NOS induction by NGF in basal forebrain cholinergic neurones: evidence for regulation of brain NOS by a neurotrophin

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Nerve growth factor (NGF) acts through trkA receptors to serve as a trophic factor for cholinergic neurones in the medial septal nucleus (MSN) and vertical limb of the diagonal band (VDB). Herein, we show that brain nitric oxide synthase (NOS), which synthesizes the neuromodulator nitric oxide, is selectively expressed in a large fraction of trkA-containing neurones in the MSN and VDB. Axotomy of these neurones gave evidence that NOS expressing cholinergic neurones innervate the hippocampus. NGF infusion induced a robust, specific increase in NOS expression in basal forebrain cholinergic neurones. These results indicate that brain NOS can be regulated by a neurotrophic factor and suggest that NGF influences forebrain function by regulating production of nitric oxide as well as acetylcholine.

Summary

Nerve growth factor (NGF) acts through trkA receptors to serve as a trophic factor for cholinergic neurones in the medial septal nucleus (MSN) and vertical limb of the diagonal band (VDB). Herein, we show that brain nitric oxide synthase (NOS), which synthesizes the neuromodulator nitric oxide, is selectively expressed in a large fraction of trkA-containing neurones in the MSN and VDB. Axotomy of these neurones gave evidence that NOS expressing cholinergic neurones innervate the hippocampus. NGF infusion induced a robust, specific increase in NOS expression in basal forebrain cholinergic neurones. These results indicate that brain NOS can be regulated by a neurotrophic factor and suggest that NGF influences forebrain function by regulating production of nitric oxide as well as acetylcholine.

Key words

NOS induction by NGF in basal forebrain cholinergic neurones, nerve growth factor, neurotrophic factor, neurotrophin, nitric oxide synthase, trkA

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Introduction

Nitric oxide (NO) is a recently described messenger molecule in the brain (for review see Bredt & Snyder 1994). Many NO effects are mediated by its ability to increase cGMP levels. In neurones within the CNS, NO is produced by a brain specific form of (NOS) (Bredt et al. 1991, Bredt & Snyder 1994). This enzyme catalyses the synthesis of NO from L-arginine via a Ca2+/calmodulin-dependent mechanism. The catalytic activity of brain NOS accounts for the nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) staining of NOS containing neurones and their fibres in the CNS (Dawson et al. 1991, Vincent & Hope 1992). Although there is an abundance of NOS containing fibres in many brain regions, NOS is localized to only a small percentage of neuronal cell bodies. A functional role for NOS has been suggested. Indeed there are data indicating that NO may act as a neurotransmitter to influence such processes as long term potentiation (LTP) in the hippocampus (Bohme et al. 1991, Schuman & Madison 1991) and long term depression in the cerebellum (Bredt & Snyder 1994). Although attractive, the data supporting these suggestions is incomplete. In hippocampus, the source of NO is as yet undefined. Studies of the localization of NOS expression and its regulation in the CNS would help to define the significance of NO production.

Basal forebrain cholinergic neurones appear to play a role in learning and memory (Coyle et al. 1983, Richardson & DeLong 1988, Olton et al. 1991), and recent work suggests that they express NOS. This was demonstrated by co-localization of NADPH-d staining and immunostaining for choline acetyltransferase (ChAT), the enzyme responsible for the synthesis of acetylcholine (ACH) (Brauer et al. 1991, Vincent & Hope 1992, Kitchener & Diamond, 1993). Nerve growth factor (NGF) is a trophic factor for basal forebrain cholinergic neurones in the (MSN) and vertical limb of the diagonal band (VDB). These neurones project to the hippocampus where NGF is produced (Korsching et al. 1991).
1985, Large et al. 1986, Shelton & Reichardt 1986, Whittemore et al. 1986). A large body of evidence suggests that NGF regulates ChAT as well as acetylcholine production in these neurons (for review see Longo et al. 1992, Rylett et al. 1993). Co-localization of NADPH-d staining with ChAT raised the possibility that NGF may also regulate NOS activity and NO production by these cells. If this was the case, it could provide a novel mechanism through which NGF could modify basal forebrain and hippocampal function.

