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The Neurotensin Agonist PD149163 Increases Fos Expression in the Prefrontal Cortex of the Rat

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Dopaminergic axons innervating the prefrontal cortex (PFC) target both pyramidal cells and GABAergic interneurons. Many of these dopamine (DA) axons in the rat coexpress the peptide neurotransmitter neurotensin. Previous electrophysiological data have suggested that neurotensin activates GABAergic interneurons in the PFC. Activation of D2-like DA receptors increases extracellular GABA levels in the PFC, as opposed to the striatum, where D2 receptor activation inhibits GABAergic neurons. Because activation of presynaptic D_2 release-modulating autoreceptors in the PFC suppresses DA release but increases release of the cotransmitter neurotensin, D2 agonists may enhance the activity of GABAergic interneurons via release of neurotensin. In order to determine if neurotensin can activate GABAergic interneurons, we treated rats with the peptide neurotensin agonist, PD149163, and examined Fos expression in PFC neurons. Systemic administration of PD149163 increased overall Fos expression in the PFC, but not in the dorsal striatum. PD149163 induced Fos in PFC interneurons, as defined by the presence of calcium-binding proteins, and in pyramidal cells. Pretreatment with the high-affinity neurotensin antagonist, SR48692, blocked neurotensin agonist-induced Fos expression. These data suggest that neurotensin activates interneurons in the PFC of the rat.

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INTRODUCTION

The dopaminergic innervation of the prefrontal cortex (PFC) plays an important role in the processing and subsequent integration of cognitive, affective, and even sensory events (Cohen *et al*, 2002; Goldman-Rakic, 2002; Miller *et al*, 2002). The targets of the prefrontal cortical dopamine (DA) innervation include both GABAergic interneurons and glutamatergic pyramidal cells (Sesack *et al*, 1995a; Verney *et al*, 1990; Seguela *et al*, 1988; van Eden *et al*, 1987). Because local circuit GABA interneurons also synapse with pyramidal cells (DeFelipe and Farinas, 1992), the cortical DA innervation can both directly and indirectly alter pyramidal cell activity and hence overall cortical output.

In vivo microdialysis studies suggest that DA increases extracellular GABA in the PFC through the activation of D2-like DA receptors (Petrie *et al*, 2002; Grobin and Deutch, 1998). Dopamine D2-like but not D1-like agonists increase extracellular GABA levels in the PFC, an effect that is blocked by D2 but not D1 antagonists (Grobin and Deutch, 1998). Although D_2 receptors are localized to GABA interneurons in the rat PFC (Le Moine and Gaspar, 1998), it seems unlikely that the activation of D_2 receptors on interneurons would drive GABA release because the net effect of D_2 receptor activation is often inhibitory (Kotecha *et al*, 2002; Hernandez-Lopez *et al*, 2000; Huff, 1996).

Dopamine D_2 receptors in the rat PFC are also present on presynaptic DA terminals, where they function as release-modulating autoreceptors (Wolf and Roth, 1987). Activation of these D_2 autoreceptors decreases DA release but *increases* release of the colocalized transmitter neurotensin (NT; see Bean and Roth, 1992). In the PFC, NT is localized exclusively to dopaminergic axons (Studler *et al*, 1988). Thus, activation of D_2 autoreceptors decreases DA release but increases NT release from mesoprefrontal cortical neurons.

The high-affinity NT receptor (NTR1) is coupled to excitatory signal transduction pathways (Yamada *et al*, 1993). *In situ* hybridization studies have revealed that many PFC neurons express NTR1 mRNA (Alexander and Leeman, 1998; Nicot *et al*, 1994). These observations, and the fact

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that D_2 autoreceptor activation in the PFC increases NT release, raise the possibility that D_2-elicited GABA release in the PFC is actually mediated by NT. Audinat *et al* (1989) reported that NT enhanced GABA-mediated synaptic noise in rat PFC pyramidal cells, suggesting that NT excites GABA interneurons.

GABAergic interneurons in the cortex can be divided into three largely non-overlapping classes based on expression of one of three calcium-binding proteins: parvalbumin (PV), calbindin (CB), or calretinin (CR) (DeFelipe, 1997; Gabbott *et al*, 1997; Rogers, 1992). In the present study, we assessed the effects of acute administration of the truncated peptide NT agonist, PD149163 (Feifel *et al*, 2003, 1999; Wustrow *et al*, 1995), on regional forebrain expression of Fos, a marker of neurons that are metabolically activated, with a particular focus on determining if the NT agonist activates prefrontal cortical GABAergic interneurons. Because the binding profile of PD149163 has not been extensively characterized, we also conducted a full screen of receptors to which PD149163 binds.

MATERIALS AND METHODS

PD149163 Binding Assay

PD149163 (Chemical Synthesis Branch of NIMH, NIH) was screened against a large number of cloned G-protein-coupled receptors, ion channels, and transporters, as previously described (Shapiro *et al*, 2003; a full description of the assay conditions can be found at http://kidb.cwru.edu/nimh/binding.php). Targets for which PD149163 had an affinity of $\leqslant 10\,000$ nM were further characterized using a full curve.

Animals

Adult male Sprague–Dawley rats weighing 275–345 g (Harlan, Birmingham, AL) were group-housed on a 12 h light–dark cycle with lights on at 0600, with food and water available *ad libitum*. All studies were performed in compliance with the NIH *Guide for Care and Use of Laboratory Animals*.

