone et al., 2004b). However, since the LC abuts the IVth ventricle, it is still possible that there was some leakage to the ventricle and diffusion to other brain structures especially since the unilateral infusion produced coordinated symmetrical behavior and affected fos expression in the contralateral LC Arguing against diffusion, however, is the finding that prior unilateral 6OHDA lesion of the LC markedly attenuated the home cage behavioral stimulation produced by unilateral 6FNE infusion. Finally, the LC showed marked opposing alterations in fos expression to oppositely-acting α_1-adrenergic receptor drugs indicating that its activity was grossly affected by these agents. Taken together these findings make it highly likely that the LC is the site of action for the behavioral actions of 6FNE infusions in the present study.

Several findings suggested that the increased home cage activity was part of a general increase in prepotent motivated behaviors. First, the hyperactivity in the home cage was found to include

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frequent climbing on the top of the cage, which, together with the frequent rearing and sniffing of the cage-top, suggests that it represented an attempt to escape from cage confinement. Escape from confinement in caged animals may remain a subthreshold prepotent response that can be readily triggered by suitable stimuli. In agreement, it is commonly observed that mice that are briefly removed from their home cages show many of the same escape-directed responses temporarily after being replaced.

Secondly, 6FNE also stimulated wheel-running in home cages so equipped and rescued operant approach behavior in the shuttle box. Both of the latter behaviors were prepotent since the provision of running wheels and the training of approach behavior would cause these responses to become dominant in their respective environments. Moreover, the conclusion that stimulation of the LC can elicit a general increase in prepotent motivated behaviors is consistent with the view that the dorsal noradrenergic system has a general or global rather than specific behavioral regulatory function (Aston-Jones and Cohen, 2005).

6FNE is a full agonist at both α_1- and α_2-adrenoceptors (Johnson and Minneman, 1986; Brasili et al., 1987). The receptor responsible for its unusual behavioral effect in the home cage, however, appears to be primarily the α_1.as TER but not ATI coinfusion was found to significantly inhibit this effect in the present study. This is consistent with the greater density of α_1- than α_2-receptors in the mouse LC (Strazielle et al., 1999). TER coinfusion also abolished home cage activity in the vehicle-treated animals, which is not surprising since α_1-receptors are known to mediate active behaviors under a variety of conditions (Stone et al., 2006b), and vehicle-treated animals show a brief reactivation of escape-like behaviors after being temporarily removed from the home cage for the infusion. The present results also suggest that the LC α_1-receptors of the mouse must be stimulated with a full agonist for their behavioral effects to be expressed. PE, a partial agonist at brain α_1-receptors, given at the same dose as 6FNE and administered alone or in combination with a selective full α_2-agonist (dexmedetomidine), did not produce behavioral stimulation in the home cage in the present experiment.

The behavioral stimulation by 6FNE was accompanied by an apparent bilateral reduction in the functional activity of the LC as fos expression in TH-containing LC neurons was markedly reduced in the nuclei of mice given the drug either in the 4th ventricle or in the contralateral LC. Furthermore, the behavioral inactivity produced by TER was accompanied by a very marked increase in fos expression in these cells. These effects resulted in significant inverse correlations between LC fos expression and measures of home cage behavioral activation. Taken together, these results therefore appear to be in agreement with the hypotheses advanced by Aston-Jones and Cohen (2005) and Weiss et al., (2005) that behavioral responsiveness is inversely related to the tonic activity of the LC and that the LC functions primarily as a stress nucleus (Korf et al., 1973; Valentino and VanBockstaele, 2007; Arnsten and Li, 2005). According to the former authors a moderate inhibition of tonic LC activity causes an enhancement of its phasic activity and of the performance of task-specific responses, which in the present studies could be interpreted to be escape from confinement and wheel-running in the home cage, and approach behavior in the shuttle box.

Despite having potentially profound effects on behavior, relatively little is known concerning the anatomy, characteristics and neurophysiology of α_1-receptors in the LC. First, although early data on the expression of α_1-receptor mRNA in LC neurons was conflicting, a more recent study has found definitive evidence for the expression of the three subtypes of these receptors (α_{1A}-, α_{1B}- and α_{1D}-) in microdissected LC tissue from both adult and juvenile rats (Osborne et al., 2002). However, α_1- receptors may be present on afferent terminals to the LC and affect transmitter release at the nucleus since presynaptic α_1-receptors have been found in various brain regions to regulate the release of NE (Aono et al., 2007), DA (Auclair et al., 2002),

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glutamate (Marek and Aghajanian, 1999) and GABA (Lei et al., 2007) and since there is a lack of correlation between α_1-receptor binding sites and the density of LC neurons (Chamba et al., 1991). Second, with respect to the identity of the endogenous catecholamine ligand for these receptors, it is not clear whether this is epinephrine derived from the n. paragigantocellularis/C1 (Pieribone and Aston-Jones, 1991), norepinephrine from either the LC itself (recurrent collaterals) (Nakamura et al., 1988) or from other noradrenergic nuclei (Maeda et al., 1991), or dopamine from either the ventral tegmental area (Deutch et al., 1986) or A13 cell group in the hypothalamus (Kitahama et al., 2007). Third, with respect to neurophysiological action(s), two opposing effects of α_1-receptor stimulation may occur in these neurons, the first being an excitatory blockade of the hyperpolarizing outward current due to activation of GIRK channels (Osborne et al., 2002) and the second an inhibition of excitatory synaptic neurotransmission which has been documented in other brain regions as discussed above. If these two effects are not mutually exclusive, the first may underlie the enhanced phasic activity while the second, may be the basis of the reduced tonic activity found in LC neurons after stimulation of α_1-receptors.

