

The Phenotypic Differentiation of Locus Coeruleus Noradrenergic Neurons Mediated by BDNF is Enhanced by Corticotropin Releasing Factor through the Activation of a cAMP-Dependent Signaling Pathway

Sabine Traver, Marc Marien, Elodie Martin, Etienne C. Hirsch and Patrick P. Michel

INSERM, Unité Mixte de Recherche 679, Experimental Neurology and Therapeutics, Paris, France (ST, E.M., E.C.H, P.P.M.); Université Pierre et Marie Curie, Paris, France (ST, E.M., E.C.H, P.P.M.); Pierre Fabre Research Center, Castres, France (M.M, P.P.M.).

Running title: CRF-mediated induction of noradrenergic neurons

Corresponding Author: Patrick P. Michel, Unité Mixte de Recherche 679, INSERM-UPMC

Bâtiment Pharmacie, Hôpital de la Salpêtrière, 47 bd de l'Hôpital, 75013, Paris, France

e-mail: ppmichel@ccr.jussieu.fr , Tel: 33142162217; Fax: 33144243658

Number of:

text pages: 22

figures: 10

references: 40

Number of words:

abstract: 247

introduction: 585

discussion: 1495

Abbreviations: BDNF: brain derived neurotrophic factor; CRF: corticotropin releasing factor; DA: dopamine or dopaminergic; DBH: dopamine- β -hydroxylase; D-PBS: Dulbecco's phosphate buffered saline; Epac: exchange protein directly activated by cAMP; ERK_{1/2}: extracellular signal-regulated kinases_{1/2}; FK: forskolin; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; LC: locus coeruleus; MAPK: mitogen-activated protein kinase; MEK: MAPK/ERK kinase; MK-801, dizocilpine; NA: noradrenaline or noradrenergic; NT-3: neurotrophin-3; NT-4: neurotrophin-4; PI3K: phosphatidylinositol-3-kinase; PKA: cyclic AMP dependent protein kinase; PNMT: phenylethanolamine N-methyltransferase; TH: tyrosine hydroxylase; Ucn: urocortin.

ABSTRACT: We have developed a model system of locus coeruleus (LC) neurons in culture, in which brain-derived neurotrophic factor (BDNF) induces the emergence of noradrenergic neurons attested by the presence of tyrosine hydroxylase (TH) and dopamine- β -hydroxylase and the absence of phenylethanolamine N-methyl-transferase. Although inactive in itself, the neuropeptide corticotropin releasing factor (CRF) strongly amplified the effect of BDNF, increasing the number of cells expressing TH and the active accumulation of noradrenaline by a factor of 2-3 via a mechanism that was nonmitogenic. CRF also acted cooperatively with neurotrophin-4, which like BDNF is a selective ligand of the TrkB tyrosine kinase receptor. The effect of CRF but not that of BDNF was prevented by astressin, a non-selective CRF-1/CRF-2 receptor antagonist. However, only CRF-1 receptor transcripts were detectable in LC cultures suggesting that this receptor subtype mediated the effect of CRF. Consistent with the positive coupling of CRF-1 receptors to adenylate cyclase, the trophic action of CRF was mimicked by cAMP elevating agents. Epac a guanine nucleotide exchange factor directly activated by cAMP contributed to the effect of CRF through the stimulation of extracellular signal-regulated kinases 1/2 (ERK_{1/2}). However, downstream of ERK_{1/2} activation by CRF, the phenotypic induction of NA neurons relied upon the stimulation of the phosphatidylinositol-3-kinase/Akt transduction pathway by BDNF. Altogether, our results suggest that CRF participates to the phenotypic differentiation of LC noradrenergic neurons during development. Whether similar mechanisms account for the high degree of plasticity of these neurons in the adult brain remains to be established.

The majority of noradrenergic (NA) neurons in the brain are localized within a single brainstem nucleus, the locus coeruleus (LC). However, because of a widespread projection system, these neurons innervate almost the entire central nervous system (Berridge and Waterhouse, 2003). Because of the ubiquitous distribution of NA, the LC-NA system plays a prominent role in a variety of brain functions and behaviors that include vigilance, attention, arousal, memory acquisition, locomotor control and response to stress (Berridge and Waterhouse, 2003). LC NA neurons are also interesting for other reasons: (1) via their neurotransmitter they influence the development and survival of other populations of neuronal cells either during development of the brain or later in life (Meier *et al.*, 1991; Marien *et al.*, 2004); (2) some of them can recover in the adult brain a phenotype that they transiently expressed during development (Bezin *et al.*, 2000); (3) they are vulnerable to neurodegenerative conditions such as Alzheimer's and Parkinson's diseases (Zarow *et al.*, 2003) and represent a potential target for pharmacological treatments of these disorders (Marien *et al.*, 2004). Because of these multiple attributes, it is vital to understand the factors and signals that control the phenotype and/or survival of these neurons.

Previous studies have suggested that a limited number of trophic peptides may be involved in the development and maturation of LC NA neurons (Reiriz *et al.*, 2002). Among these factors, TrkB ligands such as the neurotrophins BDNF and neurotrophin-4 (NT-4) may be of particular importance for two reasons: (1) both peptides increased the number of NA neurons in LC cultures maintained in serum-free conditions, i.e., in the absence of additional trophic support (Holm *et al.*, 2003); (2) a reduction in the number of these neurons was observed in TrkB null mutant mice (Holm *et al.*, 2003). Other studies have shown that cAMP dependent signaling might also play a crucial role in the maturation and maintenance of these neurons (Sklair-Tavron and Segal, 1993; Reiriz *et al.*, 2002; Rusnak and Gainer, 2005). TrkB activation by its ligands BDNF or NT-4 cannot stimulate, however, the production of cAMP

(Hanson *et al.*, 1998). This suggests that other putative trophic factors contribute to the development of LC NA cells through a mechanism that involves the cyclic nucleotide.

Corticotropin releasing factor (CRF), a neuropeptide that is crucial for the integration of multi-system responses of the brain to stress (Carrasco and Van de Kar, 2003) is a possible candidate for several reasons. (1) It elevates cAMP levels through the activation of G-protein-coupled receptors (Bale and Vale, 2004). (2) CRF afferents project to the LC, providing a neuroanatomical substrate for an interaction between CRF and LC NA neurons (Van Bockstaele *et al.*, 1996). (3) CRF type 1 (CRF1) receptors are detectable in virtually all LC NA neurons (Sauvage and Steckler, 2001) which may explain why CRF administered in the LC increases the firing rate of NA neurons and the release of NA in their terminal fields (Jedema and Grace, 2004). (4) CRF can also operate as a neurotrophic molecule, since it is highly effective in preventing neuronal apoptosis (Radulovic *et al.*, 2003) and in stimulating dendritic differentiation (Chen *et al.*, 2004).

The present study was carried out: (1) to establish whether CRF has an impact on the development of LC NA neurons; (2) to determine to what extent BDNF modulates this effect; and (3) to characterize the nature of the underlying molecular mechanisms. Our results show that CRF is highly potent in modulating the NA phenotype via a mechanism requiring the presence of BDNF.

MATERIALS AND METHODS

Peptides and pharmacological reagents

Recombinant human brain-derived neurotrophic factor, neurotrophin-3 (NT-3), NT-4 and the monoclonal anti-human BDNF antibody (MAB-248) used to neutralize the biological activity of BDNF were from R&D Systems (Minneapolis, MN). Nerve growth factor 2.5S (NGF; Grade II) was from Alomone Labs (Jerusalem, Israel). Corticotropin releasing factor (CRF), urocortin (Ucn) and astressin were purchased from Sigma/RBI-Aldrich (Saint Quentin Fallavier, France). The TrkB antibody was from Transduction Laboratories (Lexington, KY). The activator of adenylate cyclase forskolin (FK) and the inhibitors of cyclic AMP dependent protein kinase (PKA), mitogen-activated protein kinase / extracellular signal-regulated kinase (MAPK/ERK) kinase (MEK) and phosphoinositide-3-kinase (PI3K) (H-89, PD98059 and LY294002 respectively) were purchased from Calbiochem (VWR International, Fontenay/Bois). The cyclic AMP analog 8-Br-2'-O-Me-cAMP was obtained from BIOLOG Life Science Institute (Bremen, Germany). [Methyl-³H]-Thymidine and 1-[7,8-³H]-NA were from Amersham Biosciences (Orsay, France). Unless specified, other pharmacological and biochemical agents were purchased from Sigma/RBI-Aldrich (Saint Quentin Fallavier, France).