Treatments

To examine the effect of PD149163 on forebrain Fos expression, rats ($n = 4$/group) were injected with PD149163 (0.05, 0.25, or 2.5 mg/kg, i.p.) or its vehicle (water). After 2 h, the animals were decapitated and the PFC, nucleus accumbens septi (NAS), and dorsolateral striatum (CP) were dissected and stored at -80°C until assayed by Western blots.

Subsequent efforts focused on determining the effects of PD149163 on PFC neurons, using immunohistochemistry to define the type of cells in which Fos was induced. Rats ($n = 5$–7/group) were injected with 0.25 or 2.5 mg/kg PD149163 or vehicle. Other groups of rats were injected with the nonpeptide NTR1 antagonist SR48692 (1.0 mg/kg, i.p.; Sanofi Recherche, Toulouse, France) or vehicle (1% Tween 20 in distilled water), followed 30 min later by 0.25 mg/kg PD149163 or vehicle; this dose was chosen on the basis of the results from the immunoblot studies. At 2 h after administration of the NT agonist, animals were anesthetized with isoflurane (Henry Schein, Melville, NY) and their colonic temperatures were recorded as an independent confirmation of the actions of the NT agonist (Bissette *et al*, 1976). Rats were then transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and the brains were removed and post-fixed overnight before being cryoprotected in 24% sucrose in phosphate buffer. Coronal sections (42 μm) were cut through the forebrain on a freezing microtome.

Immunoblot Analyses

Immunoblot analysis of Fos protein levels was performed using a previously characterized rabbit anti-Fos antibody that was generated against the m peptide (Quinn *et al*, 1989) and recognizes both Fos and Fos-related antigens (Fras). Tissue samples were homogenized in 2% SDS and an aliquot was removed to determine protein levels (Lowry *et al*, 1951). Equal amounts of protein were run on a 10% acrylamide/0.27% methylenebisacrylamide gel overnight at 67 V and were transferred to nitrocellulose. Blots were incubated for 48 h at 4°C in the anti-Fos antibody (1 : 5000). The blots were then washed, incubated for 2 h in horseradish peroxidase-conjugated secondary antibody (Vector Labs, Burlingame, CA; 1 : 4000), and washed again before being developed using enhanced chemiluminescence.

Immunohistochemistry

Immunoperoxidase methods followed our previously described avidin–biotin methods (see Deutch and Duman, 1996). Fos was detected using a heavy-metal-intensified diaminobenzidine as the chromogen; double-label studies with MAP-2, a marker for pyramidal cells, or choline acetyltransferase (ChAT), a marker of cholinergic neurons, used a brown diaminobenzidine reaction product to mark MAP-2- or ChAT-positive cells. For immunofluorescent studies, free-floating sections were washed extensively in 50 mM Tris-buffered saline (TBS) and incubated for 60 min in TBS containing 0.2% Triton and 4% normal horse serum. Sections were then incubated for 48 h at 4°C in a solution containing two antibodies, one directed against Fos and the other against one of the three calcium-binding proteins that define cortical interneuron populations. The sections were washed and transferred to a solution containing Cy2- and Cy3-conjugated secondary antibodies (1 : 1000; Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature, then washed extensively, mounted, and coverslipped. The antibodies used included mouse anti-PV (1 : 1500; Sigma, St Louis, MO), mouse anti-CB (1 : 3000; Sigma), goat (1 : 2500; Chemicon, Temecula, CA) or rabbit anti-CR (1 : 1500; SWANT, Bellinzona, Switzerland), mouse anti-MAP2 (1 : 400; Sigma), goat anti-ChAT (1 : 1000; Chemicon) and rabbit (Oncogene, San Diego, CA; 1 : 7500) or goat anti-Fos (1 : 2000; Santa Cruz Biotechnology, Santa Cruz, CA).

Cell Counts and Data Analysis

Fos-like immunoreactive (-li) cells were counted in the deep layers of the prelimbic cortex (area 32) of the PFC, where

the mixed DA–NT innervation is most dense. The number of Fos-li neurons/mm^2 was determined, and the data were analyzed by ANOVA with *post hoc* tests when indicated.

To determine the relative induction of Fos in different types of interneurons, images of the deep layers of the prelimbic cortex were captured using a digital camera. Approximately 100 PV, 100 CB, and 50 CR-like immunoreactive cells were identified in each animal, and the percentage of these cells in which a Fos-li nucleus was present was determined. The data were analyzed by ANOVA with subsequent Newman–Keuls *post hoc* tests when indicated.

Finally, Fos expression was assessed in cholinergic neurons of the basal forebrain, which have previously been shown to express NTR1 (Alexander and Leeman, 1998; Boudin *et al*, 1996; Nicot *et al*, 1994) and to depolarize in response to NT (Cape *et al*, 2000; Matthews, 1999; Farkas *et al*, 1994). We determined the percentage of double-labeled (Fos + ChAT) cells in the horizontal limb of the diagonal band of Broca (HDB), where the cholinergic cells that project to the PFC are located (Gaykema *et al*, 1991, 1990; Luiten *et al*, 1987); in addition, we determined the numbers of Fos-li and double-labeled Fos + ChAT cells in the globus pallidus, where cells that project to the PFC are rarely encountered (Gritti *et al*, 1997).

RESULTS

Pharmacological Profile of PD149163

PD149163 selectively bound to the NTR1 receptor, with a K_i of 159 nM and no affinity for NTR2 (Table 1). With the exception of the sigma-1 and I1-imidazoline receptors, for which PD149163 displayed negligible affinities ($K_i = 5214$ and 2032 nM, respectively), the NT compound exhibited no measurable affinity for any of the other receptors, ion channels, or transporters tested (see Table 1).

