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AGE-DEPENDENT LOSS OF NGF SIGNALING IN THE RAT BASAL FOREBRAIN IS DUE TO DISRUPTED MAPK ACTIVATION.

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Abstract

The loss of Nerve Growth Factor (NGF) and its high affinity receptor TrkA has been implicated in the loss of cholinergic tone and function in Alzheimer's disease (AD) and normal aging. We employed an animal model of aging, the aged rat, which also exhibits memory loss and NGF alterations. Basal forebrain TrkA levels increased after injection of NGF in the hippocampus within one-hour in young rats, but this response was diminished in aged animals as determined by Western blot analysis. Further, NGF activated MAPK pathways without changing total ERK levels and the activation of these pathways was also diminished in aged animals. The exogenous NGF injection did not appear to activate the PI-3K pathway or alter total levels of Akt significantly. These data shed light on mechanisms of NGF signaling in the CNS, and alterations in this signaling cascade associated with age and memory loss. These findings might lead to development of novel treatment therapies for the memory loss associated with AD and other age-associated neurodegenerative diseases.

Keywords

Neurotrophin signaling; Aging; Memory; Septohippocampal cholinergic transmission

Introduction

A major research effort has focused on identifying early changes in Alzheimer's disease (AD) and mild cognitive impairment (MCI) [27]. One challenge in this endeavor is the paucity of diagnostic criteria that separate AD from other forms of dementia, and definite diagnosis thus occurs at the time of autopsy. One potential method of identifying early AD is identifying and tracking patients with MCI [11,27,31,39–41]. Many of the hallmarks of AD, such as elevated beta amyloid levels, amyloid plaques, and hyperphosphorylated tau do not correlate well with cognitive decline [3,4]. Therefore, investigators have been searching for additional biological markers. The loss of nerve growth factor (NGF) as well as its receptor has been implicated in the loss of cholinergic tone and function in AD and aging [14,33,34]. In MCI patients it was found that basal forebrain levels of TrkA decline 50% compared with age matched controls and that the number of TrkA immunopositive neurons in the basal forebrain correlate well with a number of different cognitive measures [33,34]. To further understand the mechanism behind this loss we have employed aged rats as a model that mimics the biochemical changes of NGF and TrkA as well as the memory loss found in the MCI patient [2,8,10,13,36]. Our group has been interested in identifying possible mechanisms underlying this pathology [44,45]. Here

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we show that compared to young rats, aged rats show diminished TrkA protein expression as well as second messenger activation in response to exogenous hippocampal NGF administration. This work extends previous findings from our laboratory, providing quantitative measurements with Western blots of earlier morphological observations [44,45]. Understanding these age-associated impairments in the rat may lead to important parallel findings in the human brain, and thus facilitate drug development for treatment of cognitive impairment in the MCI or AD patient.

Materials and Methods

Stereotaxic surgery

Twenty-eight adult male Fisher 344 rats (~200 g for young, and 300–400g for aged Harlan Sprague-Dawley) were used in the exogenous NGF studies. All procedures were in accordance with the NIH guidelines for animal use and care and were approved by the local Institutional Animal Care and Use Committee. Rats were anesthetized and placed into a stereotaxic apparatus [44,45]. Bregma was located, and each solution was injected with stereotaxic techniques at the following hippocampal coordinates: –4 mm bregma, +3 mm mediolateral, and –3 mm dorsoventral according to published procedures [44,45].

Intracranial injections

Four month old male Fischer 344 rats were injected with 50 µg of human recombinant NGF (R&D systems) in 10 µl of saline vehicle (n=12) into the hippocampus over a period of 10 minutes. Control rats (n=16) were injected with 50 µg cytochrome C (Sigma) dissolved in 10 µl of saline as described above. This control protein was utilized due to a similar weight and structure as NGF. Rats were sacrificed one hour post injection. This time-point was utilized due to similar time-points for rapid NGF response reported by us previously [2,44]. Brains were quickly removed and basal forebrain immediately dissected with effort to obtain both septal nucleus and diagonal band area, then placed into pre-weighed tubes.

