NOTE TO USERS

Effects of Chronic Nerve Growth Factor Treatment on Synaptic Transmission in Neurons of the Rat Medial Septum / Diagonal Band of Broca Area

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Table of Contents

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Abstract

Nerve growth factor (NGF) is a neurotrophin important for the survival, growth and function of cholinergic basal forebrain neurons. However, little is known about the function of NGF on other basal forebrain neurons such as glutamatergic or GABAergic neurons. Here, we used neurons from the rat medial septum/diagonal band of Broca (MS-DBB) to test whether chronic exposure to NGF affects the synaptic function of cholinergic, glutamatergic and GABAergic basal forebrain neurons. We isolated single rat MS-DBB neurons in culture, allowing them to establish synaptic contacts onto themselves. One group of neurons chronically received NGF while another group received none. After 2-4 weeks in culture, we used whole-cell electrophysiology to measure the amplitude of evoked autaptic EPSCs or IPSCs. Cholinergic MS-DBB neurons were identified using 192IgG-Cy3, a fluorescent $p75^{NTR}$ receptor antibody. Neurotransmitters involved in postsynaptic currents were characterized pharmacologically. In some experiments, a trk inhibitor K252a and MRL3, a function-blocking antibody for $p75^{NTR}$, were added with NGF to study the role of trkA- and $p75^{NTR}$-mediated signaling, respectively. To investigate the synaptic mechanisms underlying the changes in evoked EPSCs, miniature EPSCs were recorded. We found that NGF led to a significant increase in the amplitude of nicotinic and glutamatergic EPSCs in "cholinergic" MS-DBB neurons releasing both neurotransmitters. However, NGF did not affect autaptic currents in non-cholinergic neurons releasing glutamate or GABA. K252a prevented the NGF-induced increase in glutamatergic EPSCs but not nicotinic EPSCs. Conversely, MRL3 reduced the effect of NGF on nicotinic EPSCs but not glutamatergic EPSCs. Furthermore, chronic NGF treatment led to a 2-fold increase in the frequency of miniature EPSCs in cholinergic neurons but did not affect the amplitude of the events. The present study demonstrates, for the first time, that chronic NGF treatment dramatically increases both glutamate and ACh release from cholinergic MS-DBB neurons. The present study illustrates that NGF is crucial not only for cholinergic function but also for glutamatergic function in the septo-hippocampal pathway, which plays an important role in memory.

Résumé

Le rôle du septum médian et des bandes diagonales (SM-BD) dans la modulation de l'activité de l'hippocampe, et fonctionnellement, dans la mémoire et l'apprentissage est bien connu. Dans les trois dernières décennies, il a été démontré que ce sont les cellules GABAergiques et cholinergiques du SM-BD projetant à l'hippocampe qui seraient clés dans la fonction de la mémoire. Plusieurs études soulignent particulièrement l'importance de la relâche de l'acétylcholine des neurones du SM-DB dans la mémoire et de sa forte diminution dans la maladie d'Alzheimer. Ainsi, il semble qu'une des raisons de la baisse d'acétylcholine dans la maladie d'Alzheimer pourrait être due à une baisse des niveaux du facteur neurotrophique de croissance (NGF) dans le cerveau. Le NGF est connu comme ayant des effets bénéfiques sur la relâche d'acetylcholine dans le cerveau grâce a des actions trophiques sur les cellules cholinergiques. En conséquence, le NGF pourrait s'avéré être une molécule très importante pour augmenter les niveaux d'acétylcholine dans le cerveau et contrer les symptômes de la maladie d'Alzheimer. Une étude pilote récente chez les humains démontre d'ailleurs que le NGF pourrait avoir des effets important sur l'amélioration de la cognition chez les patients Alzheimer. Cependant, les différents effets du NGF sur les neurones cholinergiques demeurent encore mal connus. Des donnés récentes de notre laboratoire ont démontré que les cellules dites cholinergiques avaient la capacité de relâcher les neurotransmetteurs acétylcholine et glutamate de façon simultanées. Dans la présente recherche, nous avons voulu vérifier l'hypothèse que le NGF pouvait augmenter la relâche de ces deux neurotransmetteurs des neurones cholinergiques et avons également déterminé le récepteur NGF impliqué. Nous avons effectué des enregistrements électrophysiologiques patch-clamp sur des cellules du SM-DB sur des îlots d'astrocytes en cultures. Dans ces conditions, les neurones effectuent des terminaisons synaptiques sur leurs propres dendrites et corps cellulaire offrant la possibilité de mesurer la relâche de neurotransmetteur(s) pour chaque neurone activé. Les cellules cholinergiques ont été identifiées à l'aide d'un anticorps fluorescent contre le récepteur P75, un marqueur spécifique aux cellules cholinergiques. Premièrement, nous avons démontré que les cellules cholinergiques du SM-DB pouvaient relâchés l'acétylcholine et le glutamate simultanément. Les courants cholinergiques et glutamatergiques étaient médiés par des

récepteurs nicotiniques et AMPAergiques, respectivement. Deuxièmement, nous avons démontrés que le NGF (25ng/ml) appliqués de façon chronique pendant 2-3 semaines, augmentaient les courants nicotiniques et AMPAergiques de façon significative, suggérant que la relâche de l'acétylcholine et du glutamate étaient augmentés. Par contre, le NGF n'avait pas d'effet significatif sur la relâche du GABA des cellules GABAergiques ainsi que du glutamate provenant des cellules non-cholinergiques. Finalement, nos résultats démontrent que les effets du NGF sur l'augmentation de l'acétylcholine, et non ceux sur le glutamate, passaient par l'activation des récepteurs TrKA. Nos résultats démontrent pour la première fois que le NGF exerce des effets complexes sur les cellules cholinergiques du SM-DB et suggèrent que certains effets positifs du NGF sur la cognition pourraient être causés par une augmentation de la relâche du glutamate dans le cerveau.

Introduction

1. Medial Septum / Diagonal Band of Broca (MS-DBB)

1.1. Location and subdivisions

The septal area is located in the anterior and medial part of the basal forebrain. In rodents, the septal area is divided into four sub-regions: the medial septum/diagonal band of Broca (MS-DBB), lateral septum (LS), posterior septum (PS) and bed nuclei of the stria terminalis (BNST). The MS-DBB is situated in the medial part of the septal area. Shaped like an inverted Y, the MS-DBB is comprised of the medial septum (MS) and vertical and horizontal limbs of the diagonal band of Broca (DBB). The MS-DBB complex is also referred to as the "medial septal region" or the "MSDB" area. In humans, the septal region can be partitioned into two areas: the septum pellucidum with mostly fibres and glia, and the septum verum (true septum) made up of fibres, glia and neuronal cell bodies, which includes the DBB, dorsal, lateral, fimbrial, triangular and bed nuclei (Andy & Stephan, 1968).

1.2. Connectivity with other brain regions

The afferent and efferent connections of the septum have been extensively studied (Swanson & Cowan, 1977;Swanson & Cowan, 1979;Alonso & Kohler, 1982;Vertes, 1992;Vertes *et al.*, 1995). The MS-DBB receives input from the hippocampus, several hypothalamic nuclei, the brainstem and LS. It sends projections to the hippocampus, medial and lateral preoptic areas, lateral hypothalamic area, mammillary complex, the midbrain (ventral tegmental area and raphe nuclei), parataenial nucleus of the thalamus and anterior limbic area. The MS-DBB projects to the hippocampus via the dorsal fornix and fimbria, and the hippocampus projects to both the MS-DBB and LS. The LS is bi-directionally connected with some hypothalamic nuclei and sends projections to the MS, mammillary complex and the ventral tegmental area. The PS, which includes the septo-fimbrial and triangular nuclei, receives input from the hippocampus and innervates the habenular nuclei and interpeduncular nuclear complex. Lastly, the BNST receive their input from the amygdala, ventral subiculum, ventromedial nucleus and brainstem, and they send projections to the substantia innominata, nucleus accumbens, hypothalamus, preoptic areas, the midbrain, thalamus, and the amygdala.

1.3. Neuronal populations

For decades, the MS-DBB has been known to contain two neuronal populations: neurons releasing acetylcholine and gamma-aminobutyric acid (GABA) (Kimura *et al.*, 1980;Panula *et al.*, 1984). However, there has been mounting evidence for the presence of glutamatergic neurons in the MS-DBB. First, retrograde labelling and immunohistochemical studies reported the presence of a population of basal forebrain neurons, some of which project to the cortex or hippocampus, that could not be identified with either cholinergic or GABAergic markers (Gritti *et al.*, 1997;Kiss *et al.*, 1997). Secondly, septal neurons were labelled with glutamate, phosphate-activated glutaminase (PAG, a synthetic enzyme for the neurotransmitter pool of glutamate) and [^{3}H]aspartate, indirect indicators of glutamate being used as a neurotransmitter (Gonzalo-Ruiz & Morte, 2000;Manns *et al.*, 2001;Kiss *et al.*, 2002;Manns *et al.*, 2003). Thirdly, a single-cell reverse transcriptase polymerase chain reaction (sc-RT-PCR) analysis showed that a significant proportion (about 27%) of MS-DBB neurons expressed messenger ribonucleic acids (mRNAs) for vesicular glutamate transporters type 1 and/or 2 (VGLUT1/2) (Sotty *et al.*, 2003;Danik *et al.*, 2003;Danik *et al.*, 2005). The VGLUT1/2 markers have been demonstrated to reliably indicate a cell's capacity to use glutamate as a neurotransmitter (Moriyama & Yamamoto, 2004;Herzog *et al.*, 2001). Sotty and colleagues further showed that a proportion of these VGLUT1/2-positive MS-DBB cells project to the hippocampus since they were labelled with a retrograde tracer injected into the hippocampus (Sotty *et al.*, 2003). Consistent with this finding, other groups have also reported expression of mRNAs for VGLUT2 transcripts in the MS-DBB (Hisano *et al.*, 2000;Fremeau, Jr. *et al.*, 2001;Lin *et al.*, 2003) and VGLUT2 proteins in the cell bodies of some MS-DBB neurons after treatment with colchicine, an axonal transport blocker (Lin *et al.*, 2003;Hajszan *et al.*, 2004). Notably, Hajszan and colleagues provided evidence that the VGLUT2 expression in septal neurons originates from within the septum and is not due to extrinsic glutamatergic input, since most of the VGLUT2-positive axons remained intact following septal undercut and fimbria/fornix transaction, manipulations that eliminate afferent inputs to the septum (Hajszan *et al.*, 2004). Finally, in a recent paper by Colom and colleagues, a triple immunostaining analysis revealed that out of all MS-DBB neurons labelled with one or more of ChAT (choline acetyltransferase), GAD67 (glutamic acid decarboxylase 67) and glutamate antibodies,

25% were positive for glutamate, an estimate consistent with that reported by Sotty and colleagues (27%) (Colom *et al.*, 2005;Sotty *et al.*, 2003). Colom and colleagues further demonstrated that a significant proportion of these MS-DBB glutamatergic neurons project to the hippocampus, by showing that 23% of glutamate-positive neurons were co-labelled with the retrograde tracer fluorogold injected into the hippocampus. Taken together, there is sufficient evidence to confirm that there is a sizeable population of neurons releasing glutamate in the MS-DBB and that some of these neurons project to the hippocampus.

Topographic organization of the different neuronal populations within the septum is not yet completely understood. Compared to the hippocampus, the septal area is not as neatly organized according to neuronal type and input-output connectivity. Nonetheless, the distribution is neither completely random, since there are some general trends. Cholinergic and glutamatergic neurons are concentrated within the MS-DBB complex, while GABAergic neurons are present in both MS-DBB and LS but are found more numerously in the LS (reviewed in Colom, 2006). GABAergic and cholinergic neurons are morphologically similar (i.e., predominantly multipolar cells) but their distributions differ. In the DBB, cholinergic neurons are estimated to be twice as numerous as GABAergic neurons (Brashear *et al.*, 1986). Most septal cholinergic neurons are located in the MS-DBB complex with a small group also found in the LS (Kimura *et al.*, 1990). Septal cholinergic neurons are mostly medium to large in soma size with a tendency to be larger in the MS-DBB (mean 22 microns in rat, 29 microns in monkey) than in the LS (17 microns in both rat and monkey) (Brauer *et al.*, 1999;Kimura *et al.*, 1990). In comparison, GABAergic neurons are most abundant in the LS but are also found in the BNST, PS and MS-DBB. Septal GABAergic neurons are mostly small (10 microns) to medium (20 microns) in size but some larger neurons (30 microns) have also been found in the MS-DBB (Onteniente *et al.*, 1987). Recent work on the expression of two isoforms of GAD enzymes revealed that GAD65-positive neurons are more numerous in the LS, while GAD67-expressing neurons are found in large numbers in both MS and LS regions (Castaneda *et al.*, 2005). This finding suggests the presence of two distinct GABAergic systems in the septum: small to medium-sized GAD65-positive cells in the LS, and larger GAD65- and GAD67-positive cells in the MS-DBB (Castaneda *et al.*, 2005). Another

study reported that different calcium-binding proteins, parvalbumin (PV), calbindin and calretin, are expressed in distinct, largely non-overlapping, areas of the septum and that they were rarely co-expressed in the same cells (Kiss *et al.*, 1997), providing further credence to the idea that septal GABAergic population may be made up of several discrete subgroups. As for the glutamatergic neurons, they are concentrated in the MS-DBB but also present in the LS at a lower density, similar to the pattern of septal cholinergic neurons (Colom *et al.*, 2005). They are mostly small to medium-sized neurons (5-21 microns) with cell bodies of various types (e.g., oval, fusiform, round, polygonal and pyramidal), but the size and morphology of glutamatergic neurons do not seem to vary according to location in the MS-DBB (Colom *et al.*, 2005).

1.4. Intraseptal connectivity

There are local, bidirectional connections between septal cholinergic and GABAergic neurons (Leranth & Frotscher, 1989). GAD-positive terminals innervate ChAT-positive dendrites in the MS-DBB, and ChAT-positive terminals make synaptic contacts on GAD-expressing cell bodies and proximal dendrites in the MS-DBB and the LS, indicating that cholinergic and GABAergic septal neurons innervate each other (Leranth & Frotscher, 1989). Furthermore, surgical lesions of the LS dramatically reduce the number of GABAergic boutons on cholinergic cells in the MS-DBB (Leranth & Frotscher, 1989), consistent with the notion that many GABAergic neurons are located in the LS.