4. Experimental Procedure

Subjects

All experiments were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and were approved by the New York University School of Medicine IUCAC. Swiss Webster male mice (Taconic), 8-10 weeks old, were subjects. The animals were housed singly with nesting material for 5 d prior to surgery in standard size polycarbonate mouse cages (12.5 × 17 × 28 cm) at a room temperature of 22 ± 1° C under a 12 hr light/dark cycle (lights on 0500 hr). Food and water were available *ad libitum*.

Surgery

Mice, anesthetized with pentobarbital (70 mg/kg), were implanted stereotaxically with unilateral 26 ga cannula guide tubes either above the left LC, at coordinates -5.5 mm posterior to Bregma, 1.3 mm lateral and, 3.9 mm beneath the surface of the skull, approached at a 12° angle to the midline, or in the fourth cerebral ventricle (-5.9 mm to Bregma, 1 mm lateral, 3.8 mm ventral to skull surface, approached at a 13.7° angle to the sagittal suture). Unilateral implantation was used because we have shown previously that unilateral blockade or stimulation of LC α_1-adrenoceptors in the mouse produces the same acute effect on behavioral activation as bilateral blockade or stimulation (Stone et al., 2004a). All animals were given 10 days for recovery prior to behavioral testing.

Procedure

All experiments were performed between 1000 and 1400 hr. Mice were gently restrained under a layer of gauze and a 33 ga cannula connected by PE 20 tubing to a syringe pump was inserted into the cannula protruding 0.5 mm below the bottom of the guide. A total of 250 nl of solution was infused at approximately 100 nl/min over a 2-2.5 min period with the cannula remaining in place for 30 seconds after infusion. For IV ventricle infusions, 500 nl was used. The animals received either vehicle (saline), the α_1-adrenoceptor antagonist, terazosin (TER) (Sigma-RBI), the α_1-agonist, phenylephrine (PE) (Sigma-RBI), the α_2-antagonist, atipamezole (ATI) (Farmos Ltd), the α_2-agonist, dexmedetomidine (DMT) (Farmos Ltd), or the mixed α_1-/α_2-agonst, 6FNE (Sigma-RBI), singly or in combination, in doses ranging from 0.04-30 nmoles per mouse. All drugs were prepared freshly each day in saline.

Prior to all experiments (except those involving wheel running or IVth ventricle infusions) accurately implanted animals were selected by prescreening for a positive (immobile) response

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to infusion of the D2 agonist, quinpirole (Q, 2 nmoles, unilateral). As discussed above, we have shown previously that over 90% of animals with cannula tips within 0.5 mm of the LC proper show marked immobility responses to this agent at the latter dose (Lin et al., 2008). For the prescreening, animals were placed in fresh (novel) home cages immediately following Q infusion and videorecorded for 10 min. If the mouse showed 7.5 min or more of immobility (no observable movements) it was deemed "Q2-positive" and was used subsequently (4-7 d) in one of the following experiments with test drugs. Following the experimental infusion all animals were terminally anesthetized with a combination of halothane and urethane (1.8 mg/kg) and either perfused for immunohistochemical studies described below or sacrificed for brain harvesting and histological localization of cannula tips.

Lesions of the LC were produced by 0.25 μl infusions of a solution of 6-hydroxydopamine (4 mg/ml in 0.1% ascorbic acid/saline) or vehicle either unilaterally or bilaterally in lightly anesthetized animals (Nembutal, 50 mg/kg, i.p.). One hr prior to the infusions, all mice were pretreated with an i.p. cocktail of the 5HT- and DA-reuptake inhibitors, fluoxetine, 15 mg/kg, and GBR 12909, 25 mg/kg, to limit destruction to only noradrenergic neurons. Fourteen days after neurotoxin infusion, mice were infused in the LC or 4th ventricle with 6FNE at 12 or 30 nmoles, respectively, and tested for one h in the home cage for exploratory behaviors as below. The following day the animals were perfused for immunohistochemical assay of tyrosine hydroxylase to ascertain the extent of the LC lesion by cell count.

Home cage activity

Immediately following LC infusion, mice were replaced in their home cages and videorecorded for the next 60 min. Videodisks were rated blind by a trained observer for number of rearing responses, number of times the animal crossed the center of the cage (ambulation), total movement (the frequency of any observable head movement) and time spent at the feeding trough as previously described (Lin et al., 2008).

Wheel-running behavior

Five days following surgery each mouse was housed individually in a larger cage (22 × 22 × 46 cm) containing a free standing running wheel (18 cm dia, 8 cm wide) beneath a video camera and allowed 7 days to acclimate. We have found that approximately 80% of Swiss-Webster male mice at this age will eventually run in the wheel and will show robust running by 7 days.