Western blotting

Tissue samples were homogenized in lysis buffer (20mM Tris (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM Sodium pyrophosphate, 1mM B-Glycerolphosphate, 1mM sodium vanadate, and a protease inhibitor cocktail), sonicated, protein concentrations were determined by the BCA assay (Pierce). Equal amounts of homogenate proteins were separated in Tris-Acetate 4–12% precast gels (Invitrogen) and transferred to nitrocellulose membranes (BiotraceNT, Pall Corp.). The membranes were dried, washed, and blocked, and incubated overnight in primary antibodies (TrkA, gift from Dr. Louis F. Reichardt, 1:5000, p44/42 MAP kinase antibody 1:1000 Cell Signaling; Phospho-p44/42 MAPK (Thr202/Try204)(E10) mouse mAb 1:2000 Cell Signaling; Akt Ab 1:1000 Cell Signaling; Phospho-AKT (Thr308) Ab 1:1000 Cell Signaling; Phospho-AKT (Ser473) mouse mAb 1:1000 Cell Signaling). Two different phospho-specific antibodies for AKT were used along with positive (Jurkat cells activated with FBS and treated with Calyculin A) and negative control cell extracts (Jurkat cell extracts not treated with FBS). Membranes were washed, and incubated in horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000 in blocking buffer), and developed using a chemiluminescent substrate (SuperSignal west-pico for HRP, Pierce). Blot images were captured and density determined using a FluorChem imaging system (Alpha Inotech). Individual protein staining was expressed as band density of specific protein/Ponceau S protein band density. All blots were run in triplicate.

Results

Basal forebrain TrkA levels increased one hour post-NGF hippocampal injection

Following an intra-hippocampal injection of NGF or cytochrome C (as a control protein), animals (young, $n=14$ and aged, $n=14$) were sacrificed one hour post-NGF injection and basal forebrain tissue examined for levels of TrkA. Western blot analysis of TrkA protein levels in these animals showed an increase in TrkA levels in the young NGF treated animals compared to cytochrome C injected young controls (Fig. 1; $** p<0.01$). The aged NGF treated animals ($n=7$), on the other hand, did not show a significant difference in TrkA protein expression compared to aged controls ($n=7$; $p=0.128$). However, there was a significant difference between the young NGF treated animals ($n=5$) and the aged NGF treated animals ($** p<0.01$). Forebrain levels of TrkA in the aged Cytochrome C (Cyt C) injected animals versus young Cyt C injected animals ($n=9$) showed a trend toward a decrease in the aged control animals although it did not reach statistical significance ($p=0.069$).

Next, we wanted to explore signaling mechanisms. We have previously shown that the rapid response to NGF may be propagated independent of microtubules [44]. Microtubule-independent (rapid) NGF signaling has been shown to be propagated via two different second messenger systems in the PNS and in cell culture systems [9,16]. One signals through the mitogen activated protein kinase cascade (MAPK), utilizing ERK (extracellular signal-regulated kinase) phosphorylation. The other second messenger system used by NGF is through lipid signaling products of phosphatidylinositol-3 kinase (PI-3K)/AKT (protein kinase B) and subsequent generation of second messengers by phospholipase C- γ [38]. To our knowledge, these signaling mechanisms for NGF have not been evaluated in the CNS *in vivo*.

Basal forebrain tissue was analyzed for the total and activated (phosphorylated) levels of these second messenger systems. Analysis showed that while total ERK (non-phosphorylated) levels did not change with treatment or age (Fig. 2A–B), the levels of phosphorylated ERK were significantly elevated 15 min following NGF treatment, but only in the young NGF injected animals, compared to the young Cyt C injected animals (Fig. 2C–D). The mean value from basal forebrain tissue processed from aged NGF treated animals did not differ significantly from tissue from aged control (Cyt C) injected animals ($p=0.48$), suggesting that aged animals had a diminished response to NGF.