Recently, there have been several lines of evidence supporting the participation of glutamatergic neurons in intraseptal circuitry and network function. Immunocytochemical studies suggest that septal glutamatergic neurons are synaptically interconnected with local cholinergic, GABAergic and other glutamatergic neurons. VGLUT2-postive puncta have been found in close proximity to ChAT-, GAD67-, PV- and VGLUT2-positive neurons in the MS-DBB (Hajszan *et al.*, 2004;Manseau *et al.*, 2005). ChAT- and GAD67-positive puncta have also been observed close to VGLUT2/glutamate-positive neurons (Colom *et al.*, 2005). Recent electrophysiological studies provide evidence that synapses made by these septal glutamatergic neurons are indeed functional and that they play a role in network activity. Wu and colleagues found that rapid excitatory effects of nicotine on septo-hippocampal GABAergic neurons require the action of endogenously

released glutamate on group 1 metabotropic glutamate receptors (Wu *et al.*, 2003). A more direct line of evidence came from a study by Manseau and colleagues using MS-DBB slices that were pharmacologically disinhibited (Manseau *et al.*, 2005). They demonstrated that in such in vitro conditions, local glutamatergic neurons became spontaneously active as evidenced by large, repetitive, spontaneous excitatory postsynaptic potentials (EPSPs), mediated mostly by alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)/kainate receptors and to a lesser extent by N-methyl-D-aspartate (NMDA) receptors. The spontaneous EPSPs often triggered bursts of action potentials in electrophysiologically identified cholinergic, GABAergic and glutamatergic neurons, demonstrating that glutamatergic neurons of the MS-DBB form a functional network with other neurons within the MS-DBB. Using organotypic cultures of MS-DBB mini-slices in which afferent inputs from other structures are eliminated, the glutamate-mediated responses were still observed, indicating that the glutamatergic input came from within the medial septum. They also showed that glutamatergic neurons were activated by cholinergic agonists and $GABA_A$ receptor antagonists, suggesting that cholinergic and GABAergic inputs play a role in the activity of glutamatergic neurons. Finally, the large glutamate-mediated bursts were found to occur concomitantly with extracellular field potentials recorded from an in vitro intact half-septum preparation, indicating that glutamatergic neurons are part of a neuronal network in the MS-DBB that can generate large-scale synchronized activity (Manseau *et al.*, 2005). In summary, increasing evidence supports the hypothesis that an extensive, mutual and functional synaptic network exists among cholinergic, GABAergic and glutamatergic neuronal populations within the MS-DBB.

1.5. Functions

Functions of the MS-DBB area have been extensively investigated since the discovery of a selective degeneration of cholinergic basal forebrain system (CBFS) in Alzheimer's disease (AD). As part of the CBFS, the MS-DBB serves as the sole provider of cholinergic input to the hippocampus, whereas the cortex is innervated by cholinergic fibres from other areas of the CBFS, including parts of the DBB, preoptic nuclei, substantia innominata and globus pallidus, collectively referred to as the nucleus basalis magnocellularis (NBM) (Bigl *et al.*, 1982;Mesulam *et al.*, 1983). In AD patients, levels

of expression of cholinergic markers such as ChAT are significantly reduced in the hippocampus and cortex compared to age-matched controls (Bowen *et al.*, 1976;Perry *et al.*, 1977;Henke & Lang, 1983). Therefore, much emphasis has been placed on trying to understand how the MS-DBB, particularly the septo-hippocampal cholinergic neurons, affect hippocampus-related functions in aging and disease states.

Decades of research has linked the MS-DBB to many behaviours and functions, including arousal, attention, exploratory behaviour, spatial memory and learning. Berridge and colleagues demonstrated the involvement of the MS-DBB in arousal by showing that infusions of agonists of noradrenergic beta-receptors into the MS, but not into the substantia innominata, selectively increase cortical and hippocampal electroencephalogram (EEG) patterns related to arousal in halothane-anesthetized rats (Berridge *et al.*, 1996) and increased behavioural arousal to the level of normal waking in unanesthetized, resting rats (Berridge & Foote, 1996). They further showed that similar infusions of amphetamine into the MS-DBB increased both the EEG and behavioural indices of arousal in anesthetized and unanesthetized rats (Berridge *et al.*, 1999). Other studies reported that lesions of the MS-DBB in animals lead to deficits in performance of tasks involving focusing or shifting of attention (Voytko *et al.*, 1994;Stoehr *et al.*, 1997) and that the lesions also decreased basal locomotor activity and exploratory behaviour (Lee *et al.*, 1988;Poucet, 1989). The role of the MS-DBB in memory and learning has been particularly well documented due to its relevance to AD. Many studies showed that selective lesions of the MS-DBB cause a loss of hippocampal ChAT activity and impairments in Morris water maze, radial arm maze and other spatial learning tasks (Winson, 1978;Mitchell *et al.*, 1982;Hagan *et al.*, 1988;Kelsey & Vargas, 1993;McAlonan *et al.*, 1995;Berger-Sweeney *et al.*, 2001;Lehmann *et al.*, 2003).

1.6. Hippocampal theta rhythm generation

A current model states that the MS-DBB contributes to hippocampus-related functions by providing a pacemaker input for the hippocampal theta rhythm. The hippocampal theta rhythm is an oscillatory activity in the range of 3 – 12 Hz, observed in hippocampal extracellular field recordings both in vivo and in vitro. It is produced by the synchronized depolarizations of hippocampal neurons. Theta is correlated with a list of functions and behaviours similar to those associated with the MS-DBB: arousal,

attention, exploratory behaviour, voluntary motor control, spatial learning and memory, REM sleep and sensory motor integration. In 1954, Green and Arduini reported that when a resting, unanesthetized rabbit or cat shifts from a drowsy state to an alert state due to sensory stimulation (e.g., a tail pinch), in vivo field recordings in the hippocampus showed the occurrence of large-amplitude oscillations in the theta frequency band (Green & Arduini, 1954). Hippocampal theta activity has been categorized into two types according to behavioural and pharmacological criteria (Kramis *et al.*, 1975). Type 1 theta (peak frequency of 6 – 10 Hz) is observed when an animal is engaged in active 'exploratory' movements and is resistant to high doses of atropine, a muscarinic cholinergic receptor antagonist. Type 2 theta (peak frequency of 4 – 6 Hz) is present during immobility, urethane anaesthesia and movements and is abolished by atropine, thus it is thought to be mediated by muscarinic activity. Vanderwolf's work showed that Type 1 theta can vary systematically with parameters of movement such that the frequency of theta is correlated with the speed with which movement is initiated while the amplitude of theta is correlated with the magnitude of movement to be performed (Vanderwolf, 1969).

The role of hippocampal theta rhythm in memory and learning has been particularly well explored. Human EEG studies show that large-amplitude theta oscillations are correlated with certain memory tasks (Kahana *et al.*, 2001). Electrophysiological studies in animals demonstrate that bursts of high-frequency stimulation given on the positive phase of background hippocampal theta wave induce long-term potentiation (LTP), while bursts given on the negative phase of theta lead to long-term depression (LTD) or de-potentiation of previously established LTP in the hippocampus both in vivo and in vitro (Huerta & Lisman, 1993;Holscher *et al.*, 1997). These findings suggest that the phase of hippocampal theta acts as a powerful regulator of synaptic plasticity, deciding which synaptic connections are strengthened and which ones are weakened. Furthermore, the phase of theta seems to be particularly important for the representation of spatial location. O'Keefe and Recce found that location-specific 'place cells' in the hippocampus fire at a progressively earlier phase of the background theta as the animal traverses the receptive field, indicating that information about location is encoded not only in the firing rate but also in the phase of spikes in relation to the

ongoing theta rhythm (O'Keefe & Recce, 1993). Finally, manipulations that reduce or eliminate hippocampal theta, such as septal lesions, lead to deficits in performance of memory tasks such as the radial arm maze task (Winson, 1978;Mitchell *et al.*, 1982).

The neural pathway that gives rise to the hippocampal theta activity, known as the 'ascending brainstem hippocampal synchronizing pathway', includes the brainstem rostral pontine region, the supramammillary nucleus, posterior hypothalamic nuclei and the MS-DBB (Bland & Colom, 1993). At the level of the brainstem, neurons fire in a non-rhythmic, tonic fashion but by the time the ascending signal reaches the hippocampus and the cortex, neurons can fire in rhythmic bursts at theta frequencies. The MS-DBB has long been accepted as the rhythm generator in this pathway due to the evidence showing that septal or fimbria-fornix lesions effectively abolish hippocampal theta waves (Green & Arduini, 1954;Winson, 1978;Rawlins *et al.*, 1979;Mitchell *et al.*, 1982). In addition, medial septal neurons have been shown to fire in theta-band rhythmic bursts, temporally synchronized to the field hippocampal theta (Petsche *et al.*, 1962;Gogolak *et al.*, 1968). However, neurons that burst rhythmically in synchrony with hippocampal theta have also been found in some hypothalamic nuclei (Kocsis & Vertes, 1994;Bland *et al.*, 1995), alluding to the possibility that there may be multiple rhythm generators including the MS-DBB along the ascending synchronizing pathway. Nevertheless, it is clear from the lesion studies that the integrity of the medial septum is necessary for the occurrence of hippocampal theta rhythm.

The mechanism with which the MS-DBB contributes to the generation of hippocampal theta rhythm is still an unresolved issue. Since glutamatergic neurons in the MS-DBB have only recently been discovered, most studies have focused on the role of cholinergic and GABAergic MS-DBB neurons. Cholinergic neurons of the MS-DBB are known to project to both excitatory pyramidal neurons and inhibitory interneurons of the hippocampus, while the MS-DBB GABAergic afferents selectively innervate interneurons (Freund & Antal, 1988). Numerous electrophysiological studies demonstrate that many neurons in the MS-DBB fire with some type of temporal relationship with the hippocampal theta, for example firing tonically during hippocampal theta or firing in rhythmic bursts at different phases of the hippocampal theta (Gogolak *et al.*, 1968;Colom & Bland, 1991;Bland & Colom, 1993;Bland *et al.*, 1999). Selective lesions of cholinergic

neurons of the MS-DBB using 192-IgG-saporin, an immunotoxin specific to cholinergic cells of the basal forebrain, have been shown to cause dose-dependent reductions in the amplitude, but not the frequency, of hippocampal theta during wheel running and paradoxical sleep (Lee *et al.*, 1994). In another study, cholinergic and GABAergic neurons of the MS-DBB were specifically lesioned by local injections of 192-IgG-saporin and kainate, respectively, and it was found that while anesthesia-related Type 2 theta was totally abolished by either type of lesion, movement-related Type 1 theta was only attenuated but not eliminated by either lesion and only when both chemotoxins were applied together, hippocampal theta was virtually eliminated (Yoder & Pang, 2005). The caveat of this study is that although a low concentration of kainate is shown to preferentially destroy GABAergic neurons rather than cholinergic neurons, its effects on glutamatergic neurons were uninvestigated (Yoder & Pang, 2005). These findings suggest that both MS-DBB cholinergic and GABAergic neurons are essential for theta rhythm under anesthesia, but for movement-related theta the integrity of GABAergic neurons may be more crucial than that of cholinergic neurons. The MS-DBB cholinergic neurons may act as an amplifier to boost the amplitude of theta oscillations. Consistent with this idea, Borhegyi and colleagues demonstrated that there are two distinct populations of PV-positive, putatively GABAergic, MS neurons firing in regular theta-frequency bursts, tightly coupled to either the trough or the peak of hippocampal theta waves (Borhegyi *et al.*, 2004). They found that some of the theta-bursting neurons had extensive axon arbours with numerous collaterals within the MS as well as axons projecting towards the hippocampus, indicating that these septal GABAergic neurons may act locally to synchronize theta bursts within septum and also provide rhythmic disinhibition of the hippocampal circuit via projections to the hippocampal interneurons (Borhegyi *et al.*, 2004). Finally, a recent in vivo study by Simon and colleagues showed that neurochemically identified GABAergic neurons in the MS-DBB fire in rhythmic theta bursts while cholinergic neurons fire very slowly with no apparent temporal relationship with hippocampal theta in rats under anaesthesia, during wakefulness or REM sleep (Simon *et al.*, 2006). Taken together, evidence to date supports the current model that cholinergic neurons of the MS-DBB provide the excitatory tone necessary for synchronized network oscillations to occur in the septo-hippocampal circuit, while

GABAergic MS-DBB neurons provide the rhythmic drive both locally and remotely in the hippocampus to give rise to the theta-band oscillations.

1.7. Evidence for neurotransmitter co-release

Interestingly, in recent studies that reported the presence of glutamatergic neurons in the MS-DBB, it was found that a significant proportion of these VGLUT mRNA-expressing neurons also co-expressed ChAT mRNA (Sotty *et al.*, 2003;Danik *et al.*, 2005). Colom and colleagues also reported that a proportion of glutamate-positive neurons co-expressed ChAT proteins and that some of these double-phenotype neurons projected to the hippocampus (Colom *et al.*, 2005), providing further evidence that a subgroup of MS-DBB neurons are equipped with the cellular machinery to use both acetylcholine and glutamate as neurotransmitters. Whether these neurons can functionally co-release the two transmitters together has been tested electrophysiologically in a recent study by Allen and colleagues (Allen *et al.*, 2006). They grew single magnocellular basal forebrain neurons on micro-islands in culture, allowed sufficient time for the neurons to make synapses onto their own cell body and dendrites (autapses) and recorded the autaptic postsynaptic responses in these cells after evoking action potentials. They found that in 34 out of 36 autaptic neurons so tested, the evoked action potentials were followed by short-latency excitatory post-synaptic currents (EPSCs) that were blocked by kynurenic acid, a broad-spectrum ionotropic glutamate receptor blocker. By placing a voltage-clamped rat myoball expressing nicotinic receptors in contact with the neurites, they demonstrated that in 6 out of 6 neurons the glutamatergic EPSC was accompanied by a simultaneous nicotinic myoball current, providing evidence that glutamate and acetylcholine can indeed be co-released by the same cell. They further showed that the acetylcholine released into the synaptic cleft can act on the presynaptic muscarinic receptors to decrease the release of acetylcholine and glutamate in an autocrine negative-feedback fashion (Allen *et al.*, 2006). Although functional co-release of glutamate and acetylcholine from single MS-DBB neurons in slice or in vivo has yet to be demonstrated, evidence suggests that a significant proportion of MS-DBB neurons may release more than one neurotransmitter. How these multiple-phenotype neurons may influence the function of MS-DBB and its role in the hippocampal theta generation is presently unknown.