NGF acts through a receptor tyrosine kinase, trkA, whose mRNA and protein is selectively localized in cholinergic neurons in the MSN and VDB (Holtzman et al. 1992a, Steininger et al. 1993). We sought to further explore a possible interaction between NGF and NOS in the septohippocampal system. Herein, we show that NOS is co-localized in the majority of trkA containing neurons in the MSN and VDB. Axotomy of these neurons led to a dramatic decrease in trkA- and NOS-containing fibres in the hippocampus, suggesting that these neurons provide at least a portion of the neuronally derived NO in the hippocampus. Importantly, exogenous NGF markedly and specifically increased NOS mRNA, protein and activity in these cells. Our results show that brain NOS is regulated in the CNS and that NGF may influence brain function through regulation of NO.

Methods

Animal procedures and NGF injection

The brains of adult (3–4 month old) Sprague-Dawley female rats were used for all studies. For animals injected with NGF or vehicle, an intraventricular cannula was implanted into the right lateral ventricle as described (Holtzman et al. 1993) with the following modifications. (1) Alzet model 2002 miniosmotic pumps (Alza Corp.) were used, (2) cannula placement was 0.2 mm anterior to bregma, 1.1 mm lateral to the midline and 5.0 mm ventral to the skull surface, and (3) flow rate was 0.5 µL, h⁻¹ (208 ng NGF h⁻¹, 5 µg NGF 24 h⁻¹). For fimbria-fornix lesions, rats were anesthetized and a complete unilateral aspirative lesion of the right fimbria-fornix and overlying cortex was performed as described (Gage et al. 1983, Holtzman et al. 1992b).

In situ hybridization histochemistry (ISHH) and data analysis

For NOS ISHH, sense and antisense [35S] riboprobes were generated from a full length rat brain NOS cDNA (Bredt et al. 1991) subcloned into Bluescript SK⁺. In situ hybridization histochemistry (ISHH) was performed under stringent conditions exactly as described (Cunningham & De Souza, 1993). To allow comparison between subjects, all sections were hybridized and processed under identical conditions. A positive cell was defined as one in which a cluster of grains was present which was greater than or equal to 2 standard deviations above background; the latter was the mean grain count over the same area in the corpus callosum. No specific signal was seen with the sense riboprobe. Grains were counted over all positive cells in three brain regions in two coronal sections approximately 0.2 mm anterior to bregma as described (Holtzman et al. 1992a). The sections chosen for quantification were based on their anatomical location as identified it low power magnification (4X objective) of H & E stained sections. In these sections, the limbs of the anterior commissure were separated by 4.2 mm. Grains were counted on both sides of the MSN, the right striatum, and the right cortex from one section and on both sides of the MSN, the left striatum, and the left cortex from the second section. For the MSN, only cells dorsal to a line drawn between the centre of the anterior commissure were examined. In each region, a constant circle size was used to surround postive cells. Quantification of grain clusters was carried out under epifluorescence illumination at 400X magnification. Image analysis was performed with the MCID image analysis system (St Catherines, Ontario) using a Leitz Aritsoplan microscope (Leica, Foster City, CA). Values were registered by the image analysis system as the number of pixels occupied by silver grains within the circle. For analysis, data for grain counts were computed as mean ± SEM of all cells measured per region. The values for NGF and vehicle–treated subjects were then converted to a percentage of the value in vehicle-treated animals. Student’s t-test was used for statistical analysis.

Immunohistochemistry, tissue staining and cell counting

For immunohistochemistry, tissue was processed as described (Holtzman et al. 1992a), 40-µm brain sections were cut on a freezing sliding microtome. For double labelled sections, tissue was processed for immunostaining alone or immunostaining followed by NADPH-d staining (Dawson et al. 1991). The antibody to trkA (RTA, used at 1/4000) is specific for trkA. It was raised in rabbits to the baculovirus expressed extracellular domain of rat trkA. On antigen blots, it recognizes trkA but does not recognize either trkB or trkC (Clary et al. 1994). The antibody to NOS was raised in rabbits against a 160kD band on Western blots from various bacterial cultures as described (Black et al. 1993) and samples were run on a 12% SDS–polyacrylamide gel. The band corresponding to brain NOS was excised from the gel, lyophilized and 150µg of partially purified protein was used for immunization of two rabbits. The polyclonal antiserum was used at 1/4000. This antibody recognizes a 160kD band on Western blots from various brain regions, a finding similar to that seen for another antibody to brain NOS (Bredt et al. 1991). Irs staining of CNS neuronal populations completely co-localizes with
NGF induces NOS in the CNS