Effect of PD149163 on Core Body Temperature

Systemic treatment with PD149163 dose-dependently reduced core body temperature ($F_{2,18} = 29.75$; $p \leqslant 0.0001$; Table 2). The higher dose of the NT agonist reduced temperature by almost 2°C compared to vehicle-treated animals. Pretreatment with the NT antagonist SR48692 did not block PD149163-induced hypothermia, nor did it have any effect of its own on body temperature (data not shown).

Effects of PD149163 on the Density of Fos-li Cells in the PFC

Immunoblot assessment of the effects of PD1491653 on Fos expression was conducted to determine a dose range for the NT agonist, and revealed that PD149163 potently induced Fos and several lower weight Fras in the PFC in a dose-related manner (see Figure 1). The effect of PD149163 on cortical Fos expression was dose-related, with 0.25 mg/kg appearing to markedly increase Fos relative to vehicle, and the higher (2.5 mg/kg) dose resulting in a somewhat lesser induction. The NT agonist appeared to induce Fos weakly in the NAS at the 0.25 mg/kg dose, but had no apparent effect in the striatum at any dose examined.

The ability of the NT agonist to induce Fos in the PFC was confirmed and extended by immunohistochemical studies (Figure 2). ANOVA revealed a significant treatment effect on the density of Fos-li neurons in the PFC ($F_{2,16} = 4.52$; $p \leqslant 0.05$), with a significant increase in the density of Fos-li neurons in animals receiving 0.25 mg/kg, but not 2.5 mg/kg, PD149163. In contrast, analyses did not uncover any significant effect of PD149163 on Fos expression in the nucleus accumbens ($F_{2,16} = 2.47$; $p \leqslant 0.05$) or the dorsal striatum ($F_{2,16} = 0.82$; $p \leqslant 0.05$), although there was a trend toward an effect in the former site.

In a separate experiment we found that pretreatment with the selective NTR1 antagonist SR48692 blocked the ability of PD149163 to induce Fos in the PFC ($F_{3,27} = 6.79$; $p \leqslant 0.005$; Figure 3). Acute administration of SR48692 had no significant effect on the density of Fos-li neurons compared to vehicle.

Effects of PD149163 on Fos Expression in Cortical Interneurons

PD149163 increased Fos in prefrontal cortical interneurons as well as in MAP-2-li pyramidal cells (Figures 4 and 5). ANOVA revealed a significant treatment effect on Fos induction in PV- ($F_{2,17} = 6.90$; $p \leqslant 0.01$), CR- ($F_{2,18} = 5.35$; $p \leqslant 0.05$), and CB- ($F_{2,19} = 4.83$; $p \leqslant 0.05$) containing interneurons (Figure 5). *Post hoc* analyses showed that Fos was significantly induced in PV-containing interneurons in response to the low but not high dose of the NT agonist. In contrast, only the high dose significantly increased Fos expression in CB-immunoreactive interneurons.

We determined if pretreatment with the NT receptor antagonist SR48692 could attenuate Fos induction in response to PD149163 in specific populations of PFC interneurons (Figure 6). Because 0.25 mg/kg PD149163 did not increase Fos expression in CB-containing cells (Figure 5), we restricted our analysis to PV- and CR-containing interneurons. Overall ANOVAs uncovered a significant treatment effect in both PV- ($F_{3,27} = 3.80$; $p \leqslant 0.05$) and CR- ($F_{2,26} = 17.26$; $p \leqslant 0.0001$) containing interneurons. Fos expression was increased in both PV- and CR-containing interneurons by 0.25 mg/kg PD149163, and pretreatment with the NTR1 antagonist SR48692 blocked the effects of the NT agonist.

Effects of PD149163 on Fos Expression in the Cholinergic Cells of the Basal Forebrain

Fos expression in the globus pallidus was unaltered by treatment with PD149163 (data not shown). In contrast, PD149163 increased Fos expression in both cholinergic and non-cholinergic cells of the HDB (Figure 7). ANOVA revealed a significant treatment effect of PD149163 on the overall density of Fos-li cells in both the rostral ($F_{2,16} = 15.42$; $p \leqslant 0.005$) and caudal ($F_{2,15} = 9.86$; $p \leqslant 0.005$) HDB; *post hoc* analyses revealed that only the higher dose of the NT agonist increased the Fos expression in the caudal HDB, whereas both doses induced Fos in neurons of the rostral HDB. Using an antibody directed against ChAT, we found that PD149163 induced Fos expression in cholinergic neurons in the caudal ($F_{2,15} = 6.59$; $p \leqslant 0.001$) but not rostral HDB.

Table 1 Affinities of PD149163 and Reference Compounds at Various Receptors, Transporters, Channels, and Binding Sites