The notion that neurons can synthesize and release more than one neurotransmitter is not new. There has been reports of co-transmission of GABA and glycine in the cerebellum, the brainstem and the spinal cord (Jonas *et al.*, 1998;Russier *et al.*, 2002;Dugue *et al.*, 2005), acetylcholine and GABA by starburst amacrine cells in the retina (O'Malley & Masland, 1989;O'Malley *et al.*, 1992;Zheng *et al.*, 2004), norepinephrine and acetylcholine by sympathetic neurons (Furshpan *et al.*, 1976), serotonin and glutamate by mesopontine neurons (Johnson, 1994) and dopamine and glutamate by midbrain neurons (Sulzer *et al.*, 1998;Joyce & Rayport, 2000). Recently, several studies have reported the co-release of acetylcholine and glutamate from neurons of the spinal cord in the developing frog tadpole (Li *et al.*, 2004) and from the developing mouse spinal motoneurons (Nishimaru *et al.*, 2005;Mentis *et al.*, 2005). Li and colleagues provided clear functional evidence that spinal motoneurons previously known to release only acetylcholine also release glutamate, by demonstrating that in whole-cell patched spinal cord neurons, spontaneous miniature excitatory post-synaptic currents (mEPSCs) were completely blocked only when antagonists for ionotropic glutamate receptors [alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and N-methyl-D-aspartate receptor (NMDAR)] and for nicotinic acetylcholine receptor (nAChR) were jointly administered (Li *et al.*, 2004). Furthermore, they demonstrated that some mEPSCs had both the fast-rising component of nAChR and the slowly-decaying component of NMDAR, indicating that some vesicles contained both acetylcholine and glutamate. They then confirmed using paired recordings of synaptically connected spinal cord neurons that the two neurotransmitters were indeed co-released from single presynaptic neurons (Li *et al.*, 2004).

GABA and glutamate co-release in the granule cells of the hippocampus have also been reported. Gutierrez and colleagues showed that glutamatergic and GABAergic phenotypes are frequently co-expressed in developing granule cells but the GABAergic phenotype eventually disappears and only the glutamatergic phenotype remains in adults. They found that the GABAergic phenotype can be transiently re-expressed in adult granule cells by increasing glutamatergic activity, administering brain-derived neurotrophic factor (BDNF) or by inducing hyperexcitable states such as seizures (Gutierrez, 2000;Gutierrez & Heinemann, 2001;Gutierrez, 2002;Gutierrez *et al.*,

2003;Gomez-Lira *et al.*, 2005). Belousov and colleagues also found that cholinergic transmission in cultured neurons of the hypothalamus could be upregulated by chronic blockade of glutamate receptors, indicating that in the absence of glutamatergic activity, acetylcholine may act as the alternative excitatory transmitter to maintain the excitation/inhibition balance in the hypothalamus (Belousov *et al.*, 2001). In summary, these findings show that co-release of multiple neurotransmitters from one neuron may not be an uncommon phenomenon in the PNS and CNS and that the neurotransmitter phenotype of a neuron can be regulated developmentally and in an activity-dependent manner.

2. Nerve Growth Factor (NGF)

2.1. Neurotrophin family

Nerve growth factor (NGF) was the first to be identified in the family of dimeric secretory proteins called neurotrophins. The landmark discovery of NGF came about when it was observed that the neurons of dorsal root ganglia (DRG) grew neurites towards mouse sarcoma cells implanted inside an embryonic chick (Bueker, 1948). The diffusible growth-promoting factor was then isolated from the mouse sarcoma cells (Cohen *et al.*, 1954) and other sources of NGF were found such as snake venom (Cohen & Levi-Montalcini, 1956) and mouse submaxillary gland (Cohen, 1960). Following this, other members of the mammalian neurotrophin family were soon discovered: brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4/5 (NT4/5). Neurotrophins are known to promote cell survival, differentiation and growth of neurons and they have also been shown to modulate synaptic connections, neurotransmitter release and synaptic plasticity (McAllister *et al.*, 1999;Huang & Reichardt, 2001;Chao, 2003). It is now recognized that neurotrophins exert these effects by binding to three classes of receptors, the tropomyosine receptor kinase (trk) family of receptor tyrosine kinases, the pan-neurotrophin receptor $p75^{NTR}$ and sortilin. $p75^{NTR}$ is a member of the tumor necrosis factor receptor (TNFR) family that also includes two TNF receptors I, II and Fas. Sortilin is a member of the family of Vps10p-domain transmembrane receptors and has previously been shown to bind neurotensin (Mazella *et al.*, 1998). In general, each member of the neurotrophin family binds preferably to a specific trk receptor with higher affinity: NGF to trkA, BDNF and NT4/5 to trkB and

NT3 to trkC, although NT3 has been known to also bind to trkA and trkB with weaker affinity (Barbacid, 1994). All neurotrophins bind to $p75^{NTR}$ with similar affinity (Rodriguez-Tebar *et al.*, 1991), and the precursor forms of both NGF and BDNF bind to sortilin (Nykjaer *et al.*, 2004;Teng *et al.*, 2005). Most trophic effects of neurotrophins are thought to be mediated through trk receptors and apoptotic actions are attributed to $p75^{NTR}$ and sortilin, but recent evidence suggests that signalling through $p75^{NTR}$ can also promote cell survival and neurite outgrowth (reviewed in Roux & Barker, 2002).

2.2. Functions

In the periphery, NGF is a well-known trophic factor for sympathetic and sensory neurons of dorsal root and trigeminal ganglia. NGF promotes their survival, neurite outgrowth and differentiation (Levi-Montalcini, 1987). In transgenic mice lacking NGF or trkA, the peripheral sympathetic and sensory neurons are almost completely lost and the mutants die within a few months of life (Crowley *et al.*, 1994;Smeyne *et al.*, 1994). In vivo and in vitro studies have shown that NGF is required for the survival of developing sympathetic and sensory neurons, but only sympathetic neurons remain NGF-dependent throughout life and mature sensory neurons no longer need NGF for survival (Greene, 1977a;Greene, 1977b;Gorin & Johnson, Jr., 1980;Johnson, Jr. *et al.*, 1980;Eichler & Rich, 1989). In addition to promoting survival, NGF has other functions such as regulating and directing neurite growth towards the source of NGF. In a compartmentalized culture system, sympathetic neurons were shown to extend neurites only into compartments that contained NGF, and if NGF was withdrawn from the compartments to which neurites had extended, further growth was stopped and the neurons began to degenerate unless the neurites were placed back into an NGF-rich chamber (Campenot, 1977).

In the CNS, NGF exerts trophic effects on basal forebrain and striatal cholinergic neurons (Dreyfus, 1989). The most dramatic illustration of the protective role of NGF comes from studies that showed that the rapid loss of cholinergic MS-DBB neurons, induced by fimbria-fornix (FF) lesion, could be largely prevented by continuous infusions of NGF into the brain of adult rats (Williams *et al.*, 1986;Hefti, 1986;Kromer, 1987;Montero & Hefti, 1988). The protective effects of exogenous NGF on survival appear to be specific to cholinergic neurons, since GABAergic septo-hippocampal

neurons also degenerated after FF lesion but their loss could not be prevented by infusions of NGF (Peterson *et al.*, 1987;Montero & Hefti, 1988;Naumann *et al.*, 1994b). The behavioural deficits in learning a maze task associated with the cholinergic atrophy in these lesioned rats could also be ameliorated by injections of NGF (Will & Hefti, 1985). However, there is some controversy as to whether NGF is absolutely required for the survival of cholinergic basal forebrain neurons in adults. Naumann and colleagues observed that although the number of ChAT-positive neurons in the medial septum is dramatically reduced in the first three weeks following FF lesion, it recovers substantially after long survival times of up to six months, suggesting that the axotomized cholinergic septo-hippocampal neurons survive and partially regain ChAT expression over time (Peterson *et al.*, 1992;Naumann *et al.*, 1992;Naumann *et al.*, 1994a;Naumann *et al.*, 1994b). Sofroniew and colleagues found that after an excitotoxic ablation of hippocampal neurons using NMDA, which spares septo-hippocampal fibres, the number of cholinergic septal neurons identified with $p75^{NTR}$ remained unchanged for up to 500 days (Sofroniew *et al.*, 1990;Sofroniew *et al.*, 1993). In these studies, cholinergic septal neurons persisted in a severely atrophied state with marked soma shrinkage and reduced ChAT activity, suggesting that in adults NGF may not be critical for survival but play a more important role for structural integrity and function of these neurons. An alternative explanation is that adult septal cholinergic neurons require so little NGF for survival that the low level of NGF derived from intraseptal glia is sufficient to sustain them. Yet another possibility is that NGF is critical only for the survival of injured or developing septal cholinergic neurons (Arimatsu *et al.*, 1989;Arimatsu & Miyamoto, 1991).

Besides its role in survival, NGF stimulates and maintains the structural integrity, cholinergic phenotype and function of cholinergic basal forebrain neurons in both developing and adult animals. NGF increases the activity of cholinergic markers such as ChAT and acetylcholinesterase (AChE) both in vivo and in vitro, while removing NGF has the opposite effect (Honegger & Lenoir, 1982;Gnahn *et al.*, 1983;Fischer *et al.*, 1987;Martinez *et al.*, 1987;Johnston *et al.*, 1987;Hartikka & Hefti, 1988). In culture, Auld and colleagues showed that acute exposure (less than 1 hour) of basal forebrain cholinergic neurons to NGF enhances acetylcholine release without immediate increases in ChAT activity, whereas long-term exposure (days) to NGF enhances both

acetylcholine release and ChAT activity (Auld *et al.*, 2001a;Auld *et al.*, 2001b). In vivo, the function of cholinergic basal forebrain neurons naturally diminishes with age, as evidenced by reductions in their soma size and in the level of ChAT and AChE, along with deficits in spatial learning (Hornberger *et al.*, 1985;Biegon *et al.*, 1986;Luine *et al.*, 1986). Fischer and colleagues showed that continuous intracerebral infusions of NGF over four weeks can partially reverse the age-dependent deficits in rats (Fischer *et al.*, 1987). Furthermore, mutant mice heterozygous for NGF deletion expressing only half the normal level of NGF display reduced activity of cholinergic markers and deficits in memory functions, which can be corrected by prolonged infusions of NGF (Chen *et al.*, 1997). In summary, NGF serves important roles for the survival, phenotype expression and normal function of cholinergic basal forebrain neurons throughout life.

2.3. Origin of NGF in the CNS

NGF is synthesized in the hippocampus, cortex and olfactory bulb, the main target regions of cholinergic basal forebrain neurons (Korsching *et al.*, 1985;Whittemore *et al.*, 1986). A low level of NGF is also synthesized by astrocytes in the basal forebrain (Houlgatte *et al.*, 1989;Bacia *et al.*, 1992). In the dentate gyrus, NGF is expressed both by granule cells and interneurons, whereas in the hippocampus proper (CA1 to CA3 regions), NGF is synthesized specifically by GABAergic interneurons and not by the far more numerous pyramidal cells (Lauterborn *et al.*, 1993). NGF mRNA expression varies for distinct subtypes of hippocampal interneurons containing different calcium-binding proteins and neuropeptides, such that the proportion of neurons expressing NGF mRNA is highest for interneurons containing parvalbumin (PV) (82%), neuropeptide-Y (71%) and somatostatin (48%) and lowest for those containing calbindin 28k (CB) (24%), calretinin (CR) (23%), cholecystokinin (21%) and vasoactive intestinal polypeptide (10%) (Rocamora *et al.*, 1996;Pascual *et al.*, 1999). NGF mRNA is also expressed by the majority (59%) of GABAergic hippocampo-septal neurons that are CB-positive, suggesting that NGF may be transported not only retrogradely but also in an anterograde manner (Acsady *et al.*, 2000). One possible mechanism for the synthesis of NGF in some interneurons but not others is that NGF production is regulated in an activity-dependent manner, since NGF expression is highest in the highly active, fast-firing interneurons

such as PV-positive basket cells and lowest in interneurons that are relatively quiet, such as CR-positive interneurons (Rocamora *et al.*, 1996).

What could be the functional significance of the cell type-specific expression of NGF? Interestingly, a majority (80%) of GABAergic septo-hippocampal fibres terminate on interneurons expressing NGF mRNA, especially the PV-positive basket cells, suggesting that the GABAergic septal afferents prefer to synapse on NGF-positive interneurons rather than NGF-negative ones (Rocamora *et al.*, 1996). This finding is puzzling in light of the generally accepted notion that in the adult, GABAergic septal neurons do not express trkA or $p75^{NTR}$ (Batchelor *et al.*, 1989;Kiss *et al.*, 1993;Steininger *et al.*, 1993;Sobreviela *et al.*, 1994), although they most likely express trkB and trkC (Merlio *et al.*, 1992;Lamballe *et al.*, 1994;Tongiorgi *et al.*, 2000). Some in vitro evidence indicates that GABAergic septal neurons may be responsive to NGF during development. Embryonic basal forebrain GABAergic neurons in culture show high-affinity NGF binding (Dreyfus *et al.*, 1989) and a small proportion (3%) of developing GABA-positive septal neurons express $p75^{NTR}$ in culture (Arimatsu & Miyamoto, 1989;Arimatsu & Miyamoto, 1991). Calbindin-positive embryonic septal neurons have been found to increase the number and total length of neurite processes in response to NGF (Silva *et al.*, 2000). Thus, it is possible that during development, GABAergic septo-hippocampal axons target particular hippocampal interneurons releasing NGF using a yet unknown mechanism. Alternatively, a proportion of hippocampal interneurons expressing NGF mRNA also co-express NT3 mRNA (Pascual *et al.*, 1998) thus it is possible that developing GABAergic septo-hippocampal axons respond to NT3 through and trkC. The role of NGF in the function of GABAergic septal neurons in adults, however, is unclear.

In contrast, cholinergic septo-hippocampal afferents do not show such a specific pattern of innervation in the hippocampus. Early studies have reported that ChAT-positive fibres and terminals are distributed in all layers of the hippocampus, most abundantly near pyramidal and granular cell layers (Frotscher & Leranth, 1985) and terminate indiscriminately on both principal and non-principal cells (Frotscher, 1991). Recent evidence demonstrates that in the dentate gyrus cholinergic septo-hippocampal axons preferentially contact hilar interneurons rather than granule cells, and that a greater percentage of interneurons expressing neuropeptide-Y (NPY) (12%) are targeted

compared to those expressing PV (2%) (Dougherty & Milner, 1999a;Dougherty & Milner, 1999b). One proposed mechanism is that cholinergic fibres may be more attracted to NPY-containing neurons because a majority of them express mRNAs for both NGF and NT3 (Pascual *et al.*, 1999). However, the innervation pattern of cholinergic septo-hippocampal afferents is largely non-specific, and the role of neurotrophins in guiding target innervation of central neurons during development remains to be further explored.