The testing procedure occurred after an initial habituation trial and comprised 2 test days with the animals given infusions of both vehicle and 6FNE (2 or 12 nmoles) 4 d apart in counterbalanced order. Because of the greater number of infusions used in this experiment, the initial Q2 screening test was omitted. Videotapes were rated blind manually for the length of time that the animals ran in the wheel per successive 30 min periods following infusion.

Operant approach behavior

Training was carried out in a mouse shuttle-box operant chamber (Med Associates) consisting of a start box separated from a goal box by a guillotine door. A liquid dipper in the goal box was activated when the mouse made a head poke response into an illuminated hole, 2.5 cm in diameter, situated in the front wall. Reinforcement consisted of 0.01 ml of tap water. Prior to the first training session all mice were deprived of water for 22 hours. After each subsequent session the animals were given access to water in the home cage for 2 hours. After an initial 2 min habituation period, each trial was initiated by raising the guillotine door, which activated a timer and turned on the light in the nose-poke hole in the goal box. Latency to enter the goal box was recorded by the break of a photocell beam. When the mouse made a nose-poke, the dipper was raised allowing access to the cup for 2 seconds. The trial ended when the animal returned to the start box. (All animals readily learned to reenter the start box following

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reinforcement). A new trial began following a 2 min interval. Animals were trained for 5 successive days, which resulted in an asymptoting of latency scores and were then subjected to two more training days for habituation to sham injections. On the following day, independent groups received LC infusions of either the vehicle (saline), TER (2 nmoles), 6FNE (12 nmoles) or TER plus 6FNE immediately prior to placement in the shuttle chamber for the test session.

Immunohistochemistry

Terminally anesthetized mice (halothane plus urethane, 1.8 g/kg, i.p.) were perfused transcardially with 25 ml of saline followed by 45 ml of 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were postfixed for 24 hr and then submerged in 30% sucrose at 4° C for 48 h prior to sectioning at 35 μ.

For double label fos/tyrosine hydroxylase (TH) fluorescence-immunohistochemistry, LC-containing sections were sequentially washed in PBS, treated for 15 min with 0.9% H_2O_2 to reduce endogenous peroxidase, blocked with 4% normal goat serum and incubated for 24 h at room temperature with a mixture of rabbit anti-fos antibody (Oncogene Science,1:7500) and chicken anti-TH antibody (Novus Biochemicals, 1:1000) in PBS containing 1% bovine serum albumin (BSA). For antibody detection, sections were next washed as above and then incubated for 30 min with a mixture of the fluorescent secondary antibodies, Alexa-fluor 488 goat anti-rabbit IgG (excitation 495 nm, emission 519 nm, Invitrogen, 1:500) and Alexa-fluor 594 goat anti-chicken IgG (excitation 590 nm, emission 617 nm, Invitrogen, 1;500) in PBS/BSA prior to washing in PBS and mounting with Vectashield (Vector).

Images were captured with a Nikon Eclipse 50i fluorescent microscope equipped with a QImaging Retiga digital camera and a 20×/0.50 Pan Fluor objective. All sections through the full extent of the LC (Bregma -5,34 to -5.80 mm) were first screened for Alexa 594 (TH-positive cells) using filter block ET TXR (excitation 560/40, emission 630/75). Those sections containing TH-positive cells were captured first for Alexa 594 fluorescence followed by capture of Alexa 488 fluorescence using filter block ET GFP (excitation 470/40, emission 525/50). No bleed-through was observed. Total TH-positive cells having an observable nucleus were counted blind by two observers manually with ImageJ in the nonmerged Alexa 594 images. The Alexa 594 and 488 images of each section were then merged using a commercial photo processing program and adjusted for equal fluorescent intensity at the two wavelengths. Total double-labeled fos/TH cells, identified as having a TH-positive cytoplasm and a fos-positive nucleus that were both at least twice background fluorescence, were next counted manually in all sections containing TH-positive cells.

Statistics

Data were analyzed by 2 or 3 way ANOVAs for repeated measures where appropriate. All post-hoc comparisons were evaluated by the Bonferroni method. Two-mean experiments were analyzed by Student's t-test. The correlations were product-moment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