Receptor	Cold ligand	^{3}H-ligand	K_D (nM)	Assay conc. (nM)	PD149163 (nM)
NTR1	PD149163	NT	7	3	159
NTR2*	NT/levocabastine	NT	5	3	> 10 000
5-HT_{1A}	WAY 100,635	8-OH-DPAT	1	0.5	> 10 000
5-HT_{1B}	Ergotamine	GR125743	0.3	0.3	> 10 000
5-HT_{1D}	Ergotamine	GR125743	0.3	0.3	> 10 000
5-HT_{2B}	Norfenfluramine	LSD	10	5	> 10 000
5-HT_{5A}	Ergotamine	LSD	1.6	1	> 10 000
5-HT_6	Chlorpromazine	LSD	1.5	1	> 10 000
SERT	Fluoxetine	Citalopram	0.8	0.5	> 10 000
D_1	SKF38393/fluphenazine	SCH23390	0.35	0.2	> 10 000
D_{2L}	Haloperidol	N-methylspiperone	0.5	0.2	> 10 000
D_3	Chlorpromazine	N-methylspiperone	0.4	0.2	> 10 000
rD_4	Chlorpromazine	N-methylspiperone	0.5	0.2	> 10 000
D_5	Olanzapine	SCH23390	0.3	0.2	> 10 000
DAT	4′,4″-Difluoro-3a (diphen-yl-methoxy) tropane HCl	GBR12935	1	0.5	> 10 000
α_{1A}	Urapidil	Prazosin	0.2	0.2	> 10 000
α_{1B}	Corynanthine	Prazosin	0.2	0.2	> 10 000
α_{2A}	Oxymetazoline	Clonidine	2	2	> 10 000
α_{2B}	Prazosin	Clonidine	2	2	> 10 000
α_{2C}	Prazosin	Clonidine	2	2	> 10 000
β_1	Atenolol	Pindolol	0.1	0.1	> 10 000
β_2	ICI-118,551	Pindolol	0.1	0.1	> 10 000
NET	Nortriptyline/imipramine	Nisoxetine	1.2	0.5	> 10 000
m_1	Pirenzepine	QNB	0.2	0.5	> 10 000
m_2	Methoctramine	QNB	0.2	0.5	> 10 000
m_3	4-DAMP	QNB	0.2	0.5	> 10 000
m_4	Tropicamine	QNB	0.2	0.5	> 10 000
m_5	Pirenzepine	QNB	0.2	0.5	> 10 000
r$GABA_A$	GABA	Muscimol	10	3	> 10 000
rBZP	Diazepam	RO 15-1788	0.8	0.4	> 10 000
rNMDA(PCP site)	PCP/ketamine	TCP	1	0.5	> 10 000
MOR	Naloxone	Diprenorphine	0.2	0.2	> 10 000
DOR	Naltrindole	Diprenorphine	0.2	0.2	> 10 000
KOR	Naloxone	Bremazocine	4	2	> 10 000
H_1	Chlorpheniramine	Pyrilamine	3.6	1	> 10 000
H_2	me-histamine	Tiotidine	10	0.5	> 10 000
H_4	Clozapine	Histamine	10	5	> 10 000
V_1	arg8-vaso	arg8-vaso	1	0.5	> 10 000
V2	arg8-vaso	arg8-vaso	1	0.5	> 10 000
V3	arg8-vaso	arg8-vaso	1	0.5	> 10 000
CB_1	CP-55934	WIN-55,212	0.65	0.5	> 10 000
Sigma-1	Haloperidol	Pentazocine	3.6	3	5214
Sigma-2	DTG	Haloperidol	3	3.6	> 10 000
I1-Imidazoline	Naphazoline	Iodo-clonidine	0.5	0.25	2032

Experiments were performed as described in Materials and Methods, using the radioligands and unlabelled reference ligands listed above. Data represent the mean of at least four separate experiments. All studies were performed with human cloned cDNAs except where specified; *r* = rat cloned cDNA, *rat hypothalamic membranes.

DISCUSSION

Acute systemic treatment with PD149163, an NTR1 agonist that crosses the blood–brain barrier, activates both interneurons and pyramidal cells in the PFC of the rat, and is therefore consistent with the hypothesis that NT activates prefrontal cortical interneurons.

Table 2 Effect of Vehicle or PD149163 on Core Body Temperature

Treatment	Temperature (°C)
Vehicle	36.5 ± 0.2
PD149163	
0.25 mg/kg	35.5 ± 0.1*
2.5 mg/kg	34.9 ± 0.2*#

Data are presented as the mean (±SEM) core body temperature. *$p \leqslant 0.001$ relative to vehicle; #$p \leqslant 0.05$ relative to 0.25 mg/kg PD149163.

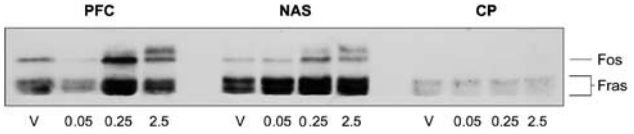

Figure 1 Representative immunoblots showing the effects of acute administration of PD149163 or vehicle on forebrain Fos levels. PD149163 increased Fos expression in the PFC and NAS, but not the CP.

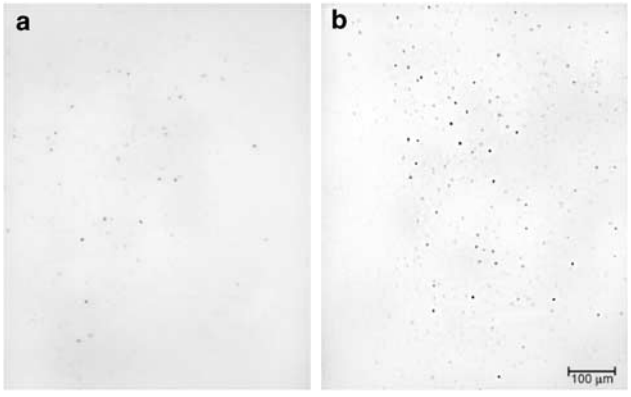

Figure 2 Effects of acute administration of PD149163 or vehicle on the density of Fos-li neurons in the PFC. Upper panel: Photomicrograph of Fos-li neurons in the PFC of an animal that received either vehicle (a) or 0.25 mg/kg PD149163 (b). Lower panel: Fos expression was highest in animals that received the low dose of PD149163. *$p \leqslant 0.05$ relative to vehicle.