LC and mesencephalic cultures

Animals were treated in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996), the European Directive N° 86/609, and the guidelines of the local institutional animal care and use committee. NA cultures from the LC were obtained from embryonic day 14 Wistar rat embryos (Janvier Breeding Center; Le Genest St Isles, France). The area dissected corresponded to the proximal rhombencephalic ring between the distal part of the mesencephalic flexure and the proximal part of the pontine

flexure (Specht et al., 1981; Holm et al., 2003). Cultures of dopaminergic (DA) neurons were obtained subsequently by dissecting out the ventral mesencephalon of the same embryos (Michel et al., 1997). After mechanical trituration, LC and mesencephalic cells were seeded at a density of 3-4 and 1.5-2.0 x10⁵ cells /10 mm well, respectively, onto polyethylenimine (1mg/ml; Sigma/RBI-Aldrich) precoated culture plates (48 wells). Cells were maintained in a culture medium consisting of MEM with Earle's salts and Ham's F12 nutrient mixture (1V:1V) (Invitrogen; Cergy Pontoise, France), supplemented with glucose (25 mM), apotransferrin (100 µg/ml), insulin (10 µg/ml), and penicillin-streptomycin. When required, 350 µl of culture medium was changed at DIV3 and 6. Since neurons were grown in an astrocyte-poor environment that favors excitotoxic stress (Michel et al., 1997), the non-competitive NMDA receptor antagonist dizocilpine (MK-801; 3 µM) was also added to the cultures before changing the medium from DIV 3 onward. Noradrenergic neurons represented ~0.2-0.4% of all cultured cells in BDNF-treated LC cultures.

Immunofluorescence staining

Tyrosine hydroxylase (TH) immunofluorescence detection was used to detect TH positive NA neurons. After fixation with a mixture of 4% formaldehyde in Dulbecco's phosphate buffered saline (D-PBS) for 15 min, cells were washed three times with D-PBS, and then incubated overnight at 4°C with a mouse anti-TH monoclonal antibody (Diasorin, Stillwater, MN) diluted at 1:5000 in D-PBS containing 0.2% Triton X-100. Subsequent incubations were performed, at room temperature, with a secondary anti-mouse IgG cyanin 3 (Cy3) conjugate (1:500; Sigma-RBI-Aldrich). In some cases, a rabbit anti-TH antibody (Pel Freez, Paris, France, 1/500) was also used for the detection of NA neurons. Astrocytes were identified with a rabbit anti-gial fibrillary acidic protein (GFAP) antibody diluted 1:100 (DAKO Corporation, Carpinteria, CA) followed by a secondary antibody Alexa fluor 488 F(ab')₂

fragment of goat anti-rabbit IgG from Molecular Probes (Montluçon, France). For phospho-Akt immunofluorescence staining, non-specific binding sites were blocked for 1h with D-PBS containing 10% horse serum. For TrkB immunodetection the blocking buffer contained 50% newborn goat serum, 1% BSA and 100 mM L-Lysine. The cultures were then incubated for two days at 4°C with a rabbit polyclonal phospho-Akt (Ser473) antibody from Cell Signaling Technology, Inc., (Beverly, MA), diluted at 1:250 in D-PBS containing 0.2% Triton X-100, or with a rabbit polyclonal antibody against the extracellular domain of TrkB (Transduction Laboratories, Lexington, KY) diluted at 1:500 in D-PBS without Triton X-100 to detect TrkB receptors bound to the plasma membrane. Both antibodies were detected with an Alexa fluor 488 F(ab')₂ fragment of goat anti-rabbit IgG. Images were acquired with the Simple-PCI software from C-Imaging Systems using a Nikon (Tokyo, Japan) TE-300 inverted fluorescent microscope equipped with an ORCA-ER digital camera from Hamamatsu (Bridgewater, NJ).

Uptake of [methyl-³H]thymidine

[methyl-³H]-Thymidine, a marker of DNA synthesis was used to label proliferating cells as described previously (Troade et al., 2002). LC cultures maintained for 3 days in the presence of test treatments were exposed chronically to 0.5 µCi [³H]-methyl-thymidine (Amersham Biosciences; 40 Ci/mmol) at 37°C in standard culture medium. After three rapid washes, the cells were allowed to recover for 1 hr in the same culture medium to remove unincorporated radioactivity. Finally, the cultures were fixed with a mixture of glutaraldehyde/formaldehyde (0.5%:4% in D-PBS) for 20 min. Positive nuclei were visualized with Hypercoat LM-1 emulsion (Amersham Biosciences) after 4 days of exposure at 4°C.

Reverse transcription and PCR

Total RNA from DIV 6 LC or mesencephalic cultures or from adult rat adrenal medulla was extracted with the RNable solution (Eurobio, Les Ulis, France). First strand cDNA was synthesized from 1 µg of total RNA using the RT kit (Qiagen). The expression of transcripts coding for TH, dopamine-β-hydroxylase (DBH), phenylethanolamine-N-methyltransferase (PNMT), CRF-1 and CRF-2 receptors, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TrkB receptors was analyzed by PCR under the following conditions: 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec with 1 µl of the reverse transcription mixture. The primers used were: rat TH forward (5'-CCTCCTTGCTCGGGCTGTAA-3') and reverse (5'-CTGAGCTTGCCTTGGCGTCA-3'), product size: 1053 bp; rat DBH forward (5'-GTGACCAGAAAGGGCAGATCC-3') and reverse (5'-CACCGGCTTCTTCTGGGTAGT-3'), product size: 599 bp (Sakurada et al, 1999); rat PNMT forward (5'-GTCTGGACAGGTCCTCATT-3') and reverse (5'-CCTCTGACACTGGAACCAC-3'), product size: 499 bp (Unsworth et al, 1999); rat CRF-1 receptors forward (5'-TCCACTACATCTGAGACCATTTCAGTACA-3') and reverse (5'-TCCTGCCACCGGCGCCACCTCTTCCGGA-3'), product size: 248 bp; rat CRF-2 receptors forward (5'-CTGCTGCAACTCATCGACCACGAAGTG-3') and reverse (5'-CCTGGTAGATGTAGTCCACTAAGTCACCAG-3'), product size: 307 bp; rat GAPDH forward (5'-TCCCAGAGCTGAACGGGAAGCTCACTG-3') and reverse (5'-TGGAGGCCATGTAGGCCATGAGGTCCA-3'); product size: 339 bp (Baigent et al, 2000); and rat TrkB receptors forward (5'-CTGGACAGCACGTCCAACAT-3') and reverse (5'-GAGAGACTTGACCTGAGCAC-3'), product size: 597 bp (Ge et al, 2004). The PCR products were visualized by electrophoresis in a 1% agarose gel containing 0.1 mg/ml ethidium bromide.

Quantification and visualization of [³H]-NA uptake

Intraneuronal accumulation of NA via active transport was monitored in LC cultures using a protocol adapted from Traver et al. (2005). Briefly, after preincubation for 10 min in 500 μ l D-PBS containing 5 mM glucose and 100 μ M ascorbic acid, the uptake was initiated by addition of 50 nM [³H]-NA (37 Ci/mmol) to the cultures and terminated after 15 min by removal of the incubation medium followed by two rapid washes with cold D-PBS. Cells were scraped off the culture wells and counted by liquid scintillation spectrometry. When the accumulation of [³H]-NA was visualized by microautoradiography, the incubation time with [³H]-NA was extended to 45 min and the concentration of NA was raised to 100 nM to improve sensitivity. After two rapid washes with D-PBS, the cultures were fixed for 20 min in D-PBS containing 0.5% glutaraldehyde and 4% formaldehyde, and dehydrated with ethanol. The incorporation of the tritiated label was detected with the Hypercoat LM-1 emulsion (Amersham Biosciences) after an exposure of 10 days in the dark at 4°C. Blockade of NA saturable transport was obtained in the presence of 0.1 μ M desipramine.

Western Immunoblotting of Akt and ERK_{1/2}

The cultures exposed to the various test treatments were recovered in Laemmli buffer supplemented with 1 mM sodium orthovanadate, 1 μ M okadaic acid and 0.5% β -mercaptoethanol. Cell samples boiled for 5 minutes were then electrophoresed through a 10% acrylamide gel and blotted onto nitrocellulose membranes. The membranes were incubated with a rabbit polyclonal phospho-Akt (Ser473) antibody or with a mouse monoclonal phospho-ERK_{1/2} (Thr202/Tyr204; clone E10) antibody and developed with the enhanced chemiluminescence detection kit (Pierce; Rockford, IL). Membranes were then stripped using the Re-Blot Plus kit from Chemicon International (Temecula, CA), incubated again with a rabbit polyclonal anti-ERK_{1/2} antibody or a mouse monoclonal anti-Akt (clone 5G3) antibody,

respectively, and developed as described previously. The two antibodies were used at a dilution of 1:1000.

Statistical analysis

Comparisons between two groups were performed with Student's t-test. Multiple comparisons against a single reference group were made by one-way analysis of variance (ANOVA) followed by Dunnett's test. When all pairwise comparisons were made, the Student-Newman-Keuls test was used. SEM values were derived from at least three independent experiments.