2.4. NGF signalling pathways

How does NGF exert its effects on cholinergic neurons? NGF binds to trkA and $p75^{NTR}$ on the cholinergic axon terminals, become endocytosed and retrogradely transported to the cell bodies of the cholinergic neurons (Large *et al.*, 1986;Johnson, Jr. *et al.*, 1987;Bronfman *et al.*, 2003). In the MS-DBB, virtually all (>95%) trkA-positive neurons co-express ChAT and $p75^{NTR}$, indicating that septal cholinergic neurons express both trkA and $p75^{NTR}$ (Sobreviela *et al.*, 1994). Co-expression of $p75^{NTR}$ sharpens the ligand specificity of trkA for NGF (Barbacid, 1994) and boosts NGF binding affinity by about 25-fold (Mahadeo *et al.*, 1994), greatly improving NGF signalling in an environment with limited amounts of NGF. Evidence from co-immunoprecipitation studies suggests that trkA and $p75^{NTR}$ may physically interact together at the level of plasma membrane, forming a receptor complex (Bibel *et al.*, 1999). Intracellular signalling cascades triggered by NGF binding to the two receptors can be both independent and linked. The functional outcome of such interactions can be synergistic or antagonistic depending on the cellular context.

The trkA signalling cascades involve ligand engagement, receptor dimerization, phosphorylation of cytoplasmic tyrosine (Y) residues and exposure of docking sites for specific adaptor proteins containing phosphotyrosine-binding (PTB) or src-homology-2 (SH-2) motifs that couple the receptor to intracellular signalling pathways, including the Ras, phosphatidylinositol-3-kinase (PI-3K) and phospholipase C (PLC)-γ1 pathways (reviewed in Huang & Reichardt, 2001). Extensive research has focused on the interaction of two tyrosine residues, Y490 and Y785, with their respective adaptor proteins, Shc and PLC-γ1. Briefly, Y785-PLC-γ1 interaction generates inositol tris-phosphate (IP_3) and diacylglycerol (DAG), which activate calcium-regulated protein

kinases, phosphatases and certain isoforms of protein kinase C (PKC) that lead to neurite outgrowth and activation of the ERK (extracellular signal-regulated kinase) cascade. Y490-Shc interaction results in the activation of Ras which stimulates PI-3K, c-Raf/ERK and p38MAPK pathways, resulting in the phosphorylation of CREB (cAMP-regulated enhancer binding protein) and other transcription factors important for neuronal survival. Y490 also interacts with FRS-2 (fibroblast growth factor receptor substrate-2), activating the adaptor protein Crk, exchange factor C3G, small G protein Rap1 and B-Raf, leading to the sustained activation of ERK and conferring neuronal differentiation. Finally, PI-3K activation generates phosphatidyl inositides, activating Akt (protein kinase B) which in turn regulates many proteins involved in cell survival, including the Bcl-2 family member BAD, IκB kinase, Forkhead transcription factors (FKHRL) and caspase-9. It is interesting to note that the signalling cascades initiated by Y490-Shc interaction lead to a transient activation of ERK and cell survival, whereas those involving Y490-FRS-2 interaction cause a prolonged activation of ERK and neuronal differentiation, implying that the two mechanisms are distinct in their kinetics and functional outcome. In fact, manipulations that inhibit endocytosis and retrograde transport have been shown to impair Rap1 and B-Raf activation and differentiation of PC12 cells but not Ras activation and cell survival, indicating that ligand-receptor internalization is necessary for differentiation but not for survival of neurons (Saragovi *et al.*, 1998;Zhang *et al.*, 2000;York *et al.*, 2000;Wu *et al.*, 2001). In agreement, Ras is distributed in the plasma membrane while Rap1 is found associated with endocytic vesicles, suggesting that trkA interaction with Rap1-expressing vesicles is required for neuronal differentiation (York *et al.*, 2000;Wu *et al.*, 2001).

$p75^{NTR}$ acts as a co-receptor for trkA, potentiating the NGF-trkA activities and also has its own signalling pathways that regulate apoptosis and cell survival (reviewed in Roux & Barker, 2002). The most widely accepted mechanism behind pro-apoptotic action of $p75^{NTR}$ is the c-Jun amino-terminal kinase (JNK) cascade, which leads to the release of mitochondrial cytochrome c and caspase-9 activation. Paradoxically, $p75^{NTR}$ also promotes cell survival by activating the NFκB and PI-3K/Akt pathway. Interestingly, the presence of trk activation can completely block $p75^{NTR}$-mediated apoptosis. Activation of trk signalling, especially the Ras/PI-3K pathway, has been shown to suppress the JNK cascade but not the survival-promoting NFκB pathway (Dobrowsky *et*

al., 1995;Yoon *et al.*, 1998;Mazzoni *et al.*, 1999). The bi-directional nature of $p75^{NTR}$ function may serve to refine neural circuitry during development by promoting survival in the presence of neurotrophins (i.e., correct target innervation) and cause apoptosis and neurite retraction in the absence of neurotrophins (i.e., incorrect target innervation or inactivity).

Finally, sortilin is a co-receptor for $p75^{NTR}$ and together they bind precursor forms of NGF and BDNF (reviewed in Nykjaer *et al.*, 2005). Sortilin binds the pro-domain while $p75^{NTR}$ binds the mature part of pro-neurotrophins (Nykjaer *et al.*, 2004). Neurotrophins are first synthesized as precursors and cleaved to yield the mature form, depending on the availability of proteases such as matrix metalloproteases (MMPs) and plasmin (Lee *et al.*, 2001;Pang *et al.*, 2004). In fact, a recent study demonstrated that such conversion is a highly regulated process, since NGF is produced mostly in the precursor form in the cortex and pro-NGF is released along with a host of proteases and their activators in an activity-dependent fashion into the extracellular space where the conversion takes place (Bruno & Cuello, 2006). NGF exists in the brain predominantly in its precursor form and pro-NGF can bind trkA and induce neurotrophic effects, albeit with five times less activity than mature NGF (Fahnestock *et al.*, 2004). Sortilin's affinity for pro-NGF increases about 20-fold with the co-expression of $p75^{NTR}$ (Nykjaer *et al.*, 2004), and pro-NGF binds to sortilin-$p75^{NTR}$ with a much higher affinity than mature NGF (Teng *et al.*, 2005). Recently, it has been shown that kainate-induced seizures can stimulate basal forebrain astrocytes to release pro-NGF, which binds to sortilin-$p75^{NTR}$ and causes apoptosis by preventing the trkA-mediated Ras/PI-3K pathway (Volosin *et al.*, 2006). Whether sortilin has downstream signaling pathways independent of $p75^{NTR}$ is presently unknown, but one possible role of sortilin is mediating the internalization and retrograde transport of proNGF.

2.5. Relevance to Alzheimer's disease

One of the key physiological markers of AD is the selective degeneration of cholinergic basal forebrain neurons with associated reductions in cholinergic activity in the hippocampus and the cortex (Bartus *et al.*, 1982;Coyle *et al.*, 1983;McGeer *et al.*, 1984;Whitehouse, 1998). For example, Henke and Lang reported that in AD patients, the activities of ChAT and AChE enzymes are dramatically reduced in the cortex (by up to

60%), hippocampus (by up to 97%), medial septum (by 55%) and the DBB area (by 65%), compared to age-matched controls (Henke & Lang, 1983). In animal models of AD, intraseptal injections of beta-amyloid peptide (Aβ) damage mostly ChAT-positive neurons with minor effects on GABAergic neurons (Harkany *et al.*, 1995). Intraventricular Aβ injections were also found to cause reductions in ChAT activity in the MS, cortex, hippocampus and lead to deficits in performance of the radial-arm maze task (Yamaguchi & Kawashima, 2001).

Accumulating evidence suggests that the cholinergic atrophy and behavioural deficits seen in AD are due to dysfunctional NGF signalling. In human AD brains, NGF mRNA levels in the neocortex are comparable to controls (Goedert *et al.*, 1986;Jette *et al.*, 1994) but NGF protein levels are increased in the hippocampus and the cortex (Crutcher *et al.*, 1993;Scott *et al.*, 1995;Narisawa-Saito *et al.*, 1996;Fahnestock *et al.*, 1996) and decreased in the basal forebrain (Mufson *et al.*, 1995;Scott *et al.*, 1995), pointing to the possibility that NGF retrograde transport may be affected in AD. In fact, trkA expression is reduced (Salehi *et al.*, 1996;Boissiere *et al.*, 1997;Mufson *et al.*, 1997;Hock *et al.*, 1998) while $p75^{NTR}$ levels are stable in AD (Goedert *et al.*, 1989;Hock *et al.*, 1998;Ginsberg *et al.*, 2006). An alternative hypothesis has been put forward recently proposing that there may be insufficient conversion of pro-NGF to mature NGF in AD, based on the finding that pro-NGF levels are twofold greater in AD cortex compared to control (Fahnestock *et al.*, 2001). It may be that both mechanisms are at work starting from early stages of AD, as both reduced trkA and increased pro-NGF levels are evident at prodromal stages of AD and become more prominent with the progression of the disease (Peng *et al.*, 2004;Ginsberg *et al.*, 2006), indicating that the two phenotypes may serve as biological markers for the onset of AD.

Consistent with the importance of NGF dysfunction in AD, a compelling animal model of AD is provided by transgenic mice expressing anti-NGF neutralizing antibodies. In these mice, the antibody levels are 2000 times higher in adults than in the newborn mice, so that the mice survive well into adulthood (Ruberti *et al.*, 2000). Aged anti-NGF mice show many classical symptoms of AD: extensive neuronal loss in the cortex, cholinergic atrophy in the basal forebrain and hippocampus, deficits in spatial learning tasks and age-dependent neurodegenerative pathology including accumulation of

β-amyloid plaques, tau hyperphosphorylation, neurofibrillary tangles in cortical and hippocampal neurons (Capsoni *et al.*, 2000;Capsoni *et al.*, 2002a). Capsoni and colleagues further showed that many of the AD-like neuropathological characteristics seen in the aged anti-NGF mice could be rescued by pharmacological treatments such as NGF agonist LT4, a thyroid hormone that increases endogenous NGF, cholinergic agonist galantamine and intranasal administration of NGF (Capsoni *et al.*, 2002b). Behavioural deficits in memory tasks observed in these mice could also be reversed with exogenous NGF (De Rosa *et al.*, 2005). The fact that many of the disease phenotypes seen in the aged anti-NGF mice are largely preventable by administration of exogenous NGF at early stages of the pathology supports the view that a lack of sufficient NGF signalling is a key factor in AD development.

Accordingly, there has been much excitement over NGF as a potential treatment for AD. In a recent report of a phase-1 clinical trial of ex vivo NGF gene therapy, autologous fibroblasts genetically modified to express human NGF were implanted into the basal forebrain of individuals with mild AD, and after an average follow-up of 22 months significant improvements in the rate of cognitive decline and increases in cortical glucose uptake were observed without long-term adverse effects (Tuszynski *et al.*, 2005). In the past, preclinical studies using animals have shown that direct intraventricular infusion of NGF is not a viable treatment option since it causes undesirable side-effects such as back pain, weight loss and Schwann cell migration into the spinal cord and medulla, due to non-selective diffusion of NGF into other areas of the brain (Tuszynski, 2002). Another promising candidate for potential AD treatment strategies is a selective partial agonist ligand for trkA, which has been shown to increase cholinergic phenotype in the brain and improve performance in memory tasks in cognitively impaired aged rats (Bruno *et al.*, 2004). Taken together, NGF is crucial for the survival, phenotype expression and normal function of cholinergic basal forebrain neurons, and dysfunction in NGF signalling likely plays an important role in the neurodegeneration and cognitive decline seen in AD.

3. Research Objectives

    3.1. Functional co-release of multiple neurotransmitters from single MS-DBB neurons

The first aim of the current research project was to determine whether a single MS-DBB neuron can functionally co-release more than one neurotransmitter. As discussed above, previous studies employing sc-RT-PCR and immunohistochemistry have provided evidence that a proportion of VGLUT- or glutamate-positive MS-DBB neurons also express ChAT, suggesting that some neurons possess the biochemical machinery necessary to use both acetylcholine and glutamate as neurotransmitters (Sotty *et al.*, 2003;Danik *et al.*, 2005;Colom *et al.*, 2005). Allen and colleagues recently demonstrated using microisland cultures that glutamate and acetylcholine can indeed be released simultaneously from single MS-DBB neurons (Allen *et al.*, 2006). We also established a microisland culture system and used 192IgG-Cy3, a fluorescently labelled antibody against $p75^{NTR}$, to identify cholinergic MS-DBB neurons. Since $p75^{NTR}$ is selectively expressed by cholinergic neurons in the MS-DBB (Hartikka & Hefti, 1988;Sobreviela *et al.*, 1994;Hartig *et al.*, 1998), 192IgG-Cy3 has been used in previous studies as a tool for identifying cholinergic septal neurons (Wu *et al.*, 2000;Alreja *et al.*, 2000). Our objective was to confirm the presence of functional co-release of acetylcholine and glutamate from single MS-DBB neurons as reported by Allen and colleagues, and to provide additional information on the occurrence of other combinations of neurotransmitters co-transmitted by MS-DBB neurons.

3.2. Effects of NGF on synaptic transmission of distinct MS-DBB neuronal populations and mechanisms underlying the action of NGF

The second objective of the project was to investigate the effects of chronic exposure to NGF on synaptic function of MS-DBB neurons releasing different neurotransmitters. As discussed above in detail, NGF is a well-known neurotrophic factor for cholinergic basal forebrain neurons. In fact, impaired NGF signalling and selective degeneration of cholinergic basal forebrain neurons are associated with Alzheimer's disease. Moreover, recent clinical-trial studies have reported that administering NGF into the basal forebrain of patients with mild AD may slow the rate of their cognitive decline (Tuszynski *et al.*, 2005). However, the effects of NGF on other populations of basal forebrain neurons, namely the GABAergic and glutamatergic neurons are virtually unknown. It remains to be shown whether the trophic effects of NGF are specific to cholinergic basal forebrain neurons and whether NGF can also affect non-cholinergic

neurons. It is an intriguing possibility to consider that NGF may be important for the function of non-cholinergic neurons and that some of the cognitive impairments associated with AD may reflect dysfunctions in these neurons. We tested this hypothesis by administering NGF to isolated MS-DBB neurons grown on microislands and examined the effects of NGF on the synaptic transmission in MS-DBB neurons releasing different neurotransmitters. The possible mechanisms underlying the action of NGF were also explored using K252a, an inhibitor for trk receptors, and MRL3, a monoclonal antibody against $p75^{NTR}$ that has been shown to inhibit NGF binding to $p75^{NTR}$, to study the role of trkA vs. $p75^{NTR}$ signalling in NGF-mediated changes in synaptic transmission.

Methods

1. Cell culture

MS-DBB neuron micro-cultures were prepared using a protocol described by Congar and colleagues (2002) with some modifications. MS-DBB neurons from 10- to 13-day old rats were plated on pre-treated glass coverslips containing microislands occupied by hippocampal astrocytes. All drugs were obtained from Sigma, St. Louis, MO, unless otherwise noted.