PD149163 Binds Selectively to NTR1

PD149163 was originally described as a reduced amide bond NT(8-13) mimetic with central activity after peripheral administration (Feifel *et al*, 1999; Wustrow *et al*, 1995). Since the pharmacology of this compound had not been

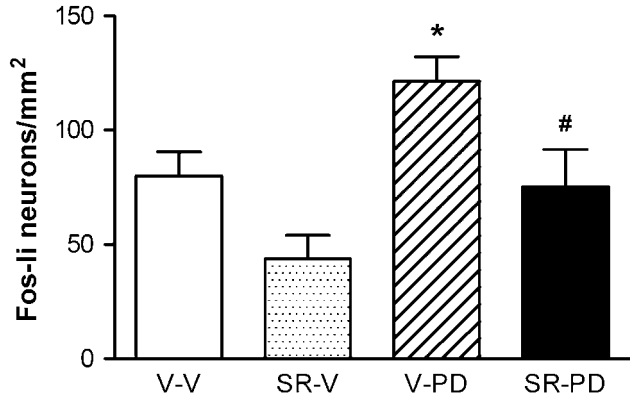

Figure 3 Effects of SR48692 pretreatment on PD149163-elicited Fos induction in the PFC. Pretreatment with the NTR1 antagonist SR48692 (SR-PD) significantly attenuated the ability of PD149163 (V-PD) to induce Fos in the PFC. SR48692 had no significant effect when administered alone (SR-V). *$p \leqslant 0.05$ compared to vehicle (V-V); #$p \leqslant 0.05$ compared to PD149163.

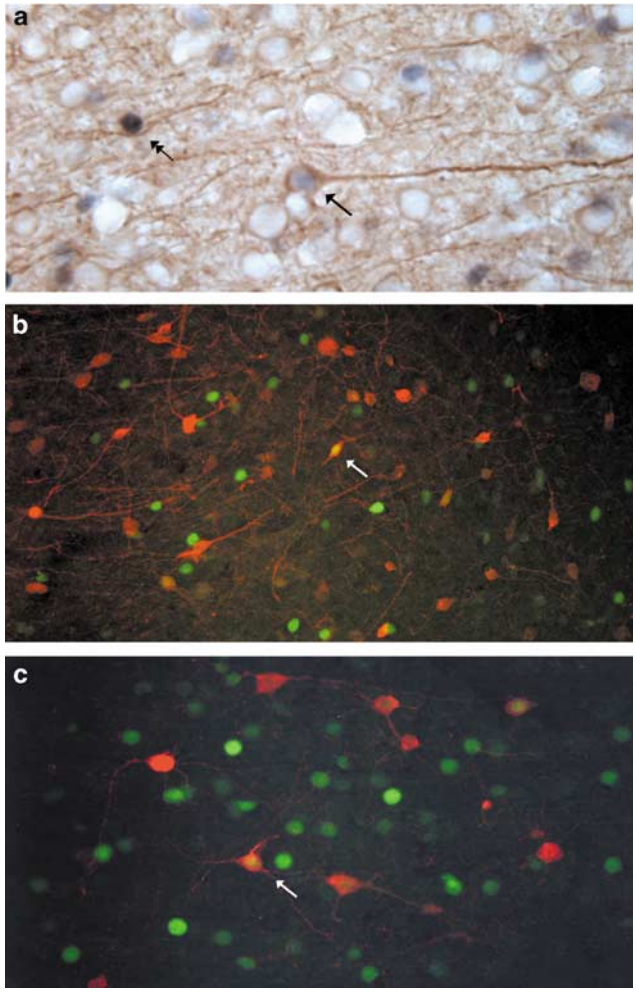

Figure 4 Photomicrographs showing the expression of Fos in PFC neurons. (a) Fos-li nucleus (gray-black) in a MAP-2-immunoreactive (brown reaction product) pyramidal cell. (b) Fos-li nucleus (green) in CB-expressing interneurons (red). (c) A PV-containing interneuron (red) expresses Fos-like immunoreactivity (green).