RESULTS

CRF induces TH⁺ neurons in BDNF-treated LC cultures

We first established that TH⁺ cells were virtually absent from ED14 rat LC cultures irregardless of their stage of maturation (Fig. 1A, Fig. 2A). TH⁺ cells became detectable when BDNF (20 ng/mL) was applied to these cultures (Fig. 1A; Fig. 2B). This concentration of BDNF was established initially as producing optimal effects in this culture model (not shown) and was used in all subsequent experiments described hereafter. When CRF was added concomitant to BDNF, the number of TH⁺ cells rose sharply (Fig.1A; Fig. 2C), in a concentration-dependent manner with an effect significant at 0.5 μM and optimal at 1 μM (Fig.1B). Higher concentrations of CRF were progressively less effective (Fig. 1B). Either in the presence of BDNF and CRF or in the presence of BDNF alone, ~30-40% of the TH⁺ neurons were intensely stained (Fig 2B,C). The other TH⁺ cells were also clearly detectable, but less intensely labeled (Fig 2B,C). The effect of BDNF on TH⁺ cells was already optimal after 1 DIV, whereas that of CRF, in the presence of BDNF, increased progressively during the first three days in culture and remained at a maximum level thereafter (Fig. 1A). It should be noted that CRF had no effect when BDNF was absent from the cultures (Fig. 1A; Fig 2D).

To characterize the phenotype of the TH⁺ neurons that were induced in LC cultures by BDNF and CRF or by BDNF alone, we performed RT-PCR to detect the expression of TH, DBH and PNMT transcripts encoding the three key enzymes involved in the synthesis of catecholamines. GAPDH was used as an internal standard. As expected from immunofluorescence experiments, TH transcripts were totally absent from control cultures but readily detectable in BDNF or BDNF/CRF-treated cultures (Fig.1C). Interestingly, mRNAs encoding DBH (the rate limiting enzyme in the synthesis of NA) were also induced by the same treatments (Fig. 1C). However, PNMT (the enzyme that converts NA into adrenaline) was undetectable (Fig.1C). Note that TH and DBH transcripts appeared to be more abundant in cultures exposed to a combined treatment to CRF and BDNF compared to cultures treated with BDNF only, as visualized on ethidium bromide-stained agarose gels (Fig. 1C).

The embryonic brains used for LC cultures served also to generate cultures from the mesencephalon. Contrasting to what we observed with LC cultures, TH⁺ neurons were detectable in mesencephalic cultures (3 DIV) in the absence of any treatment (Fig 1D). BDNF alone or in the presence of CRF failed to increase the number of TH⁺ neurons in this culture system (Fig. 1D). CRF alone was also ineffective. It is worth noting that mesencephalic cultures exposed to BDNF or BDNF/CRF contained TH but no DBH transcripts, which indicates that the TH⁺ neurons from this brain area present a stable DA phenotype (Fig. 1C). Note that TH, DBH and PNMT transcripts were all present, in adult rat adrenal medulla tissue used as a positive control (Fig. 1C).

CRF does not act as a mitogen for TH⁺ neuroblasts or their precursor cells

Next, we examined the possibility that the CRF-induced increase in the number of TH⁺ cells resulted, at least in part, from an effect of the peptide on the proliferation of TH⁺ neuroblasts in contact with BDNF. Therefore, LC cultures were exposed between 0 and 3 DIV to CRF and

BDNF in the presence of [methyl-³H]-thymidine, a marker of DNA synthesis used to label proliferating cells (Troade et al., 2002). A small percentage of LC cells (8-10%) accumulated the tritiated label in their nucleus in the presence of both peptides. However, these cells never expressed TH (Fig. 2E). A large number of the positive nuclei were in astroglial cells that expressed GFAP (Fig. 2F).

CRF induces expression of a functional NA transporter

Besides TH and DBH, NA neurons also express a high affinity NA transporter, which serves to eliminate NA from the synaptic cleft. In order to detect the population of LC neurons that accumulate NA via this transporter, we visualized the uptake of tritiated NA by microautoradiography in 8 DIV LC cultures. The specificity of the accumulation was established in the presence of a selective NA uptake inhibitor desipramine. At the concentration used (0.1 μ M) desipramine did not significantly inhibit the homologous DA transporter (Prasad and Amara, 2001; results not shown) that is expressed exclusively in DA neurons. In control cultures, virtually no cell bodies were labeled with [³H]-NA. BDNF alone induced the appearance of a population of cells that accumulated [³H]-NA (Fig. 3B,D). When added to BDNF-treated cultures, CRF (1 μ M) increased the number of neurons that incorporated the tritiated neurotransmitter by 2-3 fold (Fig. 3B,D). In a representative experiment, we counted 941 and 2818 [³H]-NA⁺ neurons, in BDNF and BDNF/CRF-treated cultures, respectively, whereas in sister cultures the corresponding numbers for TH⁺ cells were 1199 and 3240 (Fig. 3A,B). Note that in the presence of 0.1 μ M desipramine not a single cell body remained detectable in cultures exposed to both BDNF and CRF (Fig. 3D) or BDNF alone (not shown), which indicates that [³H]-NA was accumulated exclusively by NA neurons through the high affinity transporter for NA. When the accumulation was measured using liquid scintillation spectrometry, we found that the incorporation of [³H]-NA in CRF/BDNF-

treated cultures was also 2-3 fold higher compared to BDNF-treated cultures. It is worth mentioning that a low but significant level of desipramine-sensitive uptake was constantly observed in control cultures. This was probably related to the fact that a small number of nerve endings were positively labeled with [³H]-NA in these cultures even if no corresponding cell bodies were detectable (Fig. 3D).

An early exposure to BDNF is crucial for the effect of CRF at later stages of the cultures

In order to determine whether the response to CRF could also be initiated at later stages of the cultures, we postponed the treatment with the peptide up to 3 DIV. If BDNF was applied at the same time, CRF was ineffective in inducing NA neurons whereas BDNF conserved a small but significant effect (Fig. 4). However, if BDNF was present throughout the culture time, a delayed treatment with CRF starting at 3 DIV was highly effective in inducing TH⁺ cells (Fig. 4) suggesting that an early contact with the neurotrophin was a prerequisite for the effect of CRF. The effect of CRF in BDNF-treated cultures persisted, however, after its withdrawal at 3 DIV, regardless of whether BDNF was maintained or not (Fig. 4).

CRF cooperates with TrkB but not with TrkA or TrkC ligands

To determine whether CRF also cooperates with other neurotrophins, NT-4, a close homologue of BDNF that is also a selective ligand of TrkB tyrosine kinase receptors (Patapoutian and Reichardt, 2001), was added alone to the cultures (Fig. 5). Not surprisingly, CRF strongly increased the number of TH⁺ neurons in the presence of NT-4 (Fig. 5). However, when the cultures were exposed to the TrkA or TrkC ligands, NGF or NT-3, respectively, CRF was totally ineffective (Fig. 5). This was probably due to the fact that neither NGF nor NT-3 was able to induce TH⁺ neurons when added alone to LC cultures.

The effects of CRF are mediated through CRF type-1 receptors

We next characterized the receptor that was involved in the effect of CRF on TH⁺ cells. BDNF/CRF-treated cultures were maintained in the presence of astressin, a non-selective CRF-1/2 receptor antagonist (Bale and Vale, 2004). A concentration of 1 μ M astressin totally blocked the effect of CRF but not that of BDNF (Fig. 6A), indicating that the effects of the peptides were mediated by two distinct plasma membrane receptors. Only CRF-1 receptor transcripts could be amplified by RT-PCR from LC cultures suggesting that this receptor subtype alone was responsible for the effect of CRF on TH⁺ neurons (Fig. 6B). Note, however, that both CRF-1 and CRF-2 receptors were expressed in adrenal medulla tissue used as the positive control. Interestingly, Ucn a peptide homologue of CRF was able to induce NA neurons in BDNF-treated LC cultures through a mechanism that was also prevented by astressin. Consistent with the positive coupling of CRF-1 receptors to adenylate cyclase (Bale and Vale, 2004), elevation of endogenous cAMP levels by FK mimicked the effects of CRF on LC TH⁺ neurons exposed to BDNF (Fig 6A). However, at variance to what we observed with CRF or Ucn, FK also exerted an effect by itself in the absence of BDNF (Fig. 6A). Note that rolipram, a selective inhibitor of cAMP-specific phosphodiesterase PDE IV, failed to improve the effect of CRF used at an optimal concentration of 1 μ M (not shown).

The effect of CRF but not BDNF is mediated by a cAMP-dependent mechanism requiring activation of ERK_{1/2}

PKA might be a proximal target of the cAMP-dependent mechanism elicited by CRF. This is unlikely, however, since the effect of CRF was resistant to the PKA inhibitor H-89, at a concentration of 3 μ M sufficient to totally block the activation of the cAMP Response Element Binding Protein (CREB) (Troadec et al., 2002), one of the substrates of this kinase. Another potential cAMP target is an exchange protein directly activated by cAMP (Epac) that

operates as a guanine nucleotide exchange factor (Bos, 2003). The possible involvement of Epac was studied using 8-Br-2'-O-Me-cAMP, a synthetic and permeant cAMP analogue that selectively stimulates Epac but not PKA (Christensen et al., 2003). Our data show that 8-Br-2'-O-Me-cAMP (500 μ M) mimicked the effects of CRF on TH⁺ neurons in the presence of BDNF via a mechanism that was not prevented by H-89 (Fig. 7A). Most interestingly, unlike FK, 8-Br-2'-O-Me-cAMP was totally inactive in the absence of BDNF (Fig. 7A), which suggests that the modified cAMP analog reproduced the effect of CRF more closely than FK.