6FNE
: 6-fluoronorepinephrine

6-OHDA
: 6-hydroxydopamine

ATI
: atipamezole

LC
: locus coeruleus

TER
: terazosin

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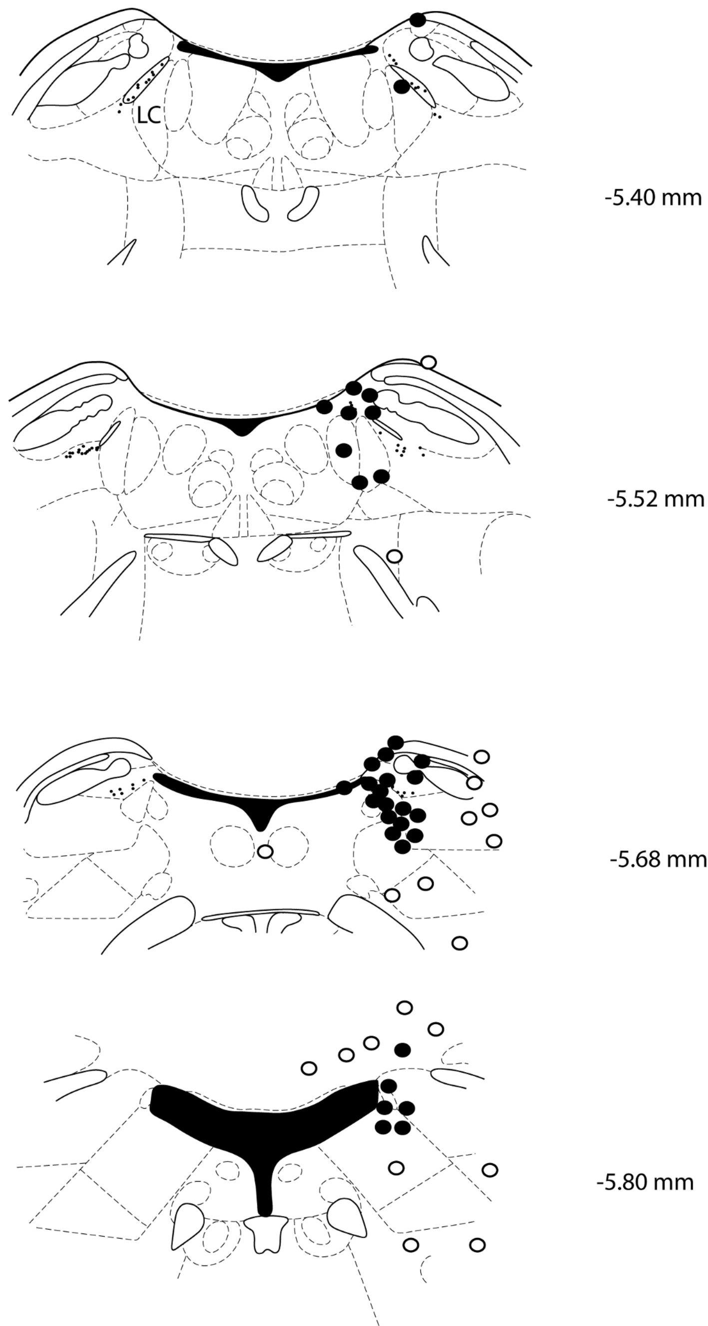

1).

Cannula tip locations (filled circles) for the 35 mice with tips within 0.5 mm of LC used in the first experiment (home cage exploration). All of these animals were Q2-positive. Open circles represent cannula tips more than 0.75 mm away from the nucleus ("outside" group) who were not Q2-positive.

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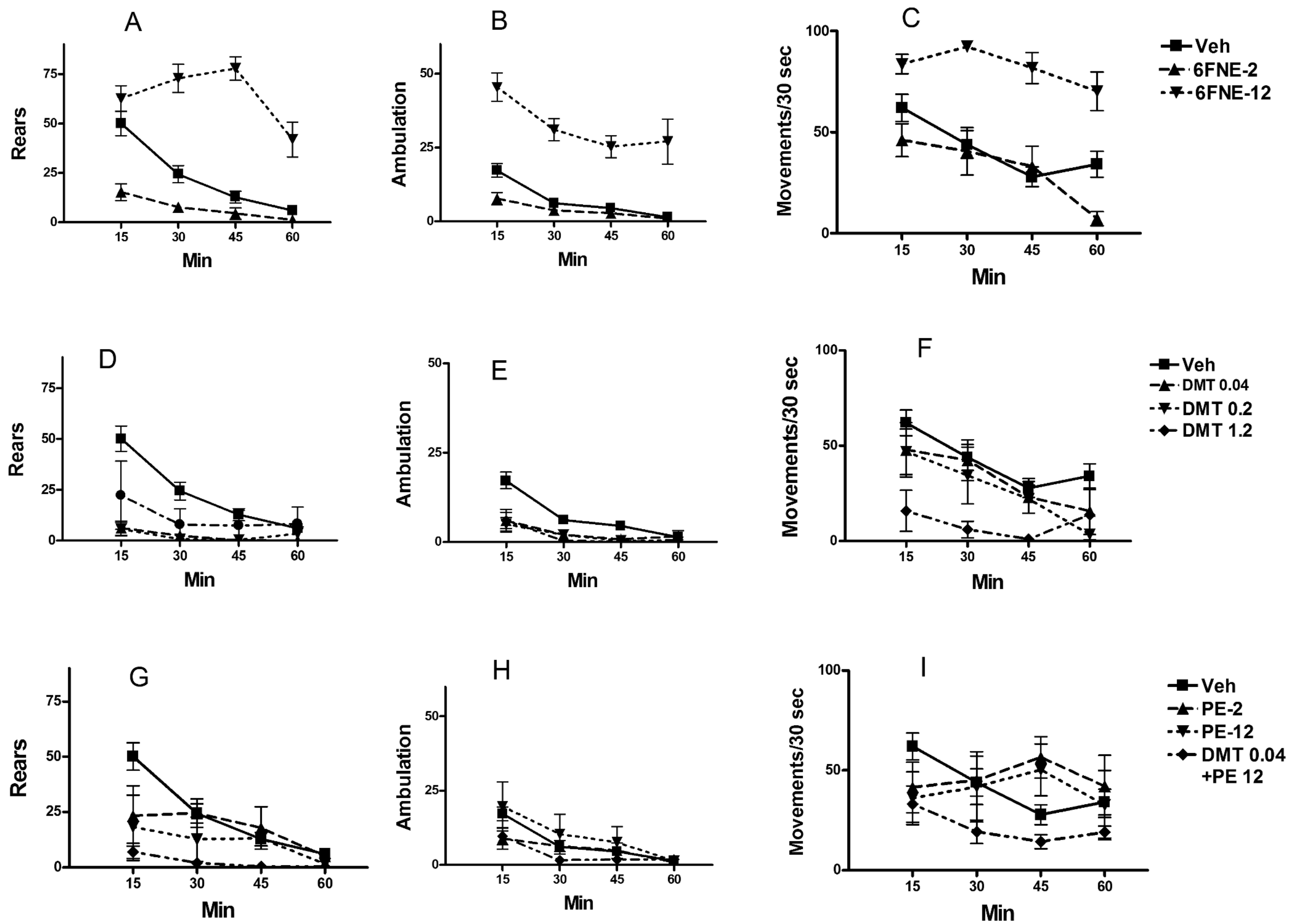