NADPH-d staining. Two weeks following unilateral fimbria-fornix aspiration, rats were killed and tissue was processed as described (Holtzman et al. 1992). Every section from the genu of the corpus callosum rostrally to the crossing of the anterior commissure caudally was collected, and adjacent sections were stained in the following order: (1) with ChAT, (2) with ChAT followed by NADPH-d staining, (3) with NOS and (4) by NADPH-d histochemistry. Sections from the fimbria-fornix through the mid-portions of the hippocampus were collected for Cresyl violet, acetylcholinesterase (ACHe) or NADPH-d staining. Hippocampal sections were also collected for trkA and NOS immunostaining and some were intensified with silver (Holtzman et al. 1992b). Complete fimbria-fornix transection was denoted by a complete loss of ACHe fibres staining in the hippocampus ipsilateral to the lesion. To assess cell number in the MSN, all ChAT and NADPH-d stained neurones were counted in the right and left MSN in three sections (200-, 600- and 1000-µm anterior to the crossing of the anterior commissure). Cells in the MSN were counted (1) if they were superior to a line between the anterior commissures and within 0.75 mm of the midline and (2) if they had a nucleus or at least one neurite. Cell counts on the lesioned side were expressed as a percentage of the number of cells on the unlesioned side after correction for cell size (Abercrombie, 1946). A total of 150–200 ChAT and 100–150 NADPH-d positive cells were present in the unlesioned MSN in the three sections examined in each animal. To assess the number of NOS immunoreactive neurones in NGF and vehicle treated rats, sections encompassing the entire MSN (from the genu of the corpus callosum to the crossing of the anterior commissure) were processed for immunostaining. The number of NOS immunoreactive neurones in the MSN were counted in every third section and numbers compared after correction for cell size (Abercrombie, 1946, Holtzman et al. 1992b). For immunostaining, conditions and reactions times were identical for tissues from NGF- and vehicle-treated subjects.

RNA isolation and Northern Blot analysis

Total RNA was isolated from cells and tissue by the method of Chirgwin et al. (1979). PC12 cells (SPC12; Schubert 1978) were grown in Dulbecco’s modified Eagle’s medium (with 3.7 g L⁻¹ NaHCO₃, 4.5 g L⁻¹ glucose, 0.584 g L⁻¹ containing 10% horse serum, 5% fetal calf serum (FCS) and an additional 2 mM glutamine. Cells were fed three times per week and split every 2 weeks. At initiation of the experiment, the medium was changed in six 30% confluent, 10-cm plastic tissue culture dishes. It was replaced with the same medium as above except that horse serum was 2% and FCS was 1%. Mouse NGF, prepared by ion exchange chromatography as described (Mobley et al. 1986), was added to each of three plates at a concentration of 50 ng ml⁻¹. Cells were harvested at 48 h for preparation of total RNA. The cDNA probes were labelled via random priming and Northern blotting and densitometry were performed as described (Holtzman et al. 1992c). The cDNA probes used were: rat NOS, the 5 kb rat cDNA (Bredt et al. 1991) and 18S rRNA, a 1.1 kb mouse cDNA (Bowman et al. 1981) was used to control for RNA loading, as described (Holtzman et al. 1992c).

NOS assay

A total of 16, 3-month-old female Sprague-Dawley rats received a 10-day infusion of mouse NGF dissolved in vehicle [artificial cerebrospinal fluid (CSF)] or the vehicle alone into the right lateral ventricle as described above. Animals were killed and dissection of rat brain regions were performed as described (Johnston et al. 1987). For assessment of NOS activity, each sample consisted of pooled tissue from two animals treated in an identical manner. NOS activity was measured by monitoring the conversion of [³H]arginine to [³H]citrulline exactly as described (Bredt & Snyder, 1990). Student’s t-test was used for statistical analysis.