Binding of cAMP to Epac could possibly lead in turn to the activation of the ERK_{1/2} signaling pathway (Bos et al., 2003). Using an antibody that recognizes selectively the phosphorylated form of ERK_{1/2}, we found that the ERK₂ isoform was activated by CRF alone. BDNF itself produced a strong activation of ERK_{1/2}, which was enhanced further by a concomitant application of CRF (Fig. 7B). In each case, the activation of ERKs was prevented by PD98059, a selective inhibitor of MEK, a MAPK kinase immediately upstream of ERK_{1/2}. In addition, MEK inhibition by PD98059 prevented induction of TH⁺ neurons by CRF and 8-Br-2'-O-Me-cAMP in the presence of BDNF. The effect of BDNF was resistant, however, to PD98059 regardless of whether CRF was present or not, suggesting that the activation of ERK_{1/2} was only crucial for the effect of CRF in the presence of the neurotrophin (Fig. 7B).

The effect of CRF results from the amplification of a BDNF-dependent mechanism

The fact that BDNF was required for the effect of CRF suggested that a common BDNF-dependent mechanism was activated downstream of ERK_{1/2} signaling. This was confirmed by the fact that an antibody that neutralized the biological activity of BDNF (MAB-248) was also able to prevent the combined effect produced by CRF and BDNF on TH⁺ neurons (Fig. 8A). This prompted us to determine whether the population of TH⁺ neurons induced by CRF also expressed functional BDNF receptors. Immunofluorescent analysis of the cultures with an

antibody raised against an extracellular epitope of the receptor (Meyer-Franke et al., 1998), showed that virtually 93% (131/140) of the TH⁺ neurons exposed to CRF/BDNF expressed TrkB on the plasma membrane (Fig. 8B). The proportion was similar (101/105; 96%) in the presence of BDNF alone. CRF did not, however, simply stimulate the synthesis of this receptor since semi-quantitative RT-PCR analysis revealed that the corresponding transcripts were not increased in BDNF-treated LC cultures exposed to CRF or FK (Fig. 8C).

The PI3K/Akt signaling pathway is involved in several of the effects of BDNF mediated by TrkB receptors (Patapoutian and Reichardt, 2001). Therefore, we examined whether this pathway was also involved in the effect of CRF in BDNF-treated NA cultures. Because the PI3K/Akt signaling pathway can also be activated by insulin (Kermer et al., 2000), which is present in our culture medium, western blot analysis of the phosphorylated (activated) form of Akt (pAkt) was performed using LC cultures maintained in a culture medium in which insulin was omitted. The absence of insulin did not reduce the trophic effect of BDNF alone or BDNF/CRF (not shown). In these conditions, basal activation of Akt was low but still detectable (Fig. 9A). In the presence of BDNF or BDNF/CRF a strong activation of Akt was observed. The signal was specific since the PI3K inhibitor LY294002 strongly reduced activation of Akt in all three experimental conditions (Fig 9A). Interestingly, detection of pAkt at the cellular level by immunofluorescence confirmed that the phosphorylation of the protein was induced in a large number of TH⁺ neurons exposed to BDNF and CRF, and that LY294002 was able to prevent this effect (Fig 9B). Most interestingly, LY294002 prevented the induction of TH⁺ neurons in the cultures by BDNF alone or in the presence of CRF (Fig. 9C). Note that ERK activation, which is crucial for the CRF effect, was unaffected by LY294002 treatment (data not shown), which indicates that the role of the PI3K/Akt pathway in the induction of NA neurons was restricted to BDNF-dependent signaling via TrkB

receptors. A schema summarizing all demonstrated steps of CRF and BDNF activation is provided in Fig. 10.

DISCUSSION

We demonstrate here that the neuropeptide CRF powerfully stimulates the induction of NA neurons in LC cultures. Importantly, the effect of CRF required the activation of CRF-1 receptors and the presence of TrkB receptor ligands such as BDNF or NT-4. CRF but not BDNF operated through a cAMP-dependent mechanism that served to activate the ERK_{1/2} signaling pathway. Downstream of ERK_{1/2} activation by CRF, however, the induction of NA neurons relied upon the stimulation of the PI3K/Akt transduction pathway by BDNF.

CRF induces a NA phenotype in the presence of BDNF in LC cultures

Consistent with previous studies, we found that BDNF had the capacity of increasing the number of TH⁺ neurons in LC cultures (Holm et al., 2003; Traver et al., 2005). CRF, which was inactive by itself, greatly improved the trophic effect of BDNF. LC cultures exposed to BDNF alone or in the presence of CRF contained not only transcripts of TH, as expected from immunocytochemical detection studies, but also of the NA synthetic enzyme DBH whereas transcripts of PNMT, the enzyme that converts NA into adrenaline, were absent. This demonstrated that TH⁺ neurons with a NA phenotype were present in these cultures. In fact, the vast majority of the TH⁺ cells induced by BDNF or BDNF/CRF were probably NA neurons, since (1) intraneuronal accumulation of [³H]-NA in these cultures was prevented by a concentration of desipramine that selectively inhibits the NA transporter localized exclusively on NA neurons, and (2) for each treatment condition, the number of cells accumulating [³H]-NA was of the same order as the number of cells immunopositive for TH. Interestingly, the effect of CRF was apparently restricted to TH⁺ neurons that originate from

the LC. Indeed, CRF neither increased the number of TH⁺ cells nor it induced DBH transcripts in mesencephalic cultures.

CRF operates via a cAMP-dependent mechanism involving CRF-1 receptors

The effect of CRF but not that of BDNF was prevented by astressin, a non-selective CRF-1/CRF-2 receptor antagonist (Bale and Vale, 2004). However, only CRF-1 receptor transcripts were detectable in LC cultures suggesting that this receptor subtype was solely responsible for the CRF effect on TH⁺ neurons. The activator of the adenylate cyclase FK and the permeant analog of cAMP 8-Br-2'-O-Me-cAMP both mimicked the effect of CRF in the presence of BDNF, which indicates that the action of CRF was also probably mediated by cAMP. This is consistent with the positive coupling of CRF receptors to the G_s protein-adenylate cyclase pathway (Bale and Vale, 2004). It also corroborates previous reports suggesting that cAMP was crucially involved the development and plasticity of LC NA neurons (Skclair-Tavron and Segal, 1993; Reiriz et al., 2002; Rusnak and Gainer, 2005). Most importantly, the effect mediated via CRF-1 receptors required concurrent activation of a specific Trk receptor. Indeed, CRF also increased the number of TH⁺ neurons if added to LC cultures with NT-4, another TrkB ligand but not with NGF or NT-3, two neurotrophins which interact preferentially with TrkA and TrkC, respectively (Patapoutian and Reichardt, 2001).

Origin of the TH⁺ neurons induced by CRF

The induction of TH⁺ neurons by CRF in the presence of BDNF may possibly result from: (1) the rescue of a subpopulation of TH⁺ cells for which CRF was a survival factor, as described previously (Radulovic et al., 2003); (2) a mitogenic effect of CRF on BDNF-induced TH⁺ cells via cAMP-dependent signaling (Ha et al., 2000); (3) the restoration of a phenotype lost transiently as the result of cell suffering or lack of appropriate trophic stimulation during the

processing of the embryonic tissue; (4) the recruitment of post-mitotic neurons which have not yet completed their differentiation. Survival promotion is unlikely to account for the effect of CRF. Indeed, CRF had no effect by itself and remained highly effective when its application was delayed provided that BDNF was applied shortly after plating. The early requirement of BDNF indicates, however, that part of the effect of the neurotrophin may be to protect LC cells that did not yet express TH as suggested previously (Holm et al., 2003). CRF is also unlikely to be mitogenic since we were unable to detect a single [³H]-thymidine-positive nucleus in the population of LC TH⁺ neurons induced by CRF and BDNF. The restoration of a NA phenotype is more plausible, since cAMP stimulates TH and DBH gene expression (Kim et al., 1994; Lim et al., 2000). Alternatively, CRF could induce the NA phenotype in a population of LC neurons that requires a cAMP-dependent signal for complete specification. This would imply, however, that BDNF was also crucially involved in this process. The two latter explanations may both apply to our model system.