2).

Effects of LC infusion of 6FNE (A-C), DMT (D-F) or PE (G-I) on various forms of active behavior in the home cage. Rearing/15 min, A,D,G; Ambulation (Cage crosses/15 min), B,E,H,; Total movements/30 sec, C,F,I. N's: Veh (19); 6FNE, 3 nmoles, (12); 12 nmoles (9); DMT all doses (6); PE, 2 nmoles (6); 12 nmoles (10); PE+DMT (6)) Vehicle group is the same for all 3 drugs. For statistics see Results.

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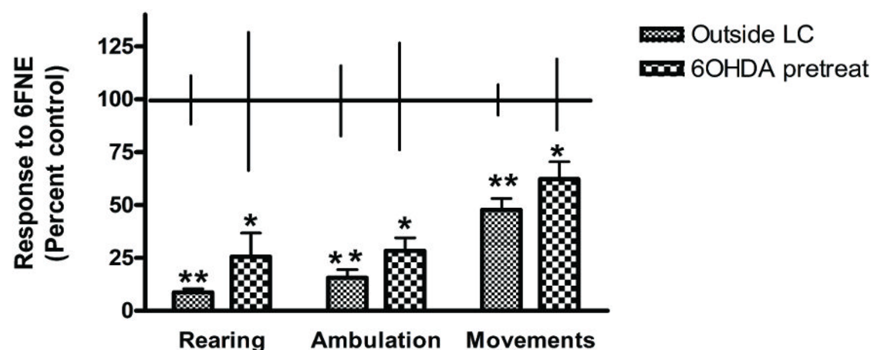

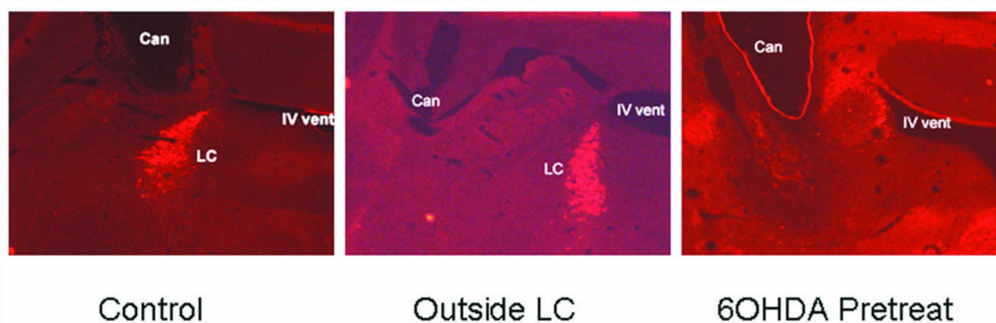

3).

Effect of cannula displacement ("outside LC") or of prior unilateral 6OHDA lesion on behavioral effect of infused 6FNE (12 nmoles) in home cage (60 min). Results are presented as percents of means of the two respective control groups, "near LC-" and sham lesioned. Means for the two control groups: rearing, 242.6, 206.0; ambulation,120.4, 52.9; movements, 327.6, 271.6 N's for groups: "near LC " (10); "outside LC" (20); sham lesioned (5); 6OHDA-lesioned, (5). Lower half of figure shows photomicrographs of TH-stained sections from a control ("near LC"), an "outside LC," and a 6OHDA-lesioned mouse. (Sham lesioned mice were similar to "near LC" controls and are not shown). Lesioned animals had significantly fewer TH-positive LC cells that were within 0.5 mm of cannula tip than sham lesioned mice ($18.1 \pm 18.1\%$, $t_8 = 3.91$, $p < 0.01$). Bar is 200 μ. * $p < 0.05$, ** < 0.01 versus respective controls.