Results

NOS was co-localized in the majority of trkA-containing neurones in the MSN and VDB

TrkA mRNA and protein are contained in basal forebrain cholinergic neurones (Holtzman et al. 1992a, Steininger et al. 1993) To determine if trkA-containing neurones produced brain NOS, immunohistochemistry for trkA and NOS were performed. Using a trkA-specific antibody (Fig. 1 A), immunostained neurones in the MSN and VDB were distributed in exactly the pattern predicted from earlier in situ hybridization studies for trkA mRNA, indicating that trkA mRNA-containing cells made the trkA receptor. To determine whether trkA-positive neurones expressed NOS, we compared the localization of trkA and NOS. TrkA and NOS were co-distributed in the MSN and VDB and both were found in magnocellular neurones (Fig. 1 A, B). To establish co-localization, immunostaining with trkA was followed by NADPH-d histochemistry. Virtually all neurones (~95%) in the MSN and VDB positive for NADPH-d staining also expressed trkA. However, not all neurones expressing trkA were positive for NADPH-d; NADPH-d staining was found in 60% of these cells (Fig. 1 C, D). In contrast to the pattern for neurones in the MSN and VDB, in the striatum trkA and NADPH-d staining did not overlap (Fig. 1 E). Interestingly, although trkA and NADPH-d staining were co-localized in other regions of the basal forebrain that project to neocortex (horizontal limb of the diagonal band, substantia innominata and nucleus basalis), the percentage of overlap was decreased in these more caudal populations (data not shown), a result in agreement with prior data for localization of ChAT and NADPH-d...
Fig. 1. NOS and trkA are co-localized in basal forebrain cholinergic neurones in the medial septal nucleus (MSN) and vertical limb of the diagonal band (VDB). Immunohistochemistry was performed through the septal region of the adult rat brain (A) with an antibody to rat trkA, or (B) with an antibody to rat brain NOS. In (A) and (B) immunostained neurones are co-distributed in the MSN. Scale bar for (A) and (B) equals 250 µM. In (C) MSN, (D) VDB and (E) striatum, immunostaining for trkA (brown, DAB) was followed by NADPH-d staining (blue). In both (C) and (D) one neurone is single labelled (thin arrow, brown only) and one is double labelled (thick arrow, blue and brown with some green from mixing of colours). Of 320 NADPH-d stained neurones assessed in the MSN and VDB, 304 (95%) were also trkA-positive (n = three animals). In contrast, of 872 trkA immunostained neurones in the MSN and VDB 523 (60%) were also reactive for NADPH-d (n = 3). In the striatum (E) trkA (brown) and NADPH-d staining (blue) were in distinct neuronal populations. Scale bar for (C), (D) and (E) equals 10 µM.
Taken together with previous work (Holtzman et al. 1992a, Kitchener & Diamond 1993), these results indicate that essentially all trkA expressing neurones in the MSN and VDB are cholinergic and that about 60% of these could also produce the neuromodulator, NO.

Fimbria-fornix lesion decreased trkA- and NOS-expressing fibres in the hippocampus

Studies in hippocampal slices suggest that NO plays a role in enhancing synaptic efficacy and modulating long term potentiation (LTP) (Bohme et al. 1991, Schuman & Madison 1991, 1994, Vincent & Hope 1992, Zhuo et al. 1993) The source of NO for these actions is unknown. One source could be a small population of hippocampal interneurones that contain NOS (Vincent & Hope 1992) Another, potentially significant source could be cholinergic terminals originating from cell bodies in the basal forebrain that project via the fimbria-forms to all regions of the hippocampus (Coyle et al. 1983, Olton et al. 1991) Cholinergic axons would be expected to contain trkA. If cholinergic terminals produce NO, they should also contain NOS. To test these assertions, we asked if fimbria-fornix transection, which has been shown to result in a loss of cholinergic fibres in the hippocampus (Gage et al. 1983, Hefti 1986, Williams et al. 1986), would result in loss of trkA- and NOS-containing fibres in this region. In the unlesioned hippocampus, many trkA- and NADPH-d-positive fibres were seen in an overlapping distribution in the fimbria, dentate gyrus, and pyramidal cell layer (Fig. 2 A, C). Following a complete unilateral aspiration of the right fimbria-fornix, there was a marked decrease in both trkA- and NADPH-d stained fibres (Fig 2 B, D). These data suggest that NGF-receptor bearing cholinergic neurones in the MSN and VDB extend terminals to the hippocampus that contain NOS.