The CRF-dependent mechanism results from the activation of ERK_{1/2} by cAMP

Since cAMP mediates the effects of CRF as the result of CRF-1 receptor activation, we investigated potential downstream targets of the cyclic nucleotide. ERK_{1/2} are part of a MAPK signaling pathway that can be activated by cAMP (Troade et al., 2002; Christensen et al., 2003). CRF induced a modest but significant activation of ERK_{1/2} when applied alone to the cultures. It also amplified the activation of ERK_{1/2} by BDNF, suggesting that the trophic effect of CRF was perhaps mediated through a ERK-dependent mechanism as reported previously (Cibelli et al., 2001). As expected, inhibition by PD98059 of MEK, the immediate upstream kinase of ERK_{1/2}, prevented ERK_{1/2} activation regardless of the treatments. PD98059 also abolished the trophic effect of CRF revealed in the presence of BDNF, but did not reduce the effect of BDNF itself. This indicates that ERK_{1/2} activation was involved exclusively in the

effect of CRF. This activation could occur through two proximal targets of cAMP: PKA and the guanine nucleotide exchange factor Epac (Mayr and Montminy, 2001; Bos et al., 2003). Epac was probably involved for two reasons: (1) The 2'-O-alkyl modified cAMP analog 8-Br-2'-O-Me-cAMP, a selective activator of Epac (Christensen et al., 2003) which does not activate PKA, mimicked the trophic action of CRF in the presence of BDNF; (2) The effects of both CRF and 8-Br-2'-O-Me-cAMP were abolished by PD98059 and resistant to the PKA inhibitor H-89. Finally, the fact that ERK_{1/2} activation produced by BDNF was not required for its effect on TH⁺ neurons suggests that different ERK_{1/2} substrates are phosphorylated depending on whether the activation occurs initially via TrkB or CRF-1 receptors.

CRF-induced ERK_{1/2} activation reinforces a BDNF-dependent mechanism

Our results demonstrate that the presence of BDNF is a prerequisite for the trophic effect of CRF on NA neurons. This explains why the activation of the PI3K/Akt signaling cascade which was critical for the effect of BDNF alone, was also essential for its cooperative action with CRF. This also indicates that PI3K activation by BDNF intervened probably downstream of the ERK_{1/2}-dependent mechanism crucial for the effect of CRF. Accordingly, the activation of ERK_{1/2} in CRF/BDNF-treated cultures was unaffected by PI3K inhibition (not shown). The key role of BDNF in the effect of CRF, may indicate that CRF served to reach optimal concentrations of the neurotrophin in the culture medium possibly via cAMP-dependent signaling (Goodmann et al., 1996). This is unlikely, however, since we used a saturating concentration of BDNF that already produced optimal effects on TH⁺ neurons (not shown). Increased synthesis of TrkB receptors by CRF might also explain the potentiation of the effects of BDNF but neither CRF nor FK increased the expression of TrkB transcripts in LC cultures. One cannot exclude, however, alternative mechanisms, such as transactivation of TrkB receptors (Rajagopal et al., 2004) or their recruitment to the plasma membrane.

Transactivation of Trk receptors in response to G-protein-coupled receptor signaling occurs generally in the absence of Trk ligand (Rajagopal et al., 2004). Such a mechanism is unlikely since the presence of BDNF was always required for the action of CRF. On the other hand, the possibility that CRF facilitated the recruitment of an intracellular pool of TrkB receptors to the plasma membrane is feasible. Indeed, cAMP has this effect in other model systems (Meyer-Franke et al., 1998; Nagappan and Lu, 2005). Consistent with this hypothesis, we observed that virtually all of the TH⁺ neurons induced by CRF treatment expressed TrkB receptors at the plasma membrane, suggesting that they acquired their NA phenotype by becoming responsive to the neurotrophin. Because TrkB ligands are believed to regulate the expression of Phox2a (Holm et al., 2003), a homeodomain transcription factor required for the development of LC NA neurons (Hirsch et al., 1998), Phox2a may conceivably represent an indirect target of CRF via the BDNF-dependent pathway.

Taken together, our results suggest that the phenotype of LC NA neurons can be controlled during development by CRF provided that BDNF is also present. The plasticity of these neurons is apparently not restricted to the developmental period (Bezin et al., 2000) since dormant NA neurons in the LC of the adult brain can also re-express NA traits when stimulated by appropriate treatments in vivo (Bezin et al., 2000). The possible involvement of CRF and BDNF in this process remains a subject for further study.

REFERENCES

Baigent SM and Lowry PJ (2000) mRNA expression profiles for corticotrophin-releasing factor (CRF), urocortin, CRF receptors and CRF-binding protein in peripheral rat tissues. *J Mol Endocrinol.* 25:43-52.

Bale TL and Vale WW (2004) CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu Rev Pharmacol Toxicol.* 44:525-557.

Berridge CW and Waterhouse BD. (2003) The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. *Brain Res Rev.* 42: 33-84.

Bezin L., Marcel D, Desgeorges S, Pujol JF and Weissmann D (2000) Singular subsets of locus coeruleus neurons may recover tyrosine hydroxylase phenotype transiently expressed during development. *Mol Brain Res.* 76:275-281.

Bos JL (2003) Epac: a new cAMP target and new avenues in cAMP research. *Nat Rev Mol Cell Biol.* 4:733-738

Carrasco GA Van de Kar LD (2003) Neuroendocrine pharmacology of stress. *Eur J Pharmacol.* 463; 235-272.

Chen Y, Bender RA, Brunson KL, Pomper JK, Grigoriadis DE, Wurst W and Baram TZ (2004) Modulation of dendritic differentiation by corticotropin-releasing factor in the developing hippocampus. *Proc Natl Acad Sci U S A.* 101:15782-15787.

Christensen AE, Selheim F, de Rooij J, Dremier S, Schwede F, Dao KK, Martinez A, Maenhaut C, Bos JL, Genieser HG and Doskeland SO. (2003) cAMP analog mapping of Epac1 and cAMP kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. *J Biol Chem.* 278:35394-35402

Cibelli G, Corsi P, Diana G, Vitiello F and Thiel G. (2001) Corticotropin-releasing factor triggers neurite outgrowth of a catecholaminergic immortalized neuron via cAMP and MAP kinase signalling pathways. *Eur J Neurosci.* 13:1339-1348

Ge Y, Belcher SM and Light KE (2004) Alterations of cerebellar mRNA specific for BDNF, p75NTR, and TrkB receptor isoforms occur within hours of ethanol administration to 4-day-old rat pups. *Dev Brain Res.* 151 : 99-109.

Goodman LJ, Valverde J, Lim F, Geschwind MD, Federoff HJ, Geller AI, Hefti F (1996) Regulated release and polarized localization of brain-derived neurotrophic factor in hippocampal neurons. *Mol Cell Neurosci.* 7:222-238.

Ha BK, Bishop GA, King JS and Burry RW (2000) Corticotropin releasing factor induces proliferation of cerebellar astrocytes. *J Neurosci Res.* 62:789-798.

Hanson MG Jr, Shen S, Wiemelt AP, McMorris FA and Barres BA. (1998) Cyclic AMP elevation is sufficient to promote the survival of spinal motor neurons in vitro. *J Neurosci.* 18:7361-7371.

Hirsch MR, Tiveron MC, Guillemot F, Brunet JF and Golidis C (1998) Control of

noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system. *Development*. 125:599-608.

Holm PC, Rodriguez FJ, Kresse A, Canals JM, Silos-Santiago I and Arenas E. (2003) Crucial role of TrkB ligands in the survival and phenotypic differentiation of developing locus coeruleus noradrenergic neurons. *Development*. 130:3535-3545.

Jedema HP and Grace AA (2004) Corticotropin-releasing hormone directly activates noradrenergic neurons of the locus ceruleus recorded in vitro. *J Neurosci*. 24:9703-9713.

Kermer P, Klocker N, Labes M and Bahr M. (2000) Insulin-like growth factor-I protects axotomized rat retinal ganglion cells from secondary death via PI3-K-dependent Akt phosphorylation and inhibition of caspase-3 In vivo. *J Neurosci*. 20:2-8.

Kim KS, Ishiguro H, Tinti C, Wagner J and Joh TH. (1994) The cAMP-dependent protein kinase regulates transcription of the dopamine beta-hydroxylase gene. *J Neurosci*. 14:7200-7207.

Lim J, Yang C, Hong SJ and Kim KS. (2000) Regulation of tyrosine hydroxylase gene transcription by the cAMP-signaling pathway: involvement of multiple transcription factors. *Mol Cell Biochem*. 212:51-60.

Marien MR, Colpaert FC and Rosenquist AC. (2004) Noradrenergic mechanisms in neurodegenerative diseases: a theory. *Brain Res Rev*. 45: 38-78.

Mayr B and Montminy M. (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB *Nat Rev Mol Cell Biol.* 2:599-609.

Meier E, Hertz L and Schousboe A. (1991) Neurotransmitters as developmental signals. *Neurochem. Int.* 19: 1-15.

Meyer-Franke A, Wilkinson GA, Kruttgen A, Hu M, Munro E, Hanson MG Jr, Reichardt LF and Barres BA. (1998) Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. *Neuron.* 21: 681-693.