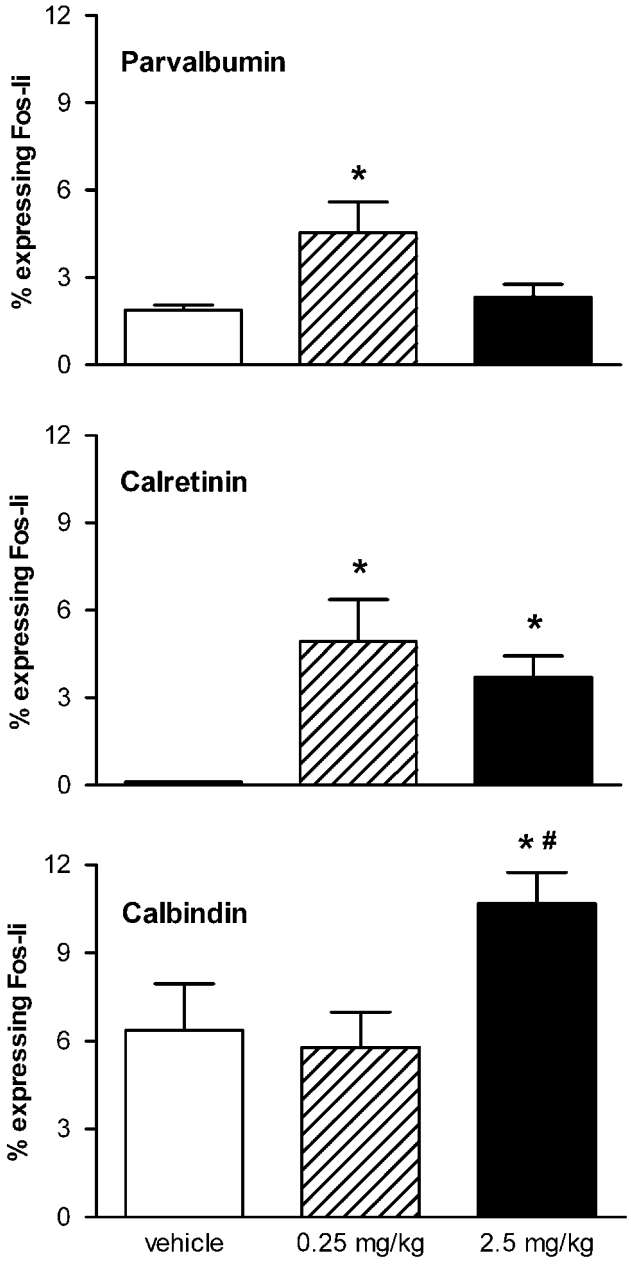

Figure 5 Effects of PD149163 on Fos expression in PV-, CR-, and CB-containing interneurons in the PFC. The low dose of the NT agonist induced Fos in PV- and CR-containing interneurons, while only the high dose of PD149163 induced Fos in CB-containing interneurons. $^{*}p \leqslant 0.05$ relative to vehicle; $^{\#}p \leqslant 0.05$ relative to 0.25 mg/kg PD149163.

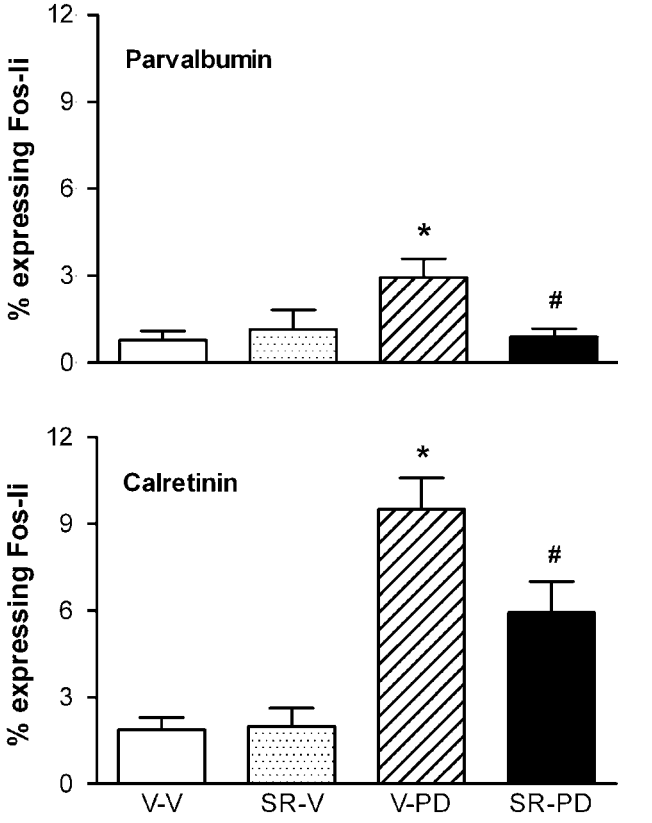

Figure 6 Effects of SR48692 pretreatment on PD149163-elicited Fos induction in PV- and CR-containing interneurons of the PFC. Pretreatment with SR48692 significantly attenuated PD149163-induced Fos expression in both classes of interneurons. $^{*}p \leqslant 0.05$ compared to vehicle; $^{\#}p \leqslant 0.05$ compared to PD149163.

comprehensively profiled, we determined if PD149163 had significant affinity for various receptors, channels, or transporters. PD149163 had modest affinity for the cloned human NTR1 ($K_i = 159$ nM), but no measurable affinity for NTR2. In addition, PD149163 did not bind to any of a wide variety of other receptors, ion channels, or transporters tested. PD149163 displayed negligible affinity (>2000 nM) for the sigma-1- and imidazoline-1-binding sites.

NT Agonist Activates Cortical Neurons

Three NT receptors have been cloned. The high-affinity NTR1 and the low-affinity, levocabastine-sensitive NTR2 are G-protein-coupled receptors (Mazella *et al*, 1996; Tanaka *et al*, 1990). NTR3, which is identical to gp95/sortilin, is a single transmembrane-spanning protein that is primarily localized to intracellular compartments (Sarret *et al*, 2003; Mazella *et al*, 1998).

Several lines of evidence suggest that the effects of PD149163 on Fos expression are mediated by NTR1. First, our analysis of PD149163 binding did not reveal any significant affinity of the agonist for NTR2 or other neurotransmitter receptors. Second, PD149163-elicited Fos induction was blocked by pretreatment with the NT antagonist SR48692, which has a high affinity (3 nM) at NTR1 but an affinity at NTR2 that is two orders of magnitude lower (Mazella *et al*, 1996; Labbe-Jullie *et al*, 1995). Third, NTR1 is expressed on both prefrontal cortical interneurons and pyramidal cells (Petrie *et al*, 2002), consistent with the effects of PD149163 being mediated by NTR1. Finally, we observed that PD149163 dose-dependently decreased the core body temperature of rats. Administration of NT decreases body temperature through activation of NTR1 receptors, with decreases in core temperature not seen in NTR1 null mutant mice challenged with NT (Pettibone *et al*, 2002; Remaury *et al*, 2002).