Fig. 2. Unilateral fimbria-forms transection decreased both trkA- and NADPH-d stained fibres in the ipsilateral hippocampus. In the hippocampus there was an abundance of trkA containing fibres in the unlesioned (A) as compared with the contralateral, lesioned (B) side. Similarly, there was an abundance of NADPH-d reactive fibres in the unlesioned (C) as compared with the lesioned (D) hippocampus. There was also a corresponding decrease in NOS-immunostained fibres in the lesioned hippocampus (data not shown). Some NADPH-d reactivity persists in scattered interneurones and their fibres which are not part of the septohippocampal cholinergic system. Scale bar equals 20 m H=hippocampus stratum oriens. F=fimbria arrows show border of fimbria and hippocampus.
NGF induced NOS in basal forebrain cholinergic neurones

The receptor tyrosine kinase trkA is critical for transducing the NGF signal (Chao 1992, Kaplan et al. 1991, Klem et al. 1991). NGF is known to act on trkA-containing basal forebrain neurones to increasing ChAT gene expression and activity (Holtzman et al. 1992a, Longo et al. 1992). The presence of NOS, another neurotransmitter synthetic enzyme in these cells, raised the possibility that like ChAT, NGF would enhance NOS activity. Using PC12 cells, a recent study showed that NGF-induced differentiation was associated with increased NOS activity and NADPH-d staining (Hirsch et al. 1993). NGF induced differentiation of PC12 cells is associated with induction of expression for many genes, as evidenced by increased levels of their mRNAs (Halegoua et al. 1991). We found that NGF increased NOS mRNA in PC12 cells. By RNA blot, NGF treatment for 48 h increased NOS mRNA 3.9-fold (mean densitometry score ±SEM as a percentage of vehicle-treated cells; NGF=394 ± 96, n=3, VEH=100 ± 12, n=3, P < 0.05). By antigen blot, a similar degree of induction was found for NOS protein (data not shown).

We next asked whether NGF would influence the expression of NOS in the CNS. Following intraventricular infusion for 10 days, NGF was found to increase markedly NOS mRNA and NOS immunoreactivity within cholinergic neurones in the MSN and VDB (Fig. 3, Table 1). NGF actions were selective in that no induction was seen outside the basal forebrain regions which contain cholinergic neurones or in the striatum, cortex or cerebellum. In addition to increasing NOS expression in individual neurones in the MSN and VDB, NGF increased the number of NOS-immunoreactive cells (Fig. 4 A). However, NADPH-d staining remained localized to cholinergic neurones, thus, the increase in cell number appears to have occurred as a result of induction or upregulation of NOS in cholinergic neurones in which it was previously undetected. To ask whether the activity of NOS was also increased, we carried out enzyme assays. As expected, NGF significantly stimulated NOS activity in the septum, the brain region containing basal forebrain cholinergic cell bodies (Table 1). In contrast, there was no significant change in striatum, cortex or cerebellum.

Fig. 3. NGF increases NOS mRNA and protein in the MSN of adult rats (A) and (B) ISHH with NOS antisense riboprobes (A) In the NGF-treated brain magnocellular neurons in the MSN contained 2.5 times the number of grams present in vehicle-treated animals (see Table 1 for quantification) (B) Vehicle-treated brain (C) and (D) Immunostaining with an antibody to NOS. There was an increase in the density of NOS immunoreactivity and the number of NOS immunostained cells in the NGF-treated (C) versus the control vehicle treated (D) brain Scale bar equals 100 µM.
**NOS expression persisted in axotomized basal forebrain neurones**