Michel PP, Ruberg M, and Agid Y (1997) Rescue of mesencephalic dopamine neurons by anticancer drug cytosine arabinoside. *J Neurochem.* 69:1499-1507.

Nagappan G and Lu B (2005) Activity-dependent modulation of the BDNF receptor TrkB: mechanisms and implications. *Trends Neurosci.* 28: 464-471.

Patapoutian A and Reichardt LF (2001) Trk receptors: mediators of neurotrophin action. *Curr Opin Neurobiol.* 11:272-280

Prasad BM and Amara SG. (2001) The dopamine transporter in mesencephalic cultures is refractory to physiological changes in membrane voltage. *J Neurosci.* 21:7561-7567.

Radulovic M, Hippel C and Spiess J (2003) Corticotropin-releasing factor (CRF) rapidly suppresses apoptosis by acting upstream of the activation of caspases. *J Neurochem* 84:1074-1085

Rajagopal R, Chen ZY, Lee FS and Chao MV (2004) Transactivation of Trk neurotrophin receptors by G-protein-coupled receptor ligands occurs on intracellular membranes. *J Neurosci.* 24:6650-6658.

Reiriz J, Holm PC, Alberch J and Arenas E. (2002) BMP-2 and cAMP elevation confer locus coeruleus neurons responsiveness to multiple neurotrophic factors. *J Neurobiol.* 50:291-304.

Rusnak M and Gainer H. (2005) Differential effects of forskoline on tyrosine hydroxylase gene transcription in identified brainstem catecholaminergic neuronal subtypes in organotypic culture. *Eur J Neurosci.* 21:889-898.

Sakurada K, Ohshima-Sakurada M, Palmer TD and Gage FH. (1999) Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. *Development* 126:4017-4026.

Sauvage M and Steckler T (2001) Detection of corticotropin-releasing hormone receptor 1 immunoreactivity in cholinergic, dopaminergic and noradrenergic neurons of the murine basal forebrain and brainstem nuclei: potential implication for arousal and attention. *Neuroscience*, 1004, 643-652

Sklair-Tavron L and Segal M (1993) Neurotrophic effects of cAMP generating systems on central noradrenergic neurons. *Brain Res.* 614:257-269.

Specht LA, Pickel VM, Joh TH and Reis DJ (1981) Light-microscopic immunocytochemical

localization of tyrosine hydroxylase in prenatal rat brain. I. Early ontogeny. *J Comp Neurol.* 199:233-253

Traver S, Salthun-Lassalle B, Marien M, Hirsch EC, Colpaert F and Michel PP. (2005) The neurotransmitter noradrenaline rescues septal cholinergic neurons in culture from degeneration caused by low-level oxidative stress. *Mol Pharmacol.* 67:1882-1891

Troade JD, Marien M, Mourlevat S, Debeir T, Ruberg M, Colpaert F and Michel PP. (2002) Activation of the mitogen-activated protein kinase (ERK_{1/2}) signaling pathway by cyclic AMP potentiates the neuroprotective effect of the neurotransmitter noradrenaline on dopaminergic neurons. *Mol Pharmacol.* 62: 1043-1052.

Unsworth BR, Hayman GT, Carroll A and Lelkes PI. (1999) Tissue-specific alternative mRNA splicing of phenylethanolamine N-methyltransferase (PNMT) during development by intron retention. *Int J Dev Neurosci.* 17:45-55

Van Bockstaele EJ, Colago EE and Valentino RJ (1996) Corticotropin-releasing factor-containing axon terminals synapse onto catecholamine dendrites and may presynaptically modulate other afferents in the rostral pole of the nucleus locus coeruleus in the rat brain. *J Comp Neurol.* 364:523-534.

Zarow C, Lyness SA, Mortimer JA and Chui HC (2003) Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. *Arch Neurol.* 60:337-341.

FOOTNOTES

This work was supported by Centre de Recherche Pierre Fabre and Institut National de la Santé et de la Recherche Médicale (INSERM). We are grateful to Merle Ruberg and Stéphane Hunot for their helpful comments on the manuscript, and to Audrey Hild for technical assistance.

Address correspondence to : Dr Patrick Michel, INSERM U679, Experimental Neurology and Therapeutics, Bâtiment Pharmacie, Hôpital de la Salpêtrière, 47 bd de l'Hôpital, 75013 Paris, France.

FIGURE LEGENDS

Figure 1: CRF stimulates the phenotypic differentiation of NA neurons in BDNF-treated LC cultures. **A**, Number of TH⁺ neurons in LC cultures as a function of the age of the culture. The cultures were treated with either BDNF or CRF or both factors concurrently, as indicated in the legend within the figure. **B**, Number of TH⁺ neurons as a function of the concentrations of CRF (0-2 μ M) in 3 DIV BDNF-treated LC cultures. **C**, RT-PCR analysis of the three enzymes of catecholamine synthesis, TH, DBH and PNMT in 6 DIV control, BDNF and BDNF/CRF-treated LC and mesencephalic cultures. Adult rat adrenal medulla tissue was used as positive control for the dopaminergic and adrenergic systems, respectively. In each lane, equal loading for each condition was controlled by using GAPDH amplification. **D**, Number of TH⁺ neurons in 3 DIV LC and mesencephalic cultures treated with either BDNF or CRF or both factors added in combination. BDNF: 20 ng/mL; CRF: 1 μ M (except in B). * $p < 0.05$, compared to corresponding control cultures. ** $p < 0.05$, compared to corresponding BDNF-treated cultures.

Figure 2: Effects of CRF and BDNF in LC cultures. **A-D**, Detection of TH-positive cells in control LC cultures (A) or in cultures exposed for 8 DIV to BDNF (B), BDNF/CRF (C) or CRF (D). **E-F**, Microautoradiographic detection of [³H]-thymidine-positive nuclei in 3 DIV BDNF/CRF-treated cultures. **E**, TH-positive cells (red label) did not incorporate the tritiated label (light blue). **F**, [³H]-thymidine-positive nuclei were found generally in GFAP⁺ cells (green label). Scale bars = 15 μ m (A-D) and 25 μ m (E-F). BDNF: 20 ng/mL; CRF: 1 μ M

Figure 3: Treatment of LC cultures with CRF induces a functional NA transporter. **A-C**, Comparison of the effects of a chronic treatment with BDNF or BDNF/CRF using either

TH immunodetection (A) or quantification of [³H]-NA uptake (B,C). In (B) the efficacy of the uptake was determined by counting the number [³H]-NA⁺ cells observed after microautoradiographic detection, whereas in (C) accumulation was quantified by liquid scintillation spectrometry. **D**, Microautoradiographic visualization of NA uptake in LC cultures exposed chronically to BDNF, BDNF/CRF or no treatment. The specificity of the uptake was determined in the presence of desipramine. Black arrows point to positive fibers in control cultures. White arrowheads show [³H]-NA-positive cell bodies. All cultures were used at 8 DIV. Scale bar: 30 μm. * p < 0.05, compared to corresponding BDNF-treated cultures. BDNF: 20 ng/mL; CRF: 1 μM; desipramine: 0.1 μM.

Figure 4: The effect of CRF depends on the time at which BDNF is first applied. LC cultures were exposed to BDNF (20 ng/mL) and/or CRF (1 μM) using treatment paradigms indicated in the figure and the number of TH⁺ cells was assessed at 6 DIV. Note that CRF remained highly effective even when applied after a 3-day delay, but only if the cultures had been in early contact with BDNF. * p < 0.05, compared to control cultures. ** p < 0.05, compared to BDNF-treated cultures.

Figure 5: CRF cooperates with TrkB but not TrkA or TrkC ligands. Number of TH⁺ neurons in 3 DIV LC cultures treated chronically with the TrkA (NGF), TrkB (BDNF, NT-4) or TrkC (NT-3) ligands, alone or in the presence of CRF. * p < 0.05, compared to control cultures. ** p < 0.05, compared to cultures treated with the corresponding TrkB ligand alone. All Trk ligands were used at 20 ng/mL. CRF: 1 μM.

Figure 6: CRF-1 receptors mediate the increase in TH⁺ cells produced by CRF. **A**, Number of TH⁺ neurons in 3 DIV LC cultures exposed to CRF (1 μM) or Ucn (1 μM) in the

presence or absence of BDNF (20 ng/mL). The CRF receptor antagonist astressin (1 μ M) prevented the effect of CRF and Ucn, but not that of BDNF, on TH⁺ cells. Similar to CRF or Ucn, the adenylate cyclase activator FK (20 μ M) amplified the effect of BDNF. * $p < 0.05$, compared to corresponding BDNF-treated cultures, ** $p < 0.05$, compared to corresponding cultures not exposed to astressin. **B**, RT-PCR amplification of the CRF-1 and CRF-2 receptor transcripts were resolved by electrophoresis on 1% agarose gels. Template mRNA were from 6 DIV LC cultures exposed to the treatments as indicated or from adult rat adrenal medulla (AM).