Round glass coverslips (12 mm in diameter) were sterilized by treating with concentrated hydrochloric acid, sterile water and 95% ethanol and flamed inside a culture hood. The surface was treated with poly-L-ornithine (0.04 mg/ml) and left for 24 hours, rinsed with water and thoroughly dried overnight. In order to make the surface nonpermissive for cell attachment, it was coated with a thin film of 0.15% solution of type-II agarose that had been briefly heated in the microwave to fully dissolve the agarose. After the agarose has completely dried overnight, the coverslips were sprayed with a 0.75 mg/ml solution of sterile liquid collagen (bovine type-I; Cohesion Technology, Palo Alto, CA) using a glass microatomizer (Kontes Glass Co., Vineland, NJ) to create microislands of permissive environment. The coverslips were sterilized for 30 minutes under UV light and placed in 12-well plates and left to equilibrate at 37°C in a 5% CO_2 incubator until further use.

Hippocampal astrocytes were obtained from 1-day-old Sprague-Dawley rat pups (Charles River Canada, St Constant, Quebec). The pups were anesthetized by placing them on ice, wiped with 70% ethanol and the brain was rapidly extracted according to protocols and guidelines approved by McGill University and the Canadian Council of Animal Care. The brain was placed into an ice-cold home-made dissociation solution containing (in mM) 90 Na_2SO_4, 30 K_2SO_4, 20 glucose, 5.8 $MgCl_2$, 0.25 $CaCl_2$, 10 HEPES and 0.001% phenol red, adjusted to pH 7.4 with NaOH. The brain was transferred to an acrylic brain matrix (World Precision Instruments, Sarasota, FL) to be cut into 1-mm-thick coronal sections. Hippocampal tissue was surgically isolated under the microscope and placed in the above-mentioned dissociation solution supplemented with 20 units/ml papain (Worthington Biochemical Corp., Lakewood, NJ) and cysteine-HCl (0.45 mg/ml). For enzymatic dissociation, tissue fragments were gently swirled in

this papain solution using a submersible magnetic stirrer (Fischer Scientific Co., Pittsburgh, PA) in a waterbath kept at 37°C for 60 minutes. The tissue fragments were then rinsed twice with a solution containing trypsin inhibitor and bovine serum albumin, both at 2.5 mg/ml, and a supplemented basal medium eagle (BME) solution hereto referred to as BME+, which contains BME, glucose (4 mg/ml), 0.1% Mito+ serum extender (Becton Dickinson Labware, Bedford, MA), 1% penicillin/streptomycin (Invitrogen, Burlington, Ontario), 1% GlutaMAX-1 (Invitrogen) and 10% heat-inactivated fetal calf serum (Invitrogen). The cell solution was triturated in BME+ delicately using borosilicate glass serological pipettes with 2-mm and 0.5-mm openings. After homogenization, the cell suspension was diluted with additional BME+ and placed in Nunclon surface cell culture flasks and maintained at 37°C in a 5% CO_2 incubator.

After sufficient time has passed allowing the cells to firmly attach to the bottom of the flasks (e.g., 48 hours), the cells were treated with cold (4°C) BME and the flasks were vigorously shaken to eliminate loosely attached cells. The remaining healthy astrocytes were fed with warm (37°C) BME+ solution and left undisturbed for 5 days in the incubator to allow proliferation and growth to approximately 90% confluence inside the flasks. On the 6th day, astrocytes from one flask was harvested and plated on the pretreated coverslips, while the astrocyte-conditioned media from the other flasks was collected to be used later as part of the culture media. For harvesting, the flask was once again vigorously shaken and the cells were rinsed twice in versene (Invitrogen) and treated with a PBS solution containing trypsin (0.5 mg/ml) to detach the cells from the bottom of the flask. After 5 minutes, additional versene was added and a glass pipette with 0.5-mm opening was used to delicately triturate the cells. The cell suspension was collected into a tube and centrifuged. The supernatant was removed and the pellet was re-suspended in 1ml of BME+. The number of living cells in 5 µl of the cell solution was counted under the microscope using trypan blue to exclude dead cells from being counted and a hemacytometer (Hausser Scientific, Horsham, PA), and the astrocytes were plated at a concentration of 60,000 living cells per ml on the pre-treated coverslips. After the astrocytes have had sufficient time to attach to the collagen microislands (i.e., after 3 hours), 1 ml of BME+ was added to each well. After 24 hours, mitotic inhibitor 5-Fluoro-2'-deoxyuridine (FDUR) (5 µM) was added to the media to inhibit astrocyte

proliferation. After additional 24 hours, MS-DBB neurons were plated on the astrocytic islands, according to the following procedure.

MS-DBB neurons were obtained from 10- to 13-day old rats, usually the siblings of the pups used for acquiring the astrocytes. The procedure is identical to that for obtaining astrocytes up to the enzymatic dissociation step with a few exceptions; rats were anesthetized by isoflurane inhalation before decapitation and the MS-DBB was collected instead of the hippocampus. The MS-DBB tissue fragments were kept in the papain solution at 37°C and stirred for 30 minutes. After being rinsed twice with dissociation solution, tissue fragments were placed in a trituration solution containing trypsin inhibitor and bovine serum albumin, both at 0.4 mg/ml, and a supplemented Neurobasal solution hereto referred to as Neurobasal+, which contains Neurobasal-A medium (Invitrogen) supplemented with 1% penicillin/streptomycin, 1% GlutaMAX-1, 2% B-27 supplement (Invitrogen) and 10% heat-inactivated fetal calf serum. The tissue fragments were gently triturated using glass pipettes with 2-mm and 0.5-mm openings. After homogenization, the cell solution was combined with Neurobasal+ solution, supplemented with trypsin inhibitor and bovine serum albumin, both at 10 mg/ml and centrifuged. The supernatant was removed and the pellet was re-suspended in 500 µl of trituration solution, and the number of living cells in 5 µl of the cell solution was counted using a hemacytometer and trypan blue. The coverslips containing astrocytic microislands were picked up one-by-one with forceps and excess media was removed from each coverslip by lightly dabbing a corner of the coverslip on a sterile filter paper, and the coverslips were transferred to new, dry 12-well plates. Taking caution not to let the coverslips dry out, neurons were quickly plated at a concentration of 80,000 living cells per ml on the pre-established microislands. After the neurons have had sufficient time to attach to the microislands (i.e., after 3 hours), 1 ml of culture media containing 33% astrocyte-conditioned media and 67% Neurobasal+ was added to each well. After 24 hours, half the media in each well was removed and replaced with an equal volume of freshly made culture media, and FDUR (10 µM) was also added at this time to inhibit proliferation of astrocytes that may have been plated along with the neurons. Culture media was changed in this fashion every 7th day for 2 to 4 weeks until the day neurons on the coverslips were taken to be recorded.

2. NGF, K252a and MLR3 treatments

A group of neurons received 25 ng/ml mouse NGF (grade I; Alomone Labs, Jerusalem, Israel) on the first day of culture and twice weekly thereafter until the day of recording. Another group of neurons received no NGF to serve as controls. In certain experiments, K252a (Alomone Labs), a trk inhibitor, was applied at 200 nM on its own or in conjunction with 25 ng/ml NGF, 30 minutes prior to the addition of NGF. In some experiments, MLR3, a high-affinity monoclonal antibody raised against the extracellular portion of recombinant human $p75^{NTR}$ by immunizing $p75^{NTR}$-knockout mice (disrupted in exon III of the $p75^{NTR}$ gene), was applied at 10 μg/ml on its own or along with NGF, 30 minutes prior to NGF addition. The MLR3 and two other similar monoclonal antibodies against $p75^{NTR}$ (MLR1, MLR2) have been characterized and confirmed to bind to human, rat and mouse $p75^{NTR}$, inhibit effects of NGF mediated though $p75^{NTR}$ and become internalized by neurons of the medial septal area in mice (Rogers *et al.*, 2006). The MLR3 antibody was kindly provided by Dr. Mary-Louise Rogers, Flinders University, Australia.

3. Electrophysiology

Electrophysiological recording was performed at room temperature on neurons maintained in culture for 2 to 4 weeks. Coverslips were transferred into a custom-made recording chamber and continuously perfused at a rate of 1 to 2 ml/min with an extracellular bathing solution containing (in mM) 140 NaCl, 10 glucose, 6 sucrose, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, adjusted to pH 7.3 with NaOH. Drugs were bath-applied using a six-channel valve controller connected to a pressure regulator. Neurons were visualized and the status of its isolation on an astrocytic island was checked using a 20x water immersion objective on an upright BX51W1 Olympus microscope (Olympus, Melville, NY) equipped with Nomarsky optics and an infrared camera. The recording was performed using an Axopatch-1C amplifier (Axon Instruments, Foster City, CA) and pClamp 10 software (Axon Instruments). The recording pipette resistance was between 4 and 6 MΩ and the intrapipette solution contained (in mM) 144 potassium gluconate, 10 HEPES, $MgCl_2$, 2 Na_2ATP, 0.3 GTP, 0.2 EGTA, adjusted to pH 7.2 with KOH and filtered at 0.2 μm. The pipette was visually guided to each isolated neuron, and the neurons were recorded using a whole-cell patch-clamp technique. Recording was

continued on a cell only if the resting membrane potential was less than -45mV and the action potentials overshot 0mV.

To measure the autaptic current, the cell was initially held at -60mV in voltage clamp, depolarized to +15mV for 1 msec to evoke an action potential, followed by a step to -85mV for 150 msec to record the postsynaptic current mediated by the synaptic action of neurotransmitters released from the autaptic neuron onto its own dendrites and soma. To determine the reversal potential of the autaptic current, the protocol was repeated with the cell held at different membrane potentials between -85 to -25mV for 150 msec after the action potential. Once the presence of functional autaptic connections was verified, the chemical identity of the neurotransmitter was investigated using the following protocol. The neuron was depolarized to fire an action potential as described above and the membrane potential after the spike was held at -80mV for 150 msec and the traces were repeated every 60 seconds to avoid neurotransmitter depletion. Once a stable baseline has been established for the amplitude of autaptic current, drugs were bath-applied to determine whether the autaptic current was due to the release of acetylcholine, glutamate and/or GABA. D-tubocurarine was used at 10 μM to block currents mediated by nicotinic receptors, and kynurenic acid and 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX) were used at 1 mM and 50 μM, respectively, to block ionotropic glutamatergic currents. (-)-Bicuculline methiodide or (-)-bicuculline methochloride (Tocris, Ellisville, MO) was used at 50 μM to block currents mediated by $GABA_A$ receptors.

An autaptic current was classified as "inward" current or excitatory postsynaptic current (EPSC) if the reversal potential was more positive than -40mV or if the current was fully inhibited by blockers of glutamatergic and/or nicotinic currents. The current was characterized as "outward" current or inhibitory postsynaptic current (IPSC) if the reversal potential was more negative than -50mV or if the current was completely blocked by bicuculline. The current was categorized as being both inward and outward if the current was fully blocked only by co-administering kynurenic acid/DNQX and bicuculline, or tubocurarine and bicuculline, or all three types of blockers together. In experiments where glutamatergic and nicotinic current amplitudes were compared across treatment conditions, the difference between the peak amplitude of the current before and during a particular drug application (the average of 2-3 points for each value) was taken

as the size of the particular current. In some cases, one antagonist was applied followed by washout of the drug, and then the antagonist was administered in combination with a second drug and if some current is left over during the first drug but eliminated by the combination, the remaining component was considered to be a current blocked by the second drug. Results from the pharmacological experiments were included in the analysis only if the current amplitude after the washout of an antagonist changed less than 30% from the baseline value before drug application.

To investigate the mechanisms underlying the NGF-mediated changes in the amplitude of EPSCs, miniature EPSCs (mEPSCs) were recorded from the autaptic neurons. For these experiments, a slightly different extracellular bathing solution was used with higher K^+ and Ca^{2+} concentrations: (in mM) 140 NaCl, 10 glucose, 6 sucrose, 5 (instead of 3) KCl, 2.8 (instead of 2) $CaCl_2$, 2 $MgCl_2$, 10 HEPES, adjusted to pH 7.3 with NaOH. The intrapipette solution was not changed. Initially, the neuron was held in voltage clamp, evoked to fire an action potential to check for the presence of an autaptic current. After the reversal potential of the autaptic current was determined, the neuron was perfused for at least 5 minutes with extracellular solution containing 0.5 μM tetrodotoxin and 10 μM bicuculline to block action potentials and to prevent miniature IPSCs from contaminating the traces. The neuron was then held at -70mV in voltage clamp for recording of spontaneous mEPSCs. Preliminary experiments revealed that the autaptic neurons displayed very little spontaneous events (much like Congar *et al.*, 2002), making it extremely difficult to obtain enough data for the analysis of frequency and amplitude of the events. Thus, later experiments were carried out in the presence of a Ca^{2+} ionophore inonomycin (2.5 μM) to increase the frequency of the miniature events. Ionomycin has been shown to induce transmitter release by increasing calcium influx into neurons (Capogna *et al.*, 1996;Congar *et al.*, 2002). We observed that ionomycin did increase the frequency of mEPSCs in the autaptic neurons. After the frequency of miniature events increased and reached a stable value in the presence of ionomycin, a fixed number of consecutive mEPSCs were selected from each recording and analyzed using MiniAnalysis 6.0.3 software (Synaptosoft, Leonia, NJ).

4. $p75^{NTR}$-labelling and visualization

Twenty-four hours before recording, neurons were treated with 0.3 μg/ml 192IgG-Cy3 (Advanced Targeting Systems, San Diego, CA), a fluorescently tagged monoclonal antibody against $p75^{NTR}$ to identify cholinergic MS-DBB neurons. $p75^{NTR}$ is selectively expressed by cholinergic neurons in the MS-DBB (Hartikka & Hefti, 1988;Sobreviela *et al.*, 1994;Hartig *et al.*, 1998), making 192IgG-Cy3 a reliable tool for visualizing living cholinergic neurons during recording (Wu *et al.*, 2000;Alreja *et al.*, 2000). The antibody was administered 24 hours prior to recording, so that the action of NGF which is applied chronically starting from the first day of culture for 2 to 4 weeks would be least affected by the brief exposure to the labeled antibody. During recording, isolated neurons were placed at the centre of the visual field and illuminated at a 530-nm excitation wavelength using a fluorescence system (DeltaRam; PTI, Monmouth Junction, NJ) controlled by dedicated software (Felix; PTI). The presence of the red Cy3 labeling in the cell soma was visually verified. Depending on the experiment, either labeled, unlabeled or both types of neurons were selected for recording.