An interesting property of NOS-containing neurones in the striatum is their relative resistance to injury from excitatory amino acids and in Huntington’s disease (Beal et al. 1986). We asked whether NOS-containing basal forebrain neurones might have similar properties. Cholinergic neurones in the MSN degenerate following axotomy via fimbria-fornix transection (Gage et al. 1983, Hefti 1986, Williams et al. 1986). Therefore, we determined whether the subset of cholinergic neurones in the MSN that express NOS were less vulnerable to axotomy than non-NOS-containing neurones. Compared with the unlesioned side, only 21% of ChAT-positive neurones were present in the lesioned MSN 2 weeks following unilateral fimbria-fornix transection. The decrease in ChAT-positive cells is comparable to that reported in other studies using these methods (Gage et al. 1988). In contrast, 64% of NADPH-d reactive neurones remained (Fig. 4 B). NOS immunostaining showed the same result (data not shown). The persistence of NOS raised the possibility that cells containing NOS were less vulnerable. We noted, however, that in the lesioned MSN, the number of NOS-positive cells was actually greater than the number of ChAT-positive cells, a result opposite that in the normal MSN. Two possibilities were suggested. The first was that NOS was being expressed in a different population of neurones. This was unlikely since the presence of NOS and NADPH-d staining was in the same distribution as that seen prior to axotomy. The second possibility was that NOS was being detected in cholinergic neurones that were no longer ChAT-positive. It has been shown that ChAT immunostaining underestimates the number of cells that remain viable after axotomy. Many cholinergic neurones express too little ChAT to be detected shortly after axotomy and only later go on to die (O’Brien et al. 1990, Tuşyński et al. 1990). Thus, the apparent savings of NOS expressing cells may have resulted from the fact that NADPH-d was a more sensitive marker than ChAT. In accordance with this view, we found that the majority of NADPH-d reactive neurones no longer expressed detectable ChAT immunostaining (data not shown). In addition, many of the remaining NADPH-d reactive neurones were shrunken and atrophic and were significantly smaller than in unlesioned controls (mean cell area±SEM, lesioned=118.8 ±3.43, n=127 neurones, n=4; unlesioned=150.5 ±4.20, n=133, n=4; P < 0.001). Finally, among the remaining neurones

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### Table 1. Assessment of NOS mRNA and activity levels

<table>
<thead>
<tr>
<th>Brain region</th>
<th>NOS mRNA (Data expressed per neurone (% of vehicle±SEM))</th>
<th>NOS activity (cpm 0.5 µg wet weight±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vehicle (n=3)</td>
<td>NGF (n=3)</td>
</tr>
<tr>
<td>Septum (MSN)</td>
<td>100±6 (n=331)</td>
<td>249±10 (n=393)</td>
</tr>
<tr>
<td>Striatum</td>
<td>100±6 (n=155)</td>
<td>100±6 (n=160)</td>
</tr>
<tr>
<td>Cortex</td>
<td>100±5 (n=153)</td>
<td>100±6 (n=159)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>7666±644</td>
<td>7777±731</td>
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**Fig. 4. Regulation of NOS and ChAT in the NGF-treated and fimbria-fornix lesioned MSN.** (A) There was an increase in the number of detectable NOS immunoreactive neurones in the MSN 10 days following the intraventricular infusion of NGF (mean±SEM, NGF = 1933±83, N = 4; VEH = 1511±61, N = 4; Student’s t-test, P < 0.01). (B) In the lesioned MSN 2 weeks following fimbria-fornix transection, 21±2.6% of ChAT-immunostained neurones and 64±6.0% of NADPH-d stained neurones remained as compared with the unlesioned side. The decrease for ChAT was significantly greater than for NADPH-d (n = 4, Student’s t-test, P < 0.001).
immunostained for ChAT in the lesioned MSN, there was no increase in the percentage of NADPH-d reactive cells compared to the intact side. These data argue against a protective effect of NOS in axotomized basal forebrain cholinergic neurones. Instead, our findings suggest that NOS continues to be expressed in atrophic neurones that are destined eventually to die.

**Discussion**

The studies reported here are the first to show that a neurotrophic factor, NGF, can regulate brain NOS in the CNS. While the brain form of NOS is 50–60% homologous to the highly regulated macrophage and endothelial NOS, regulation of the gene for brain NOS has been thought to be constitutive (Dawson et al. 1992). Our findings show that brain NOS can be regulated and suggest that NGF and other neurotrophins may enhance NOS expression in responsive neuronal populations in the developing, mature and injured CNS.

The significance of NGF actions on NOS in basal forebrain cholinergic neurones is yet to be defined. NGF can stimulate ChAT activity as well as ACh synthesis and release in the hippocampus (Longo et al. 1992, Rylett et al. 1993). A possible mechanism for enhancing the release of ACh as well as other neurotransmitters is suggested by our findings. NO facilitates ACh release both in the basal forebrain in vivo (Prast & Philipu 1992) and in PC12 cells in vitro (Hirsch et al. 1993). In addition, NO may stimulate release of glutamate and noradrenaline from brain synaptosomal preparations (Montague et al. 1994). Our data suggest that through increased NOS synthesis and NO production, NGF could enhance release of ACh as well as, perhaps, other neurotransmitters. NGF has been previously shown to improve spatial memory in aged rats (Fischer et al. 1991, Haley et al. 1992). NGF may augment NO-mediated functions not directly involving ACh. For example, prior studies showed that direct electrical stimulation of the MSN resulted in an increase in hippocampal cGMP which was not mediated by ACh (Segal & Guidotti 1981). This effect could have been mediated by NO, which is known to stimulate guanylyl cyclase and increase cGMP levels (Bredt & Snyder 1994). Since NO is freely diffusible, NO produced in the axons of basal forebrain cholinergic neurones could diffuse to hippocampal neurones to influence their function.