Figure 7: CRF operates via a cAMP-dependent signaling pathway that involves activation of ERK_{1/2}. **A**, Number of TH⁺ neurons in 3 DIV LC cultures exposed to BDNF, CRF, or the specific activator of Epac, 8-bromo-2'-O-methyl-cAMP in the presence or absence of BDNF. The inhibitor of MEK, PD98059, but not the inhibitor of PKA, H89, prevented the increase in TH⁺ cells produced by CRF or 8-bromo-2'-O-methyl-cAMP in BDNF-treated cultures. * $p < 0.05$, compared to corresponding BDNF-treated cultures, ** $p < 0.05$, compared to corresponding cultures without inhibitor. **B**, Western blot analysis of pERK_{1/2} in 1 DIV LC cultures exposed acutely to the test treatments as indicated in the legend within the figure. BDNF and CRF were added for 10 min and the treatment with PD98059 was initiated 30 min earlier. Protein loading was controlled by staining blots with an antibody against total-ERK_{1/2}. BDNF: 20 ng/mL; CRF: 1 μ M; 8-bromo-2'-O-methyl-cAMP: 500 μ M; PD98059: 20 μ M; H89: 3 μ M.

Figure 8: Role of BDNF and TrkB receptors in the effects of CRF. **A**, Number of TH⁺ neurons in 3 DIV LC cultures exposed chronically to BDNF or BDNF/CRF in the presence or the absence of the neutralizing anti-BDNF antibody MAB-248 (10 μ g/mL). * $p < 0.05$,

compared to BDNF-treated cultures. ** $p < 0.05$, compared to corresponding cultures exposed to the same treatments without the antibody. **B**, Immunofluorescent detection of TrkB receptors (green) and TH (red) in 3 DIV CRF/BDNF-treated cultures. Scale bar = 15 μm . **C**, RT-PCR analysis of TrkB receptors in cultures exposed to CRF, BDNF, BDNF/CRF, FK and BDNF/FK or no treatment (Con). In each lane, equal loading was controlled by GAPDH amplification. BDNF: 20 ng/mL; CRF: 1 μM ; FK: 20 μM .

Figure 9: Role of the PI3K/Akt signaling pathway in the cooperative effect of CRF and BDNF. **A**, Western blot of p-Akt in 1 DIV cultures exposed to BDNF or BDNF/CRF in the presence or the absence of the PI3K inhibitor LY294002. The cultures were maintained in an insulin-free medium and BDNF and CRF were applied for 10 min. The PI3K inhibitor LY294002 was added 30 min prior to initiating the treatments. Protein loading was controlled by staining blots with an antibody against total-Akt. **B**, Double immunofluorescence detection of p-Akt (green) and TH (red) in 3 DIV LC cultures treated chronically with BDNF/CRF in the presence or absence of LY294002. Scale bars = 20 μm (top and bottom panels) and 10 μm (middle panel). **C**, Number of TH⁺ neurons in 3 DIV culture exposed to BDNF or BDNF/CRF in the presence or the absence of LY294002. BDNF: 20 ng/mL; CRF: 1 μM ; LY294002: 10 μM . * $p < 0.05$, compared to BDNF-treated cultures. ** $p < 0.05$, compared to corresponding cultures exposed to the same treatments without the inhibitor LY294002.

Figure 10: Schematic representation of the mechanisms that may control the acquisition of the NA phenotype in LC neurons. **A**, A subset of LC neurons acquires the NA phenotype by a mechanism controlled by BDNF and its cognate receptor TrkB leading to activation of PI3K/Akt-dependent signaling. The effect of BDNF can be prevented either by treatment with a monoclonal antibody (MAB-248) that neutralizes the biological activity of the trophic

peptide or with LY294002 which blocks PI3K activity. It is mimicked by another TrkB ligand NT-4. **B**, A distinct population of LC neurons can also acquire the NA phenotype by becoming responsive to BDNF through a mechanism that requires activation of CRF-1 receptors by CRF or Ucn. Astressin, a competitive antagonist at the CRF receptor site prevents the action of both CRF and Ucn but has no effect against BDNF. The effect of CRF occurs via activation of a cAMP-dependent signaling pathway that involves the guanine nucleotide exchange factor EPAC and subsequently ERK_{1/2} signaling. Note that the effects of both CRF and 8-Br-2'-O-Me-cAMP, a selective activator of EPAC, which mimics the effect of CRF, are resistant to PKA inhibition by H89. The precise mechanism by which CRF-induced ERK_{1/2} activation makes LC neurons responsive to BDNF remains to be determined but the recruitment of an intracellular pool of TrkB receptors to the plasma membrane is a candidate mechanism. PD98059: inhibitor of MEK, the immediate upstream activator kinase of ERK_{1/2}.

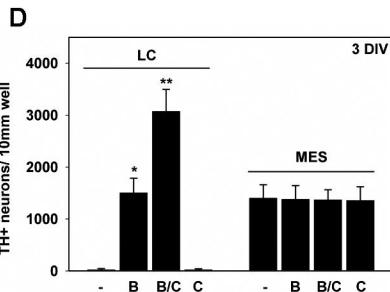
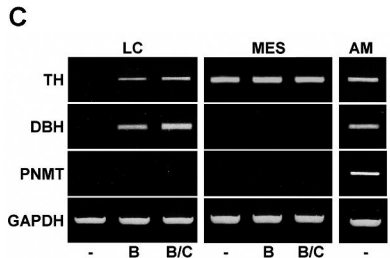
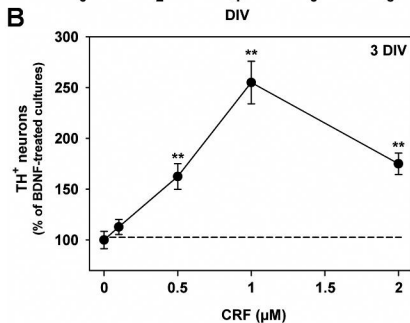
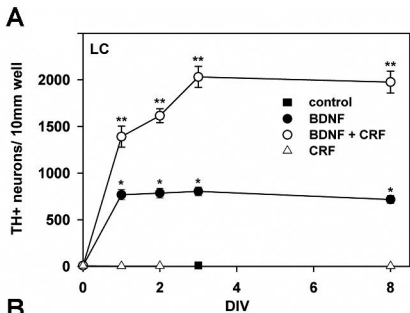
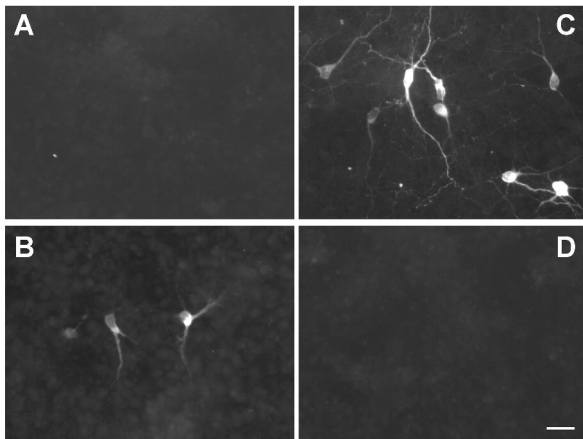