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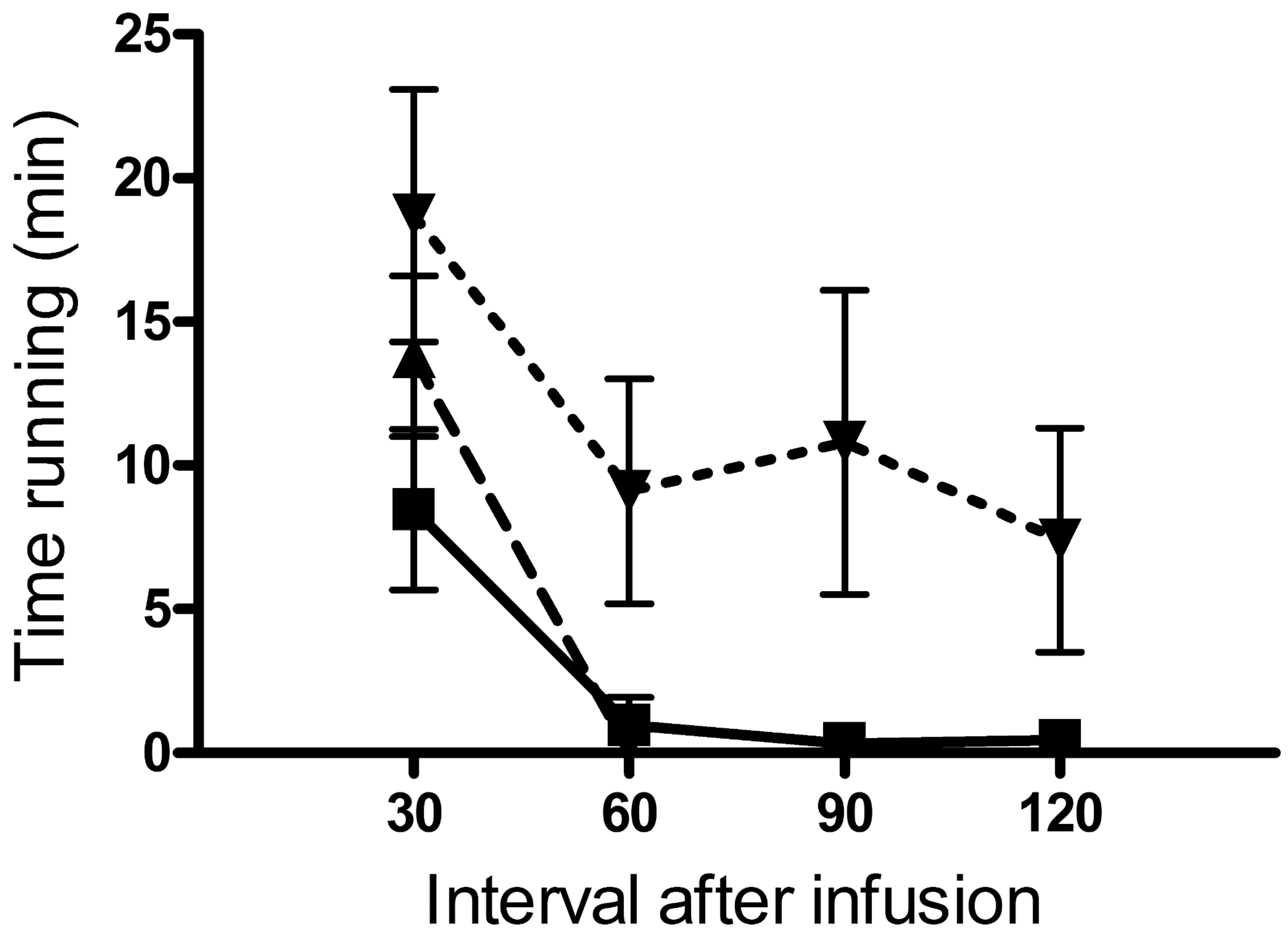

4).

Effect of 6NFE infusion on wheel running in home cage. Mice previously trained to run in wheels in the home cage were infused 2 or 12 nmoles of 6FNE in the LC and measured for running times in the following 2 h. N = 5-7/gp. For statistics see Results.

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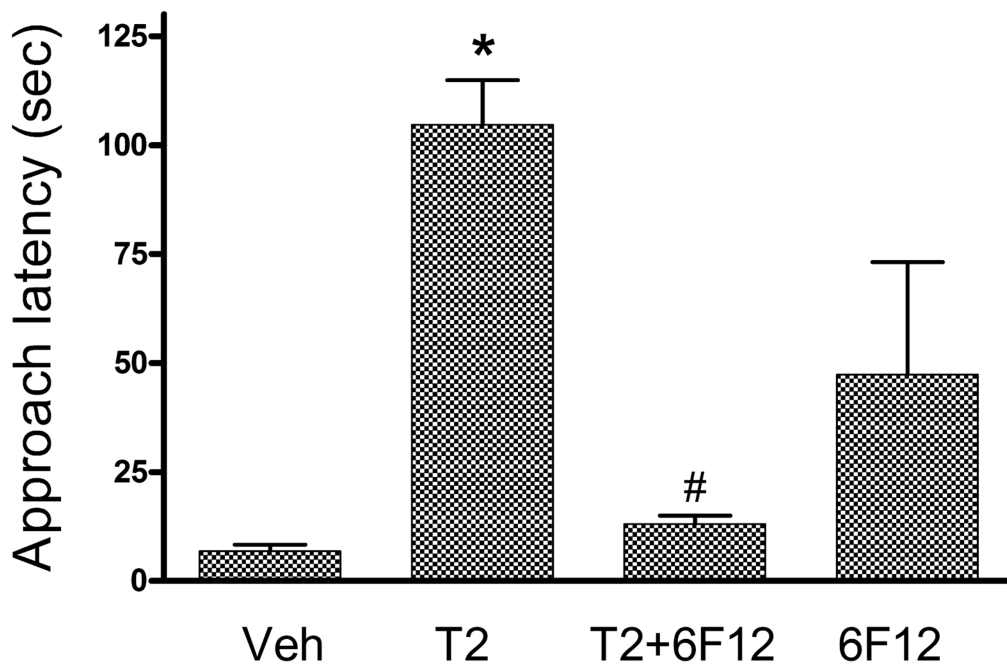