In order to determine whether the PI-3 kinase/AKT pathway is also activated in the basal forebrain in response to NGF, we analyzed basal forebrain tissue for total and phosphorylated AKT. We found no differences in total AKT levels between treatment and age groups (Fig. 3A–B), even though there was a trend towards elevated total Akt in the young NGF injected group (Figure 3A). Using multiple antibodies to different phosphorylation sites on AKT, we found no expression of phosphorylated AKT at either phosphorylation site (Only pSer-473 data shown; Fig. 3C). Positive control Jurkat cell extracts (Cell Signaling) were used to confirm antibody specificity and sensitivity. Thus, it does not appear that phosphorylation of AKT plays a major role in the observed rapid basal forebrain response to injected NGF at this time point and with this dose of NGF.

Discussion

These studies show that basal forebrain TrkA levels increased significantly within one hour post-injection in response to an exogenous hippocampal injection of NGF, and that this response is attenuated in aged animals. Exogenous NGF activates MAPK pathways in the basal forebrain without altering total ERK levels and that the activation of these pathways is also diminished in aged animals. We were unable to detect changes in the PI-3K pathway or total AKT levels after NGF injection, in these studies.

NGF signaling in the CNS

Studies from the PNS [9], have shown that NGF and BDNF signals can be transported in a rapid fashion, possibly via a phosphorylation cascade, from the terminals to the cell body of peripheral neurons. In this report, we show that a hippocampal NGF injection elicits significant elevations in TrkA protein levels in the basal forebrain within one hour. We have previously demonstrated that the NGF signal can reach the basal forebrain within 15 minutes of a hippocampal injection, despite pretreatment with a microtubule inhibitor, colchicine [44]. In the present study, given the distance the signal must travel, and the known rate of microtubule transport [18], we therefore believe that this one-hour signal is communicated to the basal forebrain largely by microtubule-independent signaling of activated second messengers possibly similar to mechanisms that exist for the rapid signaling of other trophic factors such as EGF [43]. Our data strongly support that such a rapid signaling cascade exists for NGF in the central nervous system (CNS). However, participation of a microtubule dependent (rapid) signaling systems in the septohippocampal cholinergic transmitter system has not been ruled out.

The second messenger systems of NGF signaling (i.e. MAPK and the PI-3K) appear to elicit different effects in cell culture systems [6]. The MAPK cascade plays a greater role in axonal growth while the PI-3K cascade appears to function more in neuronal survival [28], although there seems to be considerable crosstalk between the pathways. Our current data showed that short-term exposure of BFCNs to NGF via an intraparenchymal injection into the ipsilateral hippocampus activated the MAPK cascade without altering total ERK levels. The hippocampal NGF injection did not increase the levels of p-AKT (as detected by multiple antibodies to different phosphorylation sites on AKT as well as positive and negative controls) at this time point, although total AKT levels were marginally increased in the young, but not in the aged, NGF treated subjects. Whether both cascades are activated at later time points or whether the same distinction of roles observed in the PNS carries over to the CNS, remains to be determined in future experiments.

MAPK signaling in the CNS

ERK activity is believed to play a significant role in learning and synaptic plasticity, particularly in the hippocampus. There are currently 20 known activators of ERK, including NGF [42]. Both early and late as well as NMDA-dependent and NMDA-independent long-term potentiation (LTP) have been shown to be dependent on ERK activation [5,12,15]. Studies have also established that cholinergic input from the basal forebrain enhances hippocampal plasticity in the form of LTP [1]. Since ERK plays such an important role in other molecular processes, such as LTP, the NGF activation of ERK (especially the rapid effects described in the present study) may not only have trophic effects on cholinergic neurons, but may also play a physiological role in cholinergic neurotransmission in the hippocampus and cerebral cortex, perhaps via the cholinergic role in theta rhythm and thus induction of LTP [21,23,35]. Since hippocampal LTP is diminished in the aged rat [26,30], and cholinergic function plays a role in hippocampal LTP, it is thus feasible that altered NGF signaling, and hence the altered ERK activation, may play a role in age-related memory loss.