Results

1. Effects of NGF on synaptic transmission of cholinergic MS-DBB neurons

First we asked: does chronic exposure to NGF affect the synaptic function of cholinergic MS-DBB neurons? To answer this question, MS-DBB neurons obtained from 10- to 13-day old rats were grown on astrocytic microislands for 2-4 weeks, until they established synaptic connections onto their own soma and dendrites. One group of neurons received NGF (25 ng/ml) chronically while another group received none. Using whole-cell electrophysiology, we recorded evoked autaptic currents as a measure of the extent of synaptic connections established by each neuron. To record evoked autaptic currents, the cell was first held in voltage clamp at -60mV, then briefly depolarized to +15mV to evoke an action potential, causing the release of neurotransmitters that act on receptors on the cell's own postsynaptic membrane to produce excitatory or inhibitory postsynaptic currents (EPSCs or IPSCs). The peak amplitude of the autaptic current following an action potential was measured for each neuron while the membrane was held at -85mV (Figure 1B). 192IgG-Cy3 was used to visually identify cholinergic MS-DBB neurons at the time of recording (Figure 1A). We found that cholinergic neurons that were exposed to NGF exhibited much larger EPSCs compared to those grown without NGF, as shown in the examples in Figure 1B. When the peak amplitudes of EPSCs from all cholinergic neurons recorded over five cultures were normalized and pooled together for analysis, there was a highly significant, 4-fold increase in the size of evoked EPSCs due to NGF (Figure 1C). This indicates that chronic exposure to NGF significantly increases excitatory synaptic transmission from cholinergic MS-DBB neurons.

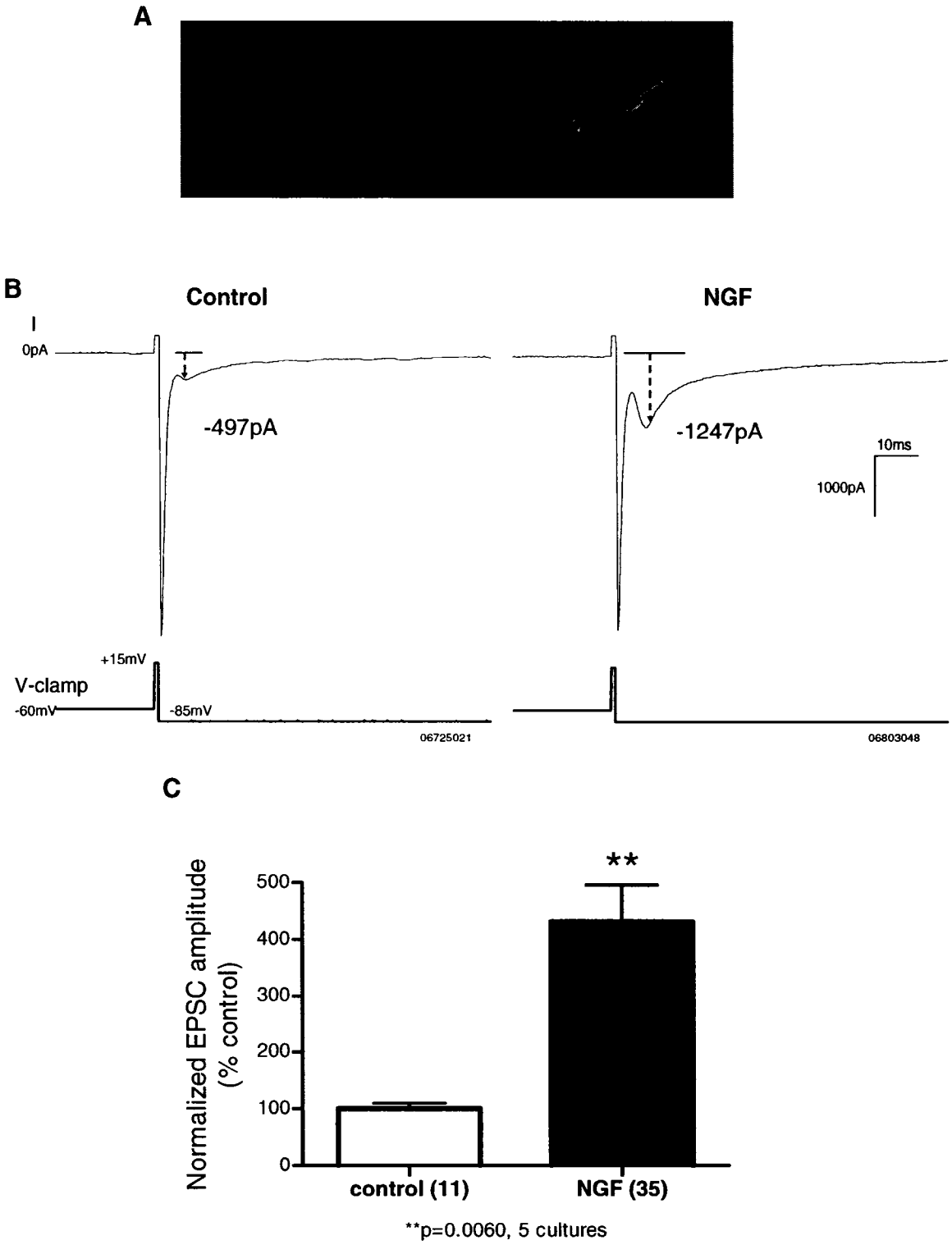


Figure 1. Effects of NGF on synaptic transmission of cholinergic MS-DBB neurons. **A.** Cholinergic neurons were visually identified at the time of recording by using 192IgG-

Cy3, a fluorescent antibody against $p75^{NTR}$. The presence of the red Cy3 labeling in the cell soma was checked using a fluorescence system to identify cholinergic neurons before patching. **B.** Examples of evoked EPSCs recorded from cholinergic MS-DBB neurons grown in control vs. NGF conditions. **C.** Chronic exposure to NGF induced a 4-fold increase in the amplitude of evoked EPSCs in cholinergic MS-DBB neurons. The effect of NGF was highly significant over 5 cultures. Numbers in brackets are number of neurons recorded.

2. Effects of NGF on synaptic transmission of non-cholinergic MS-DBB neurons

We next wanted to know whether NGF has similar effects on the synaptic function of non-cholinergic MS-DBB neurons. This time, we selected neurons that were not labeled with 192IgG-Cy3 and compared the peak amplitudes of evoked autaptic currents in neurons grown in control vs. NGF conditions. We found that NGF had no significant effect on the size of EPSCs or IPSCs displayed by non-cholinergic neurons (Figure 2A). For pharmacological characterization of neurotransmitters involved in the EPSCs and IPSCs, neurons were held at -80mV after an evoked action potential, so that both EPSCs and IPSCs were negative-going at this membrane potential. The neuron in Figure 2B had an EPSC that was fully blocked by DNQX, a blocker for kainate/AMPA receptors, indicating that the neuron released glutamate. The neuron in Figure 2C exhibited an IPSC that was fully blocked by bicuculline, a $GABA_A$ receptor antagonist, indicating that the neuron released GABA. Therefore, NGF has no significant long-term effects on synaptic transmission of non-cholinergic neurons releasing glutamate or GABA.

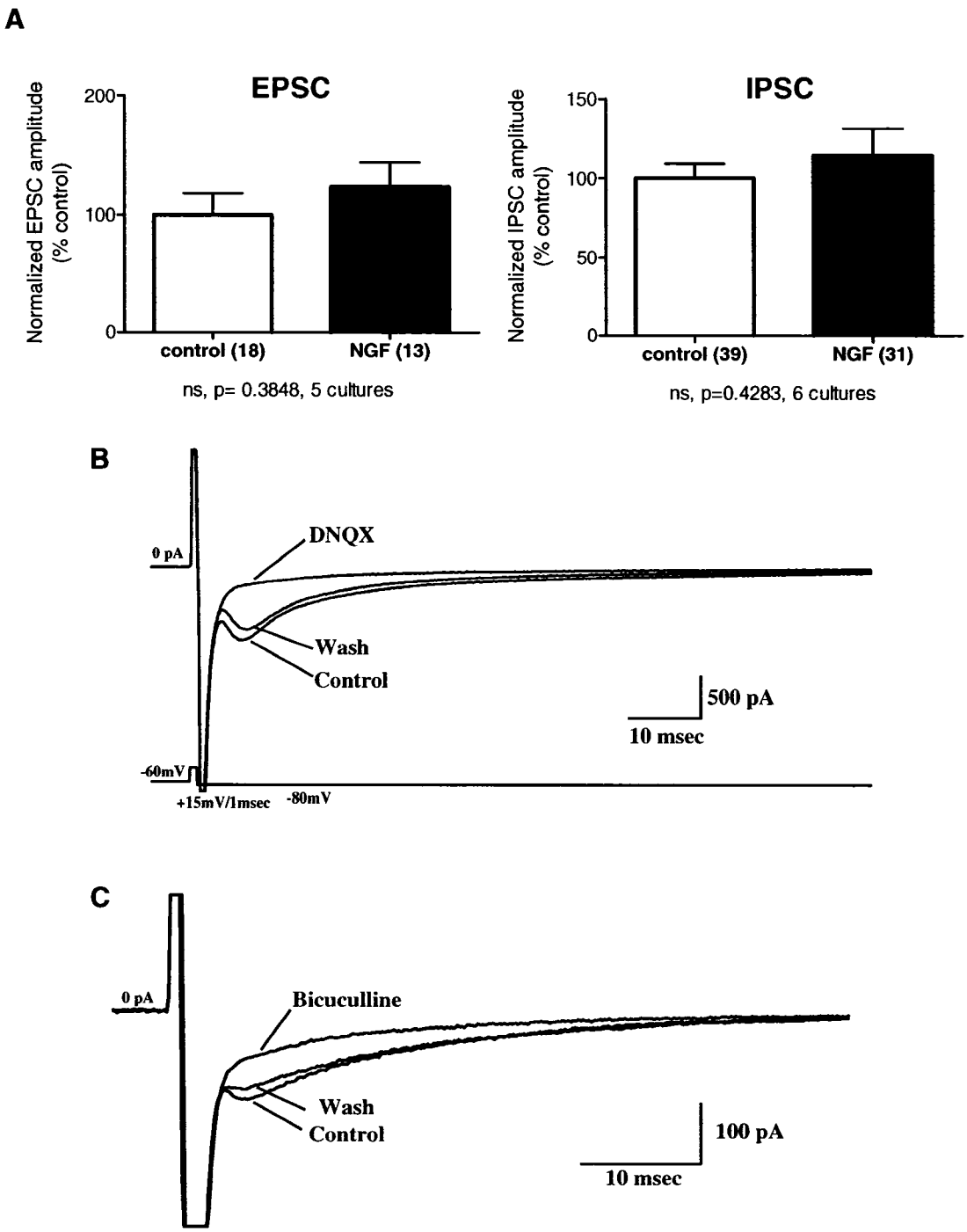


Figure 2. Effects of NGF on synaptic transmission of non-cholinergic MS-DBB neurons. **A.** Chronic exposure to NGF had no significant effects on the amplitude of evoked EPSCs or IPSCs in non-cholinergic MS-DBB neurons, not labeled with 192IgG-Cy3. Numbers in brackets are number of neurons recorded. **B.** EPSCs in non-cholinergic neurons were mediated by glutamate release. This is an example of a neuron displaying a

glutamatergic autaptic current. In voltage clamp, the membrane was initially held at -60mV, then briefly depolarized to +15mV to evoke an action potential, followed by a step to -80mV for 150msec to record the postsynaptic current. The EPSC was completely and reversibly blocked by 50 μM DNQX, a kainate/AMPA receptor blocker. **C.** IPSCs in non-cholinergic neurons were mediated by GABA release. This is a typical neuron with a GABAergic autaptic current. The IPSC was completely and reversibly blocked by 50 μM bicuculline methiodide, a $GABA_A$ receptor blocker. Both neurons were cultured without NGF for 25 days before recording and were not labelled with 192IgG-Cy3.

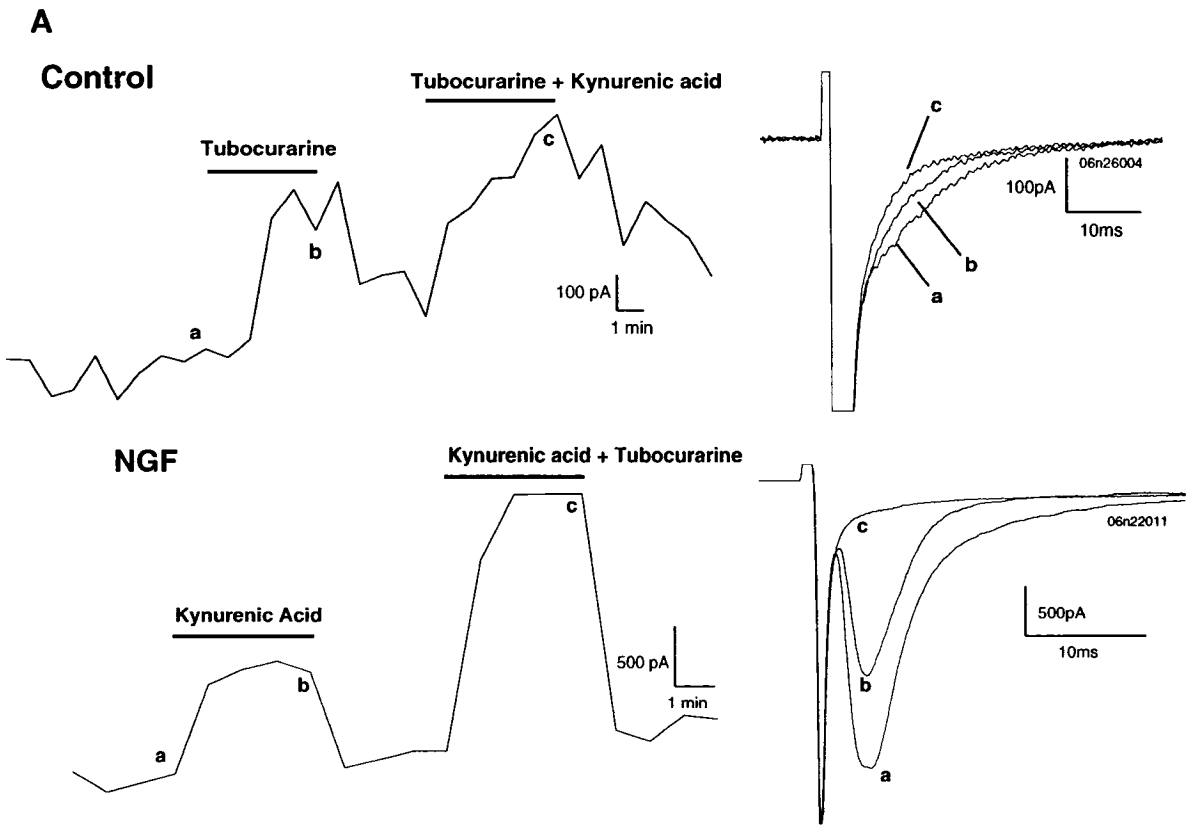
3. Effects of NGF on cholinergic and glutamatergic transmission in cholinergic MS-DBB neurons

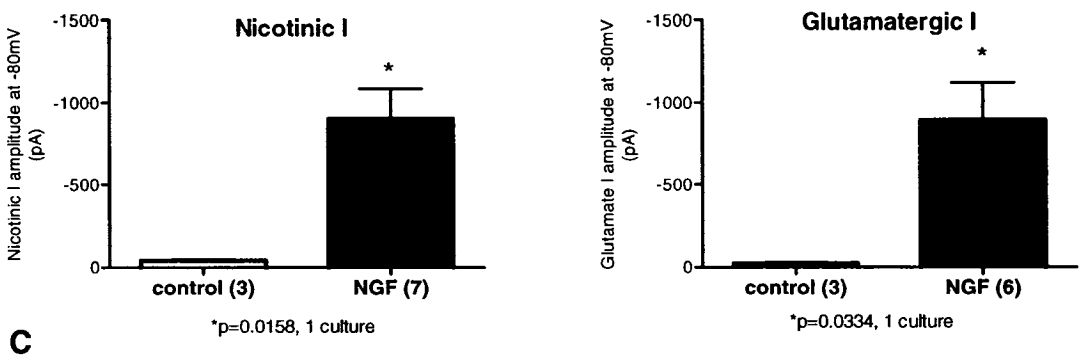
So far, we have demonstrated that chronic exposure to NGF selectively increases excitatory synaptic transmission from cholinergic MS-DBB neurons, while having no significant effects on glutamate or GABA transmission from non-cholinergic MS-DBB neuron. Since there is some evidence showing that cholinergic basal forebrain neurons can release both acetylcholine and glutamate in culture (Allen *et al.*, 2006), we next investigated whether cholinergic MS-DBB neurons in our cultures can also release two neurotransmitters simultaneously and if so, how this transmission is affected by NGF.