5).

Effect of 6FNE infusion on latency of a learned operant approach response. Mice previously trained to make a head-poke response for a water reward in a shuttle box were infused in the LC either vehicle, terazosin (T, 2 nmoles), 6FNE (6F, 10 nmoles) or T+6F) and measured for response latencies over the following 30 min., 6F. N = 5-7. * $p < 0.001$ versus Vehicle, # < 0.001 versus T2.

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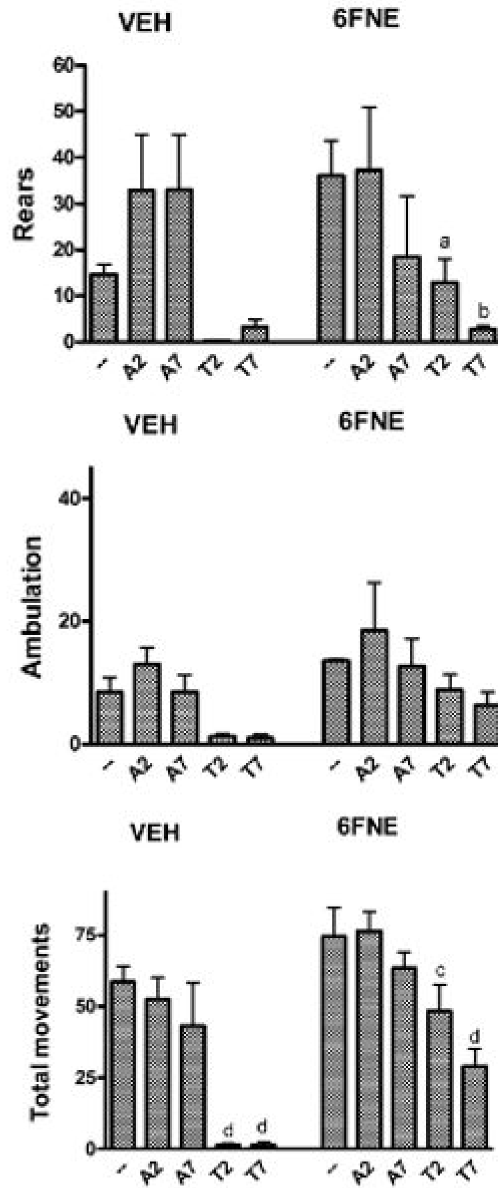

6).

Effect of α_1- and α_2-adrenoceptor antagonists on behavioral effects of vehicle or 6FNE-12 in home cage. Values are mean number of rears and ambulations per 15 min periods and movements per 30 sec intervals averaged over the first 30 min of test. Numbers below columns are dosages in nmoles. T, TER; A, ATI. N's of groups: Veh alone (15); Veh-ATI (6); Veh-TER (6); 6FNE alone (10); 6FNE-ATI (6); 6FNE-TER (6). The Veh alone and 6FNE alone groups were the same as the vehicle and 6FNE-12 groups in Fig. 2.