Evidence that NO acts as a neurotransmitter in the hippocampus includes the following: (1) exogenously generated NO has been shown to enhance synaptic efficacy in hippocampal slices (Bohme et al. 1991, Zhuo et al. 1993); (2) inhibition of NOS in slice preparations blocked induction of LTP (Bohme et al. 1991, Haley et al. 1992, O'Dell et al. 1991, Schuman & Madison 1991), and (3) in vivo inhibition of NOS appeared to impair spatial learning (Bohme et al. 1993), a task requiring the hippocampus and its afferent and efferent projection systems (Olton et al. 1991). Although it is possible that NO acts as a hippocampal neurotransmitter in vivo, a key criterion for this yet to be established, is the source of NOS for NO production. Arguments have been made for NO production in hippocampal pyramidal cells (Schuman & Madison 1991, 1994, Zhuo et al. 1993). However, we failed to localize NOS mRNA or protein in CA1 pyramidal cells (see also Vincent & Hope 1992). Thus, the source of NOS for the LTP generated in vivo has not been defined. One possibility is that NOS is present in pyramidal cells but was not detected because of very low levels of mRNA, protein, and enzymatic activity. Another possibility is that NOS is expressed in hippocampal pyramidal neurones in slice preparations but not in vivo. A recent study has shown that NADPH-d staining was not detected in these neurones in freshly made slices but was markedly induced after 30 min (Divac et al. 1993). If confirmed, these findings would define a source for NOS relevant to in vivo studies; however, they do not address the source of NOS relevant to findings in vivo (Bohme et al. 1993). Our data show that NOS is present in the hippocampus in cholinergic terminals and raise the possibility that this source of NO contributes to hippocampal neurotransmitter functions, including possibly those subserving LTP and learning.

Both in vivo and in vitro, certain NOS containing neurones appear to be relatively resistant to injury. NADPH-d containing neurones survive in the striatum in Huntington's disease while most other neurones die (Ferrante et al. 1985). In addition, these neurones are resistant to hypoxic-ischemic injury (Ferriero et al. 1988, Uemura et al. 1990) as well as NMDA agonists (Koh et al. 1986). How certain NOS-containing cells escape cell death is uncertain. It is possible that NOS expression confers this property, but the mechanism for this has not been deduced. Alternatively, NOS-containing neurones may contain high levels of NADPH, a NOS cofactor, which could limit free radical toxicity. Our data indicate that NADPH-d is a sensitive marker for surviving basal forebrain cholinergic neurones following axotomy, but that NOS-expressing neurones do not appear selectively resistant to this injury. The results of our experiments suggest additional consideration. Recent experiments in vitro suggest that, depending on redox conditions, NO may be toxic to cells (Lipton et al. 1993). Persistence of NOS in atrophic basal forebrain neurones is evidence for continued NO production. Whether this would predispose these or surrounding neurones to NO-mediated injury is unclear, but this is an interesting possibility.

If NO plays a role in processes modulated by basal forebrain cholinergic neurones (Olton et al. 1991), our findings may have implications for proposed clinical trials of NGF in the treatment Alzheimer's disease. Dysfunction and loss of basal forebrain cholinergic neurones may contribute to cognitive impairment in Alzheimer's disease (Coyle et al. 1991).
1983, Olton et al. 1991). Beyond the benefits derived from an improved trophic state and increased ACh release, NGF effects on NO production by cholinergic neurones could enhance other NO-mediated hippocampal functions. Moreover, NO diffusion to surrounding cells raises the possibility that NGF treatment could indirectly influence the function of post-synaptic neurones not innervated by basal forebrain cholinergic neurones. Through enhanced NO production, NGF may have widespread actions in the AD hippocampus.

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