Although SR48692 blocked PD149163-induced Fos expression in the PFC, the NT antagonist did not block PD149163-elicited hypothermia. This observation is consistent with the findings of Dubuc *et al* (1994), who noted

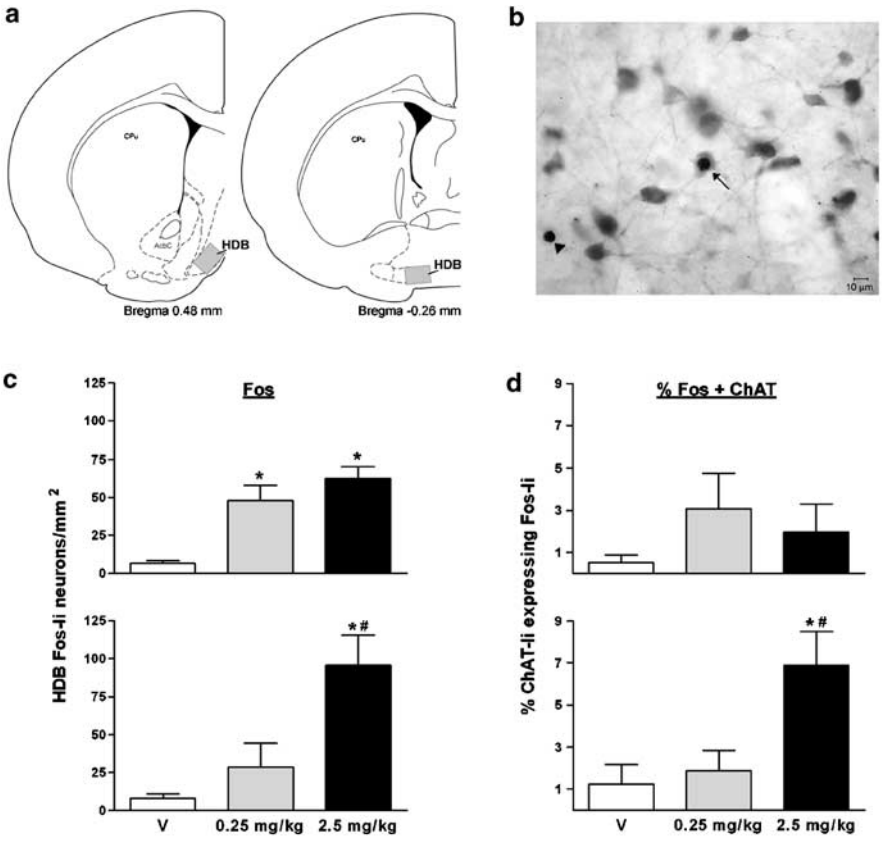

Figure 7 Effects of acute administration of PD149163 on the density of Fos-li neurons in the HDB. (a) Schematic illustration of the two areas of the HDB in which Fos-li neurons were counted. The red box indicates the approximate location of the counted cells. (b) Fos-li nucleus (arrowhead) in a ChAT-li cholinergic neuron (arrow). (c) PD149163 caused a dose-related increase in the density of Fos-li neurons in the rostral (upper panel) and caudal (lower panel) HDB. (d) Neurons immunoreactive for ChAT expressed Fos-li in the caudal (lower panel) but not rostral (upper panel) HDB. $^{*}p \leqslant 0.05$ compared to vehicle, $^{\#}p \leqslant 0.05$ compared to 0.25 mg/kg PD149163.

that SR48692 had no effect on NT-induced hypothermia in rats or mice at the same doses that blocked NT-induced rotational behavior. Since NTR1 null mutant mice do not display a hypothermic response to NT, it is unclear as to why SR48692 does not antagonize PD149163-induced hypothermia. Previous reports have noted that nonpeptide NT antagonists do not block the full spectrum of NT-mediated effects (Leonetti *et al*, 2002; Nalivaiko *et al*, 1998; Pinnock and Woodruff, 1994), leading to the suggestion that there may be an additional NT receptor that has not yet been cloned.

PD149163 binds weakly to both imidazoline-1 and sigma-1 sites. The functional significance of these binding sites remains unclear (Langa *et al*, 2003; Eglen *et al*, 1998; Maurice and Lockhart, 1997). Although acute administration of the sigma ligand E-5842 has been shown to increase Fos in the medial PFC (Guitart and Farre, 1998), the observation that SR48692 blocks PD149163 effects on Fos expression argues for a role of NT receptors but not the sigma-1 site.

The hypothermia elicited by PD149163 may serve as a stressor. Because stress induces overall Fos expression in the PFC, it is possible that indirect stress effects account for the observed immediate-early gene response to PD149163. However, this is very unlikely because the high dose of the NT agonist, which elicited a lower body temperature than the lower dose, did not significantly increase overall Fos expression in the PFC.

Effects of PD149163 on GABAergic Interneurons

The ability of PD149163 to activate PFC interneurons is consistent with previous *in vitro* electrophysiology studies showing that NT increases GABA-mediated spontaneous postsynaptic potentials in the PFC (Audinat *et al*, 1989). NT has also been shown to activate GABAergic neurons in the striatum, with a resultant increase in extracellular GABA levels (Ferraro *et al*, 1998).