Figure 1

TH+



TH+ / [³H]-thym⁺

GFAP+ / [³H]-thym⁺

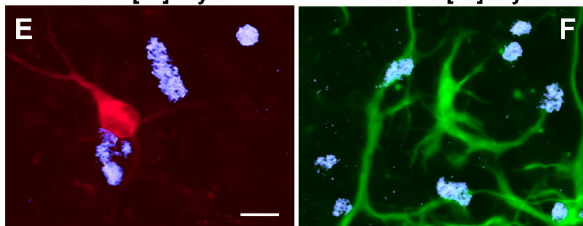


Figure 2

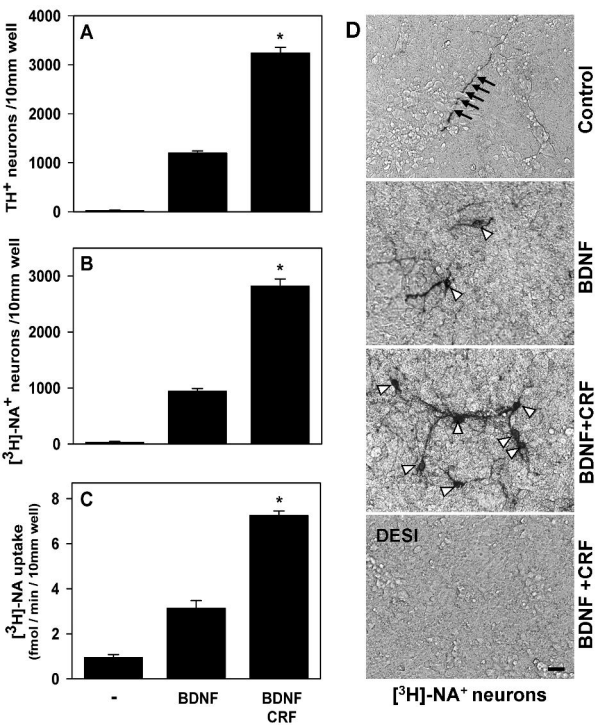


Figure 3

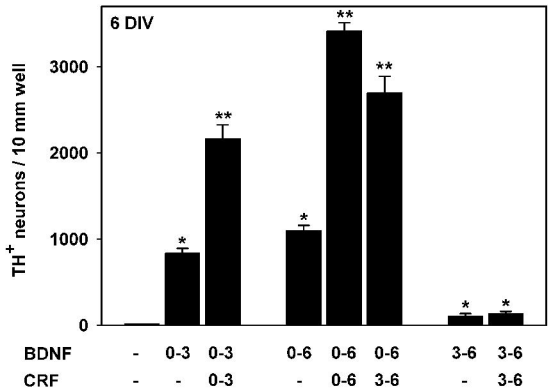


Figure 4

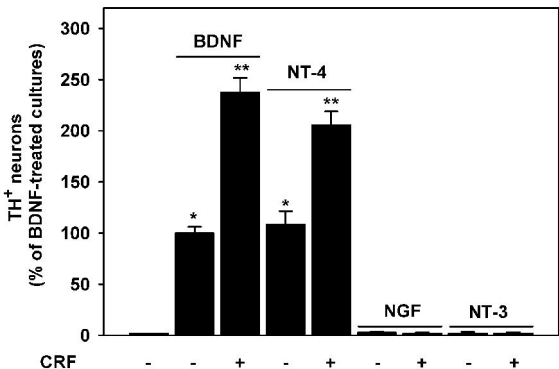


Figure 5

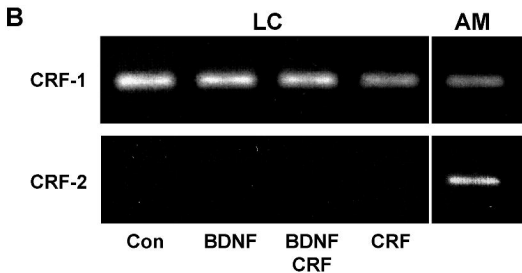
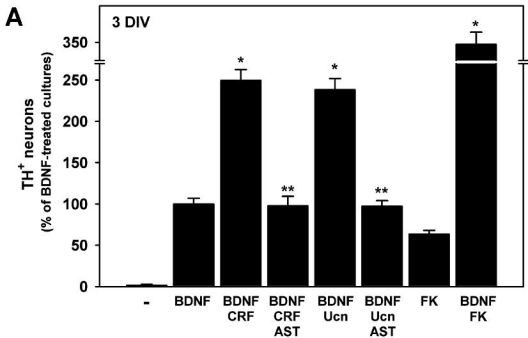


Figure 6

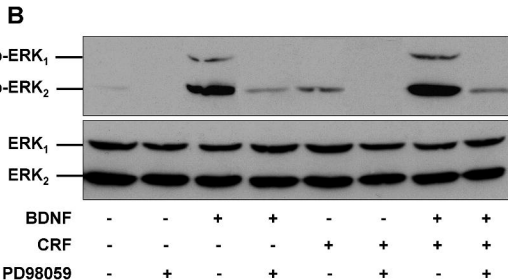
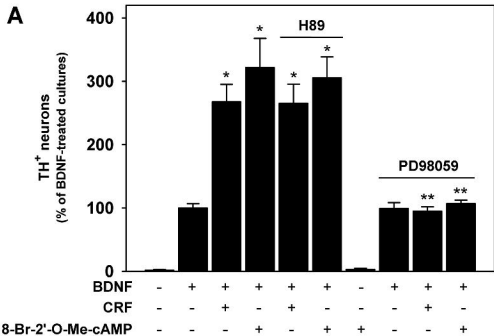


Figure 7

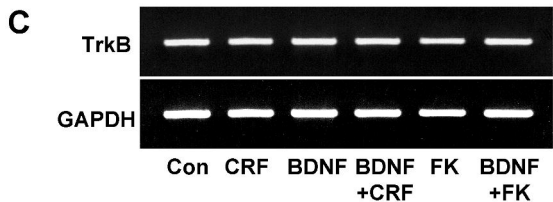
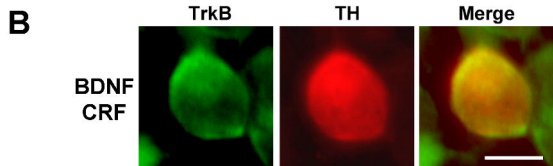
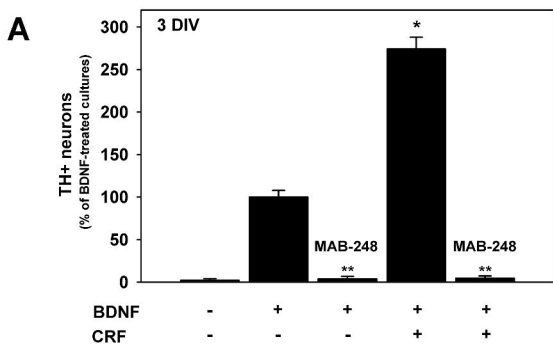


Figure 8

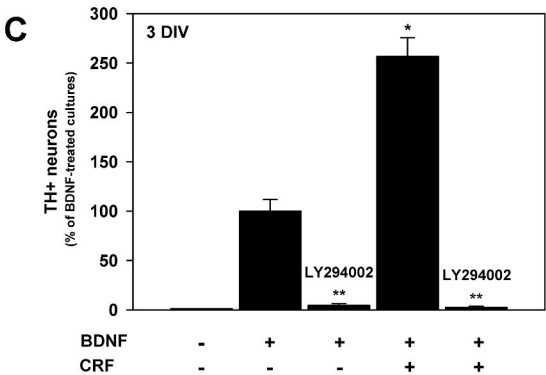
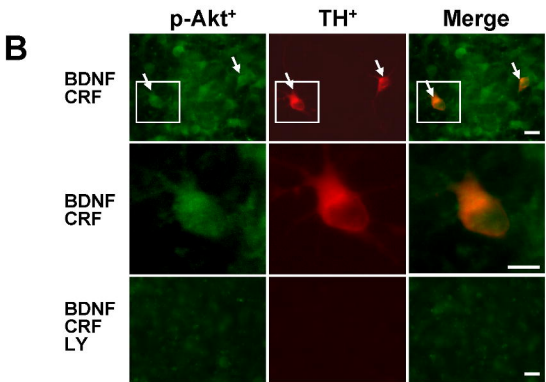
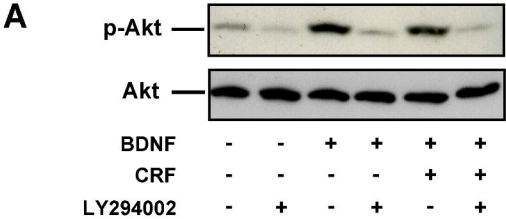


Figure 9

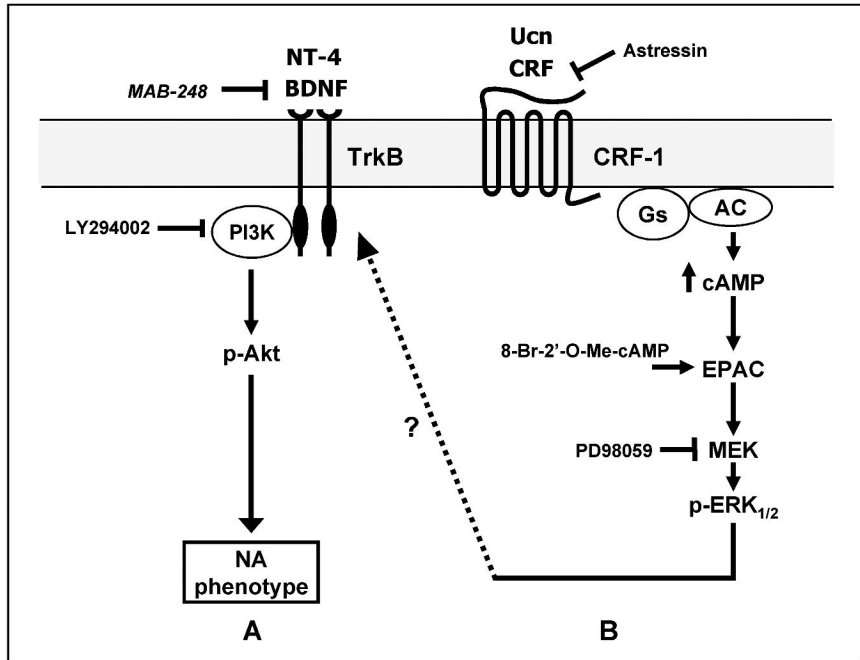


Figure 10