Age dependent changes in NGF signaling

We observed a diminished response to exogenous NGF in the aged rat, particularly with regard to levels of TrkA and ERK activation suggesting that rapid NGF signaling is disrupted in the aged rat. In support of this theory, retrograde microtubule-dependent NGF transport has previously been shown to be diminished in aged compared to young animals [36]. Other investigators [13] showed that basal forebrain cholinergic neurons receiving less retrograde NGF were also the first neurons to atrophy in the aged rat, providing direct evidence for NGF dependence of the aged cholinergic neurons, and providing evidence for a link between NGF

loss and memory performance of these animals. In contrast, other studies suggest that basal forebrain NGF protein levels do not correlate well with impaired behavior during aging in the rat [19]. Thus, other factors may influence behavior in terms of the cholinergic innervation of the hippocampus, such as NGF signaling. We have previously shown that the response level of NGF-stimulated p-ERK immunostaining in the basal forebrain of aged rats correlates with spatial reference memory as tested in the Morris water maze, suggesting that this signaling system is important for spatial memory [45].

Evidence suggests that defective retrograde transport of NGF in AD patients may be one of the initial events leading to cholinergic dysfunction [32]. Second messenger activation is likely to play a role in targeting the endocytic vesicle to dynein [29], hence the loss of ERK responsiveness in the aged animal we observed may explain why aged rats have decreased retrograde transport of NGF. Since ERK activation is dependent on endocytosis of the TrkA/NGF receptor complex [20], a loss of endocytic capability may be responsible for the blunted ERK activation in the aged animals. This is interesting considering that both aged animals and AD patients appear to have disrupted endocytic pathways [37].

In conclusion, our results have demonstrated that in young rats, TrkA and phospho-ERK are rapidly upregulated in the basal forebrain after a hippocampal NGF injection, but this response is blunted in aged animals. Thus, our data and others have shown that trophic molecules not only have significant bearing on the long-term survival of neurons during development and maintenance of phenotype, but also appear to play a role in continuous and rapid regulation of neuronal activity, gene expression and protein synthesis in the adult neuron. Novel treatment strategies for age-related neurodegenerative disease may be able to target the loss of these rapid growth factor effects or their downstream signaling pathways.

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Abbreviations

NGF

Nerve Growth Factor

AD

Alzheimer's disease

MCI	Mild cognitive impairment
Trk	Tyrosine Kinase receptor
ACh	Acetylcholine
ChAT	Choline acetyltransferase
MAPK	mitogen activated protein kinase
ERK	extracellular signal-regulated kinase
PI3K	phosphatidylinositol-3 kinase
IP	intraperitoneally
HRP	horseradish peroxidase
BFCNs	basal forebrain cholinergic neurons

Figure 1. Young, but not aged, NGF injected animals show increases in the Basal forebrain TrkA levels

A, TrkA was determined by Western blotting of young (n=14) and aged (n=14) animals injected with either NGF (n=7 aged, n=5 young) or a control protein, Cyt C (n=9 young, n=7 aged). The young NGF injected animals showed a robust increase in band intensity compared to the aged NGF injected animals (** p < 0.01). **B**, Representative blot showing the protein from each group. Ponceau S stain was used to ensure equal loading of protein.

Figure 2. Basal forebrain ERK and phospho-ERK levels by western blot analysis 1 hour post-NGF injection into the hippocampus

A, Total ERK protein levels did not change between the four groups. **B**, Representative bands of non-phosphorylated ERK showing no differences between the four groups in the bar graph. **C**, phosphorylated ERK levels increased in the young NGF injected animals (n=5) compared to young Cyt C injected controls (n=9; ** p<0.01). Aged NGF injected animals (n=7) did not differ from Cyt C injected controls (n=7). **D**, Representative bands from each of the four groups in the bar graph shown in C.

Figure 3. Basal forebrain AKT and phospho-AKT levels by western blot analysis 1 hour post-NGF injection into the hippocampus

A. Staining density for total non-phosphorylated AKT levels for each of the different treatment groups. No significant differences were found between groups ($p=0.43$). **B.** Representative bands showing total AKT levels. **C.** Membrane blotted for phosphorylated AKT shows no staining for samples taken from young Cyt C injected [25], young NGF injected (YN), aged Cyt C injection (AC), or aged NGF injected (AN) animals. Positive control (+; Jurkat cell extracts activated to express P-AKT) and negative control (-; Jurkat cell extracts without AKT activation) cell extracts were loaded to show antibody specificity and assay integrity.