PD149163 significantly increased Fos expression in PV- and CR-containing interneurons. However, the percentage of PV- and CR-containing cells in which Fos was induced was relatively low. Nonetheless, induction of Fos in the PV population of interneurons is likely to exert significant effects. PV interneurons are the most common class of interneuron in the cortex, comprising 60–70% of the total pool of GABAergic interneurons. PV-containing interneurons have a wide axonal arbor, in contrast to the narrow (columnar) axonal arbor of many other interneurons, and regulate hundreds of pyramidal cells. Moreover, chandelier cells, a morphological subtype of PV-containing interneuron, synapse onto the axon initial segment of pyramidal cells (Freund *et al*, 1983; Somogyi, 1977), placing them

in a position to regulate powerfully pyramidal cell activity and hence overall cortical output. Thus, it is likely that activation of even a small percentage of PV-containing cells will exert a relatively broad effect on cortical function. Additional electrophysiological studies will be required to elucidate the physiological correlates of PD149163-induced Fos expression in the PFC.

Dose-Related Induction of Fos in PFC GABA Interneurons

We observed that a low but not high dose of PD149163 induced Fos in PFC neurons, irrespective of the type of neuron; a similar dose-related effect emerged upon examination of Fos in PV-containing interneurons. Previous reports have also noted an inverted U-shaped dose–response relationship of NT agonists on baseline and amphetamine-disrupted prepulse inhibition of the startle response (Shilling *et al*, 2003; Feifel *et al*, 1997). The mechanisms that account for such dose-dependent effects of NT agonists are not known. It is possible that differential activation of pre- and postsynaptic NT receptors may contribute to the dose-related effects of PD149163 on Fos expression in PFC neurons, including PV-containing interneurons.

The most parsimonious model accounting for the ability of systemically administered PD149163 to increase PFC Fos is through direct actions of the agonist on cortical cells. NTR1 mRNA and protein have been observed in PFC neurons, particularly those in the deep layers of the infralimbic and prelimbic cortices (Alexander and Leeman, 1998; Boudin *et al*, 1996; Nicot *et al*, 1994). Preliminary data suggest that both interneurons and pyramidal cells express NTR1 in this region (Petrie *et al*, 2002).

We cannot, however, exclude the possibility that trans-synaptic actions reflecting an initial action of the NT agonist on some afferent population to the PFC is responsible for the increase in Fos expression in PFC interneurons. We therefore assessed if PD149163 increased Fos expression in basal forebrain neurons, including cholinergic cells of the basal forebrain that project to the PFC. These cholinergic neurons express NTR1 and are depolarized in response to NT application (Cape *et al*, 2000; Matthews, 1999; Farkas *et al*, 1994).

We observed that PD149163 increased Fos expression in neurons, irrespective of phenotype, in both the rostral and caudal parts of the HDB. In contrast, the effect of PD149163 on cholinergic cells in the HDB was observed only in the caudal HDB. It is interesting to note that those HDB cholinergic cells that project to the PFC are most dense in the caudal HDB (Gaykema *et al*, 1991, 1990; Luiten *et al*, 1987), suggesting that the NT agonist may be selectively activating those cholinergic cells that innervate the PFC.

The high (2.5 mg/kg) dose of PD149163 increased Fos expression in the basal forebrain cholinergic cells. We observed a significant increase in the percentage of CR-containing PFC interneurons expressing Fos at the high dose, whereas the lower dose activated PV-li interneurons. This parallel suggests that the actions of PD149163 at the higher dose on CR interneurons may reflect trans-synaptic activation from basal forebrain cholinergic cells, but that the induction of Fos in PV cells, the major type of cortical interneuron in the rat, may be a direct action.

Implications

Several studies have reported that cerebrospinal fluid levels of NT are reduced in schizophrenic patients, particularly those with prominent negative symptoms (Garver *et al*, 1991; Lindstrom *et al*, 1988; Widerlov *et al*, 1982); NT levels tend to normalize in response to antipsychotic drug (APD) treatment (Sharma *et al*, 1997; Breslin *et al*, 1994). APDs exert behavioral effects similar to those seen in animals receiving central administration of NT (see Kinkead *et al*, 1999). Accordingly, considerable interest has been generated in NT as an endogenous APD (Nemeroff, 1980) with potential therapeutic value in schizophrenia.

The regional pattern of Fos induction by PD149163 is similar to that of an atypical APD, with pronounced effects in the PFC but not dorsolateral striatum. Activation of both prefrontal cortical interneurons and pyramidal cells is consistent with the actions of atypical APDs such as clozapine (Deutch and Duman, 1996). This is particularly interesting because the number and/or function of GABA interneurons in the PFC may be compromised in schizophrenia (Hashimoto *et al*, 2003; Benes *et al*, 2000; Lewis *et al*, 1999; Akbarian *et al*, 1995). Our data suggest that NT agonists activate PFC interneurons and could thereby compensate for a prefrontal cortical GABAergic deficit in schizophrenia.

GABAergic neurons in primate species receive DA inputs (Sesack *et al*, 1995b). However, in contrast to rodents, the laminar distributions of DA and NT axons in primates differ, suggesting that the two transmitters are not colocalized in the PFC of primates, including humans (Gaspar *et al*, 1990). *In situ* hybridization analysis has, however, revealed that tyrosine hydroxylase and NT mRNAs are colocalized in some ventral tegmental area neurons (Bean *et al*, 1992). Moreover, it is possible that NT may not be present at detectable levels under basal conditions, but may be rapidly induced and become obvious after appropriate challenges (Deutch and Zahm, 1992; Merchant *et al*, 1991; Eggerman and Zahm, 1988). Finally, NT is clearly present in axons of the human PFC, regardless of NT presence in dopaminergic axons, and thus NT may be positioned to activate GABAergic neurons and thereby potentially ameliorate negative symptoms and cognitive deficits arising from PFC.

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