To explore these questions, careful pharmacological experiments were carried out to examine whether more than one neurotransmitter is responsible for the excitatory autaptic currents observed in cholinergic MS-DBB neurons. Once again, only neurons labeled with 192IgG-Cy3 were selected for recording. Kynurenic acid was used to reveal glutamatergic components of the EPSCs, and tubocurarine was used to reveal nicotinic components of the EPSCs. If one drug was found to only partially block the current, two drugs were administered together to verify that the current became completely blocked (Figures 3A).

Remarkably, NGF increased both nicotinic and glutamatergic currents expressed by cholinergic MS-DBB neurons (Figure 3B), demonstrating that chronic exposure to NGF increases both acetylcholine and glutamate transmission from cholinergic MS-DBB neurons. We also found that the majority of cholinergic MS-DBB neurons in our study released glutamate (78% of neurons in control, 86% in NGF condition). Most $p75^{NTR}$-expressing cholinergic neurons found in the control condition exhibited small inward currents that were completely or at least partially blocked by kynurenic acid or DNQX. In some cases, there were small components of the autaptic current remaining during the application of glutamate receptor antagonists and the current was completely abolished only by co-administering kynurenic acid and tubocurarine (Figure 3A) or tubocurarine and bicuculline together. In one instance, a cholinergic neuron showed an outward current that was fully blocked by bicuculline alone. Interestingly, exposure to NGF seemed to increase the proportion of neurons expressing nicotinic current alone or nicotinic and glutamatergic currents together (Figure 3C). These findings demonstrate

that the majority of cholinergic MS-DBB neurons do functionally release glutamate with or without NGF and that some of these neurons also co-release acetylcholine and/or GABA.


A
Control
Tubocurarine + Kynurenic acid
Tubocurarine
a
b
c
100 pA
1 min
06n26004
100pA
10ms
NGF
Kynurenic acid + Tubocurarine
Kynurenic Acid
500 pA
1 min
06n22011
500pA
10ms


B
Nicotinic I
Nicotinic I amplitude at -80mV (pA)
-1500
-1000
-500
0
control (3)
NGF (7)
*p=0.0158, 1 culture
Glutamatergic I
Glutamate I amplitude at -80mV (pA)
control (3)
NGF (6)
*p=0.0334, 1 culture

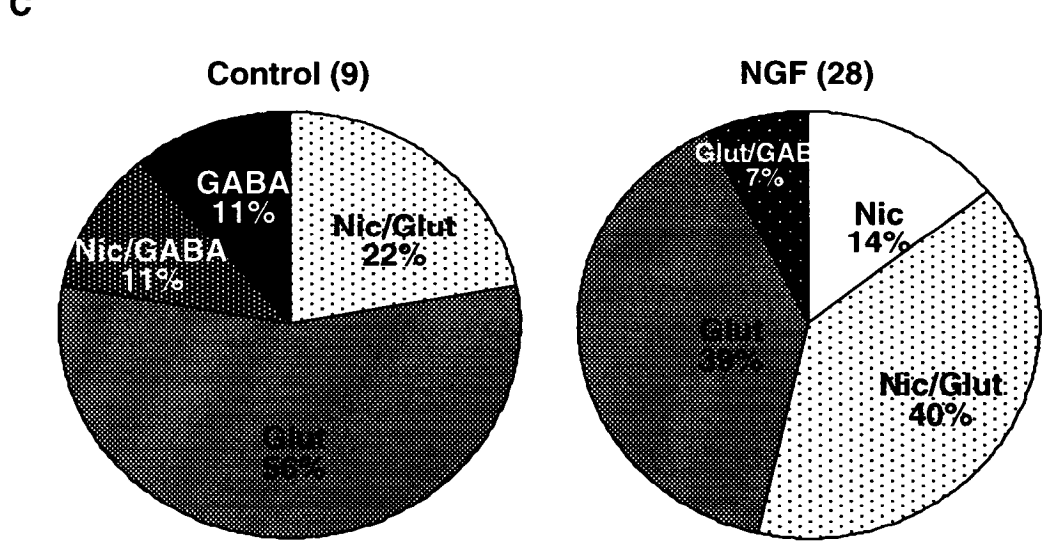

C
Control (9)
GABA 11%
Nic/Glut 22%
Nic/GABA 11%
Glut 56%
NGF (28)
Glut/GAB 7%
Nic 14%
Glut 39%
Nic/Glut 40%

Figure 3. Effects of NGF on cholinergic and glutamatergic transmission in cholinergic MS-DBB neurons. **A.** Examples of $p75^{NTR}$-labelled cholinergic neurons (one in control and one in NGF condition) exhibiting inward currents that were fully blocked by co-administration of tubocurarine (10 μM) and kynurenic acid (1mM). Notice the difference in inward current amplitudes between the neurons grown in control vs. NGF conditions. **B.** Chronic exposure to NGF increased the amplitude of both nicotinic and glutamatergic currents in cholinergic MS-DBB neurons. **C.** Most cholinergic neurons grown in control or NGF conditions displayed glutamatergic currents alone or in conjunction with nicotinic or GABAergic currents.

4. Role of trkA- vs. $p75^{NTR}$-mediated signaling in the effects of NGF on synaptic function of cholinergic MS-DBB neurons

Now that we have determined that NGF increases both acetylcholine and glutamate transmission from cholinergic MS-DBB neurons, we next investigated the mechanisms with which NGF exerts these effects. For example, which NGF receptor is responsible for mediating these effects on cholinergic MS-DBB neurons, which express both trkA and $p75^{NTR}$?

In order to study the role of trkA, we used K252a, a widely used inhibitor for trk receptor activity. To examine the role of $p75^{NTR}$, we did a preliminary study where we used MLR3, a high-affinity monoclonal antibody for $p75^{NTR}$ that has been shown to inhibit $p75^{NTR}$-mediated effects of NGF (Rogers *et al.*, 2006). For both K252a and MLR3, we administered to a group of MS-DBB neurons either alone or in conjunction with NGF.

To our surprise, inhibiting trkA activity with K252a had no discernible effects on the NGF-induced increase in the amplitude of inward currents and nicotinic currents (Figure 4A,B) but led to a marked reduction in the effect of NGF on glutamatergic currents (Figure 4C). Conversely, inhibiting $p75^{NTR}$-mediated signalling with MLR3 had little effect on NGF-mediated increase in inward and glutamatergic currents (Figure 4D,E) but showed a tendency to reduce the increase in nicotinic currents (Figure 4F), although this was only a preliminary study and the effects were only marginally significant.

These findings suggest that chronic exposure to NGF increases both acetylcholine and glutamate transmission in cholinergic MS-DBB neurons, but the mechanism with which NGF enhances the two types of excitatory transmission is different. The action of NGF on glutamate transmission is mediated by trkA receptors, while the effect of NGF on acetylcholine transmission seems to be mediated mostly by $p75^{NTR}$.

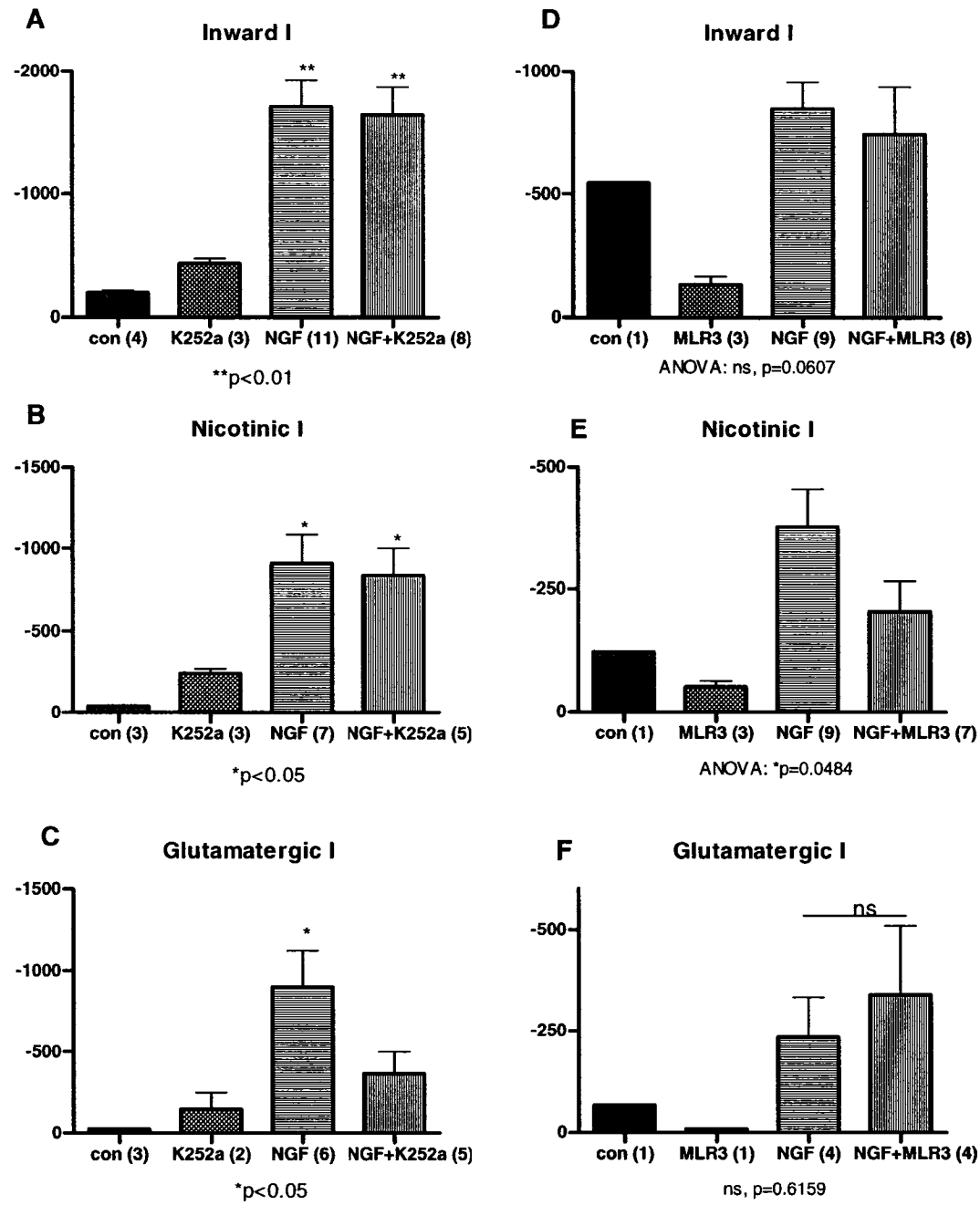


Figure 4. Role of trkA- vs. $p75^{NTR}$-mediated signaling in the effects of NGF on synaptic function of cholinergic MS-DBB neurons. Application of K252a (200nM), a trk inhibitor, had no significant effect on NGF-induced increases in EPSCs (**A**) or nicotinic currents (**B**) but significantly reduced the effects of NGF on glutamatergic currents (**C**). Conversely, administration of MLR3 (10μg/ml), a function-blocking monoclonal

antibody for $p75^{NTR}$, had no significant effect on NGF-mediated increases in EPSCs (**D**) or glutamatergic currents (**F**) but showed a tendency to reduce the effects of NGF on nicotinic currents (**E**).

5. Synaptic mechanisms underlying the effects of NGF on synaptic transmission of cholinergic MS-DBB neurons

We have demonstrated that chronic exposure to NGF increases transmission of acetylcholine and glutamate from cholinergic MS-DBB neurons. However, we have not clarified whether the effects of NGF on synaptic function are due to changes in presynaptic (transmitter release), postsynaptic (receptor expression) or both components of neurotransmission. Furthermore, NGF has been shown to increase the cell size of cholinergic septal neurons, and the increase in neurotransmission that we see in cholinergic MS-DBB neurons may be an indirect effect of increased cell size.

To investigate these issues, we first looked at whether cholinergic MS-DBB neurons respond to NGF by increasing cell size in our cultures. We took the membrane capacitance of the cell as a measure of cell size and found that NGF significantly increased the membrane capacitance of cholinergic MS-DBB neurons (Figure 5A). The mechanism with which NGF modulates cholinergic cell size appears to be mediated by trkA signalling, since K252a significantly reduced the effect of NGF on the membrane capacitance. Then we normalized the amplitude of EPSCs to reflect variability in cell size by dividing the peak amplitude of the current by the membrane capacitance of the cell. As illustrated in Figure 5B, we found that a simple increase in cell size could not account for the increase in neurotransmission induced by NGF in these neurons. These results indicate that the enhancement of acetylcholine and glutamate transmission in cholinergic MS-DBB neurons induced by NGF is more likely mediated by the formation of extensive synaptic connections rather than by a gross increase in the size of the neuron.