[a] $p<0.05$, [b] <0.01, [c] <0.001, [d] < 0.0001 versus corresponding veh group

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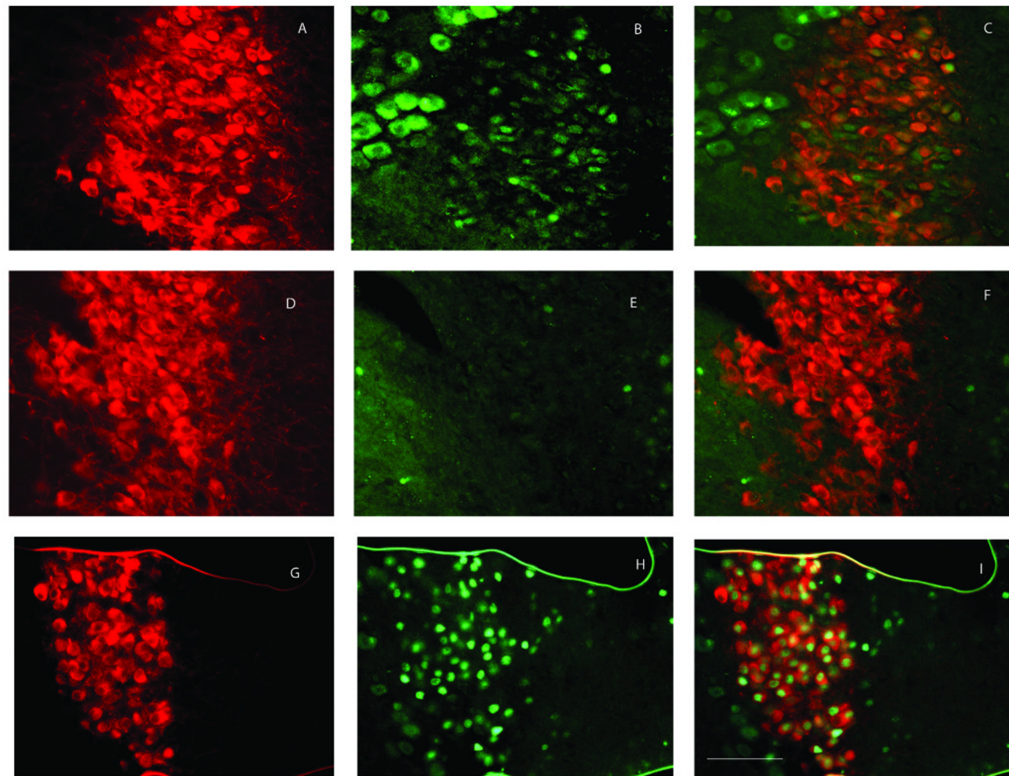

7).

Double-label fluorescent immunohistochemistry of fos and tyrosine hydroxylase in the LC after IVth ventricular infusion of Vehicle (A,D,G), 6FNE 30 nmoles (B,E.H), or TER 10 nmoles (C,F,I,) in representative animals. Left column, TH images; middle column, fos images; right column merged images. Note in B some nonspecific staining of cytoplasm of adjacent mesencephalic trigeminal nucleus neurons by fos antibody. Bar is 200 μ.

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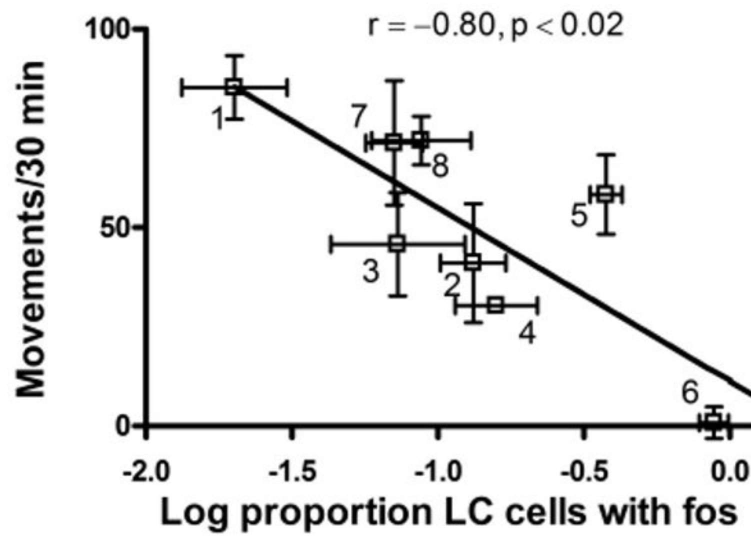

8).

Correlation of movement in the home cage with the log_{10} proportion of double-labeled fos/TH cells in LC. Numbers denote the following treatments (nmoles): 1- 6FNE (30); 2 - Veh; 3- 6FNE (30) + ATI (10); 4 - 6FNE (30) + TER (10); 5 - ATI (10); 6 -TER (10); 7 - DMT (0.056); 8 - DMT(0.1).

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Table 1

Fos expression in TH-positive LC neurons after infusion of agents in 4th ventricle

	Total No. TH	No. Fos+TH	(Fos+TH)/TH
Veh	650.4 ± 76.7	84.4 ± 10.3	0.164 ± 0.015
6FNE-30	674.3 ± 84.5	11.0 ± 5.8	0.026 ± 0.011
TER-10	922.3 ± 192.1	819.3 ± 183.0	0.881 ± 0.025
ATI-10	761.3 ± 85.7	288.3 ± 39.1	0.384 ± 0.044
6FNE+TER	825.6 ± 127.6	137.9 ± 31.0	0.224 ± 0.089
6FNE+ATI	825.0 ± 47.8	90.8 ± 44.3	0.109 ± 0.049
DMT-0.056	699.2 ± 80	52.0 ± 8.4	0.079 ± 0.014
DMT-0.1	945.3 ± 157	95.0 ± 35.7	0.100 ± 0.030

Mice were infused in the 4th ventricle with 6FNE (30 nmoles), DMT (0.056-0.1 nmoles), or TER or ATI (10 nmoles) in the presence or absence of 6FNE 70 min prior to perfusion for immunohistochemistry of tyrosine hydroxylase (TH) and fos in the full LC. Values represent the total number and SEM of neurons positive for TH and the number of double labeled fos/TH neurons. The ratio of double labeled to total TH is presented in last column. N = 5-6 mice/group. For statistics see Results.