Secondly, miniature EPSCs from cholinergic MS-DBB neurons were recorded in the presence of TTX, bicuculline and ionomycin. During analysis, the length of time between the onset of two consecutive miniature EPSCs (interevent interval) and the amplitude of each event were measured and calculated for 13 consecutive miniature EPSCs for each cell. Performing a Kolmogorov-Smirnov test on the miniature EPSCs recorded from 2 control and 3 NGF-treated neurons revealed that NGF induced a significant increase in the frequency of miniature events but did not affect their amplitude. This finding demonstrates that NGF increases acetylcholine and glutamate

release from cholinergic MS-DBB neurons but does not alter the number of postsynaptic nicotinic and glutamatergic receptors, indicating a presynaptic site of synaptic modulation.

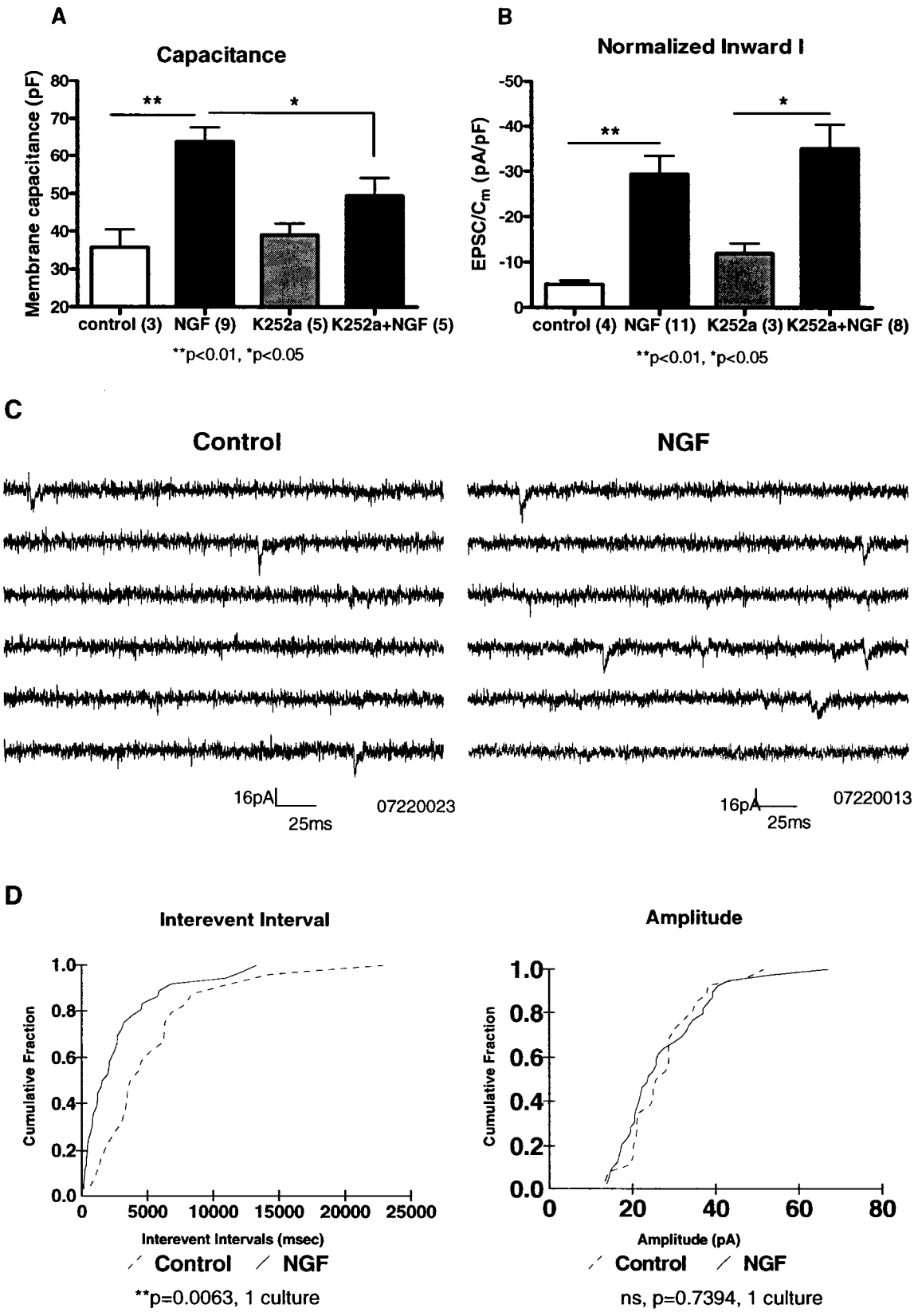

A
Capacitance
Membrane capacitance (pF)
80
70
60
50
40
30
20
**
*
control (3)
NGF (9)
K252a (5)
K252a+NGF (5)
**p<0.01, *p<0.05
B
Normalized Inward I
EPSC/Cm (pA/pF)
-50
-40
-30
-20
-10
0
**
*
control (4)
NGF (11)
K252a (3)
K252a+NGF (8)
**p<0.01, *p<0.05
C
Control
NGF
16pA
25ms
07220023
16pA
25ms
07220013
D
Interevent Interval
Cumulative Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
5000
10000
15000
20000
25000
Interevent Intervals (msec)
Control
NGF
**p=0.0063, 1 culture
Amplitude
Cumulative Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Amplitude (pA)
Control
NGF
ns, p=0.7394, 1 culture

Figure 5. Synaptic mechanisms underlying the effects of NGF on synaptic transmission of cholinergic MS-DBB neurons. **A.** Chronic exposure to NGF increased the cell size of cholinergic MS-DBB neurons, as indicated by the significant increase in the membrane capacitance. K252a significantly reduced this effect of NGF. **B.** EPSC amplitudes were normalized to compensate for the differences in cell size by dividing the current amplitude by the membrane capacitance. The positive effect of NGF on excitatory currents remained robust after normalization. Numbers in brackets are number of neurons. **C.** Unfiltered traces showing the presence of miniature EPSCs in cholinergic MS-DBB neurons grown in control vs. NGF conditions. **D.** Kolmogorov-Smirnov test on miniature EPSCs recorded from 2 control and 3 NGF-treated neurons revealed that NGF induced a significant increase in the frequency of miniature events but did not affect their amplitude.

Discussion

The most important finding in this study is that chronic exposure to NGF increased both acetylcholine and glutamate release from cholinergic MS-DBB neurons. The enhanced synaptic function of these neurons was not simply due to NGF-induced cell growth but a result of increased number of synapses and greater neurotransmitter release. This finding confirms previous studies that showed NGF-induced increases in the expression of cholinergic markers and in the level of acetylcholine release by cholinergic neurons of the basal forebrain (Auld *et al.*, 2001a;Auld *et al.*, 2001b). What is surprising here is that NGF also increased glutamate release from cholinergic MS-DBB neurons while having no significant effect on glutamate release from purely glutamatergic MS-DBB neurons.

Cholinergic septal neurons provide important excitatory input to the hippocampus for the generation of hippocampal theta activity, which is crucial for learning and memory. Our finding suggests that in pathological conditions where NGF signalling is impaired (e.g., Alzheimer's disease), both cholinergic and glutamatergic input from the septum to the hippocampus may become reduced before the cholinergic basal forebrain neurons are actually lost, leading to memory deficits during early stages of the disease. Whether cholinergic septal neurons do release both acetylcholine and glutamate in vivo remains unknown and this question can be answered by performing paired recordings of synaptically connected neurons in the septum, a technically very daunting task. However, our study demonstrates that single cholinergic MS-DBB neurons can simultaneously release acetylcholine and glutamate in culture and that the presence of NGF is crucial for promoting and maintaining both cholinergic and glutamatergic transmission in these neurons.

The majority of cholinergic MS-DBB neurons examined in our study released glutamate, and chronic exposure to NGF enhanced both acetylcholine and glutamate release from these neurons. Allen and colleagues established a micro-island culture of basal forebrain neurons much like our own and found that 6 out of 6 neurons that released glutamate simultaneously also released acetylcholine, consistent with our results (Allen et al., 2006). In the literature, the proportion of MS-DBB neurons that co-express cholinergic and glutamatergic markers varies depending on the techniques used. Sotty

and colleagues found using sc-RT-PCR that more than 60% of ChAT mRNA-expressing neurons were positive for VGLUT transcripts (Sotty et al., 2003). Colom and colleagues used immunohistochemistry and reported that only 4.8% of septal neurons expressing ChAT protein were positive for glutamate (Colom et al., 2005). The difference between these studies may be due to the fact that sc-RT-PCR is a very sensitive method for detecting mRNA expression whereas immunohistochemistry is used to detect protein expression. Not all neurons expressing transcripts for a particular protein may accumulate sufficient levels of the protein for detection using immunocytochemistry. In addition, co-expression of multiple transmitter phenotypes may be more common during development than in adults (Gutierrez et al., 2003;Danik et al., 2005), and multiple phenotypes can be transiently re-expressed in adult hippocampal granule cells by administering BDNF or by inducing hyperexcitable states such as seizures (Gutierrez, 2000;Gutierrez & Heinemann, 2001;Gutierrez, 2002;Gutierrez *et al.*, 2003;Gomez-Lira *et al.*, 2005). Therefore, one explanation for the discrepancy between studies on the proportion of MS-DBB neurons expressing multiple phenotypes may be that the transmitter phenotype of a neuron can be regulated by multiple factors, such as the developmental stage, excitability state and exposure to neurotrophins.

Our results demonstrate that the trophic effects of NGF on synaptic function are specific to cholinergic MS-DBB neurons and that GABAergic and glutamatergic MS-DBB neurons are unaffected by NGF. This finding supports previous studies that show that NGF selectively promotes survival of cholinergic septo-hippocampal neurons while not affecting GABAergic septo-hippocampal neurons (Peterson *et al.*, 1987;Montero & Hefti, 1988;Naumann *et al.*, 1994b). Some in vitro evidence indicates that GABAergic septal neurons may be responsive to NGF during early development. For example, calbindin-positive embryonic septal neurons increase the number and total length of neurite processes in response to NGF (Silva *et al.*, 2000). Embryonic basal forebrain GABAergic neurons in culture show high-affinity NGF binding (Dreyfus *et al.*, 1989) and a small proportion (3%) of developing GABA-positive septal neurons have been shown to express $p75^{NTR}$ in culture (Arimatsu & Miyamoto, 1989;Arimatsu & Miyamoto, 1991). The septal neurons used in these studies were either embryonic or early in development, whereas neurons in our study were more "mature" as they were obtained

from rats at postnatal days 10-13 and grown in culture for 16-30 days. The fact that GABAergic and glutamatergic MS-DBB neurons in our study were not affected by NGF provides further evidence that at least in adults, NGF promotes synaptic function of only cholinergic MS-DBB neurons. Indeed, in the MS-DBB, virtually all (95%) neurons positive for trkA co-express ChAT and $p75^{NTR}$ (Sobreviela *et al.*, 1994), indicating that NGF receptors are selectively expressed by cholinergic MS-DBB neurons, enabling them to respond to NGF.

The most intriguing finding in our study is that chronic exposure to NGF increased glutamate transmission through trkA and acetylcholine transmission through $p75^{NTR}$ in the same cholinergic MS-DBB neurons. How and why NGF enhances the release of two different neurotransmitters in the same neurons through two separate mechanisms is puzzling yet fascinating. Several studies have reported that NGF can rapidly (within minutes) increase glutamate and acetylcholine release from synaptosomes obtained from the rat hippocampus and visual cortex (Knipper *et al.*, 1994a;Knipper *et al.*, 1994b;Sala *et al.*, 1998;Raiteri *et al.*, 2003). In these brain regions, trkA and $p75^{NTR}$ are located predominantly on the cholinergic afferents from the basal forebrain (Rossi *et al.*, 2002), thus the effects of NGF likely reflect its action on these cholinergic nerve terminals. These studies also found that the NGF-induced glutamate release from visual cortex synaptosomes was mostly trkA-dependent (Sala *et al.*, 1998;Raiteri *et al.*, 2003). This is in agreement with our study since we found that the NGF-induced increase in glutamate release from cholinergic MS-DBB neurons was mediated mostly by trkA. Numakawa and colleagues reported that in developing cerebellar granule cells, NGF can induce rapid glutamate release via a $p75^{NTR}$-mediated pathway involving ceramide production (Numakawa *et al.*, 1999;Numakawa *et al.*, 2003). Cerebellar granule cells express trkB and $p75^{NTR}$ but lack trkA, which may explain why the mechanism for NGF action on glutamate release in these neurons differs from that in cholinergic MS-DBB neurons that express both trkA and $p75^{NTR}$.

It is unclear why the NGF-induced increase in acetylcholine transmission in our study was mediated mostly by $p75^{NTR}$, in contrast to previous studies that have reported the principal involvement of trkA. Auld and colleagues reported that both long-term (24-96 hours) and short-term (less than 1 hour) exposures to NGF increase acetylcholine

release from cultured embryonic basal forebrain neurons via mostly trkA signaling (Auld *et al.*, 2001a;Auld *et al.*, 2001b). One factor that could explain the difference between their results and ours is that exposure to NGF was much longer in our study (2-4 weeks) than theirs (maximum 4 days). Such chronic exposure to NGF may alter the cellular distribution and/or function of NGF receptors expressed by neurons, thereby possibly changing the mechanism of NGF action. In fact, Hartikka and Hefti showed that in high-density embryonic septal cultures, exogenous NGF stimulates the staining intensity of both ChAT and $p75^{NTR}$ labeling in immunocytochemistry (Hartikka & Hefti, 1988). In addition, the age of basal forebrain neurons used in Auld's study was also much younger; neurons were taken from rat embyos and cultured for a maximum of 8 days. Our cultures may therefore represent a more "mature" neuronal population and it is possible that the mechanisms with which neurons respond to NGF may change with development. Interestingly, Numakawa and colleagues found that developing cerebellar granule cells that rapidly release glutamate in the presence of NGF lose this sensitivity to NGF as they become mature neurons, but continue to respond to BDNF via trkB (Numakawa *et al.*, 2003), suggesting that the mechanisms with which neurons respond to neurotrophins can change with development.

Conclusion

The ability of a single neuron to express multiple transmitter phenotypes adds another layer of complexity and versatility to the already sophisticated mechanisms with which synaptic networks function. Evidence is accumulating in support of the notion that co-release of multiple transmitters from one neuron may not be an uncommon phenomenon in the brain and that the expression of a particular phenotype in a neuron may be regulated developmentally and in an activity-dependent manner. Results from the current study illustrate that the majority of cholinergic MS-DBB neurons grown in culture establish functional glutamatergic synapses, and that chronic exposure of these neurons to NGF, a well-known trophic factor for cholinergic basal forebrain neurons, potentiates both acetylcholine and glutamate. Dysfunction in NGF signalling has been strongly linked to the cholinergic atrophy and cognitive decline seen in AD patients, and NGF has emerged as a new prospective treatment for AD. The previously unidentified effects of NGF on MS-DBB neurons as reported here provide new insights into the understanding of the development of AD and the use of NGF as a potential treatment.

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