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Delayed treatment with Nerve Growth Factor (NGF) reverses ECMA-induced cholinergic lesions in rat brain reaggregate cultures

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Low concentrations of the alkylating cholinotoxin, ethylcholine mustard aziridinium (ECMA) produce selective cholinergic lesions in sub-populations of cholinergic neurones in developing rat whole-brain reaggregate cultures *in vitro* [1, 2]. Nerve Growth Factor (NGF) has significant and relatively specific positive neurotrophic effects on cholinergic neurones in the developing, lesioned adult and ageing brain [2–5]. These properties appear to be mediated through activation of phosphoinositide turnover and protein kinase C [6]. We have previously reported that although NGF significantly elevates choline acetyltransferase (ChAT) activity in brain reaggregate cultures after several days exposure (in a thyroid hormone dependent manner) it does not appear to reverse ECMA-induced lesions if added simultaneously with the neurotoxin [2]. It was postulated that this may be due to alkylation of NGF receptor-associated proteins on cholinergic neurones, precluding retrograde NGF transport to, and action on nuclei in the cholinergic perikarya [7]. We have now investigated whether high concentrations of NGF are effective if addition is delayed until several days following ECMA lesioning, when both the ECMA alkylating capacity has diminished and *de novo* receptor protein synthesis has occurred. The effects of GM1 ganglioside have also been examined since it appears to promote the functional recovery of injured neurones and the possible interaction of NGF with its receptor [8].

Methods

Foetal rat whole brain reaggregate cultures were prepared as described previously [2] in a serum-supplemented (S+) DMEM-based culture medium. The freshly prepared cholinotoxin, ECMA, was added at 8 days *in vitro* (8 DIV). B-NGF (7S sub-unit, Sigma Chemical Co., Poole, U.K.) was added by two different protocols by either, Protocol A: three times during the culture period at a final concentration of 50 ng/mL at 8, 10 and 12 DIV (with or without GM1 ganglioside; final concentration 100 μg/mL); or Protocol B: at 10, 12 and 14 DIV (delayed NGF addition experiments). Both treatment protocols involved three NGF additions at 48 hr intervals over a similar period assuming an ECMA degradation time of approximately 6–8 hr [9]. Partial-culture medium changes were performed on alternate days from 8 DIV onwards. Cultures were harvested and washed at 15–16 DIV and assayed for protein, ChAT activity and muscarinic receptor binding (mAChR: defined as the specific binding of [^{3}H]quinuclidinylbenzilate-QNB in the presence of 100 μM atropine) as previously described [2].

Results

NGF at low concentrations of 5 ng/mL produces only fairly modest 45–50% increases in ChAT activity in control reaggregates, an effect which appears to be dependent on the presence of thyroid hormone ($L\text{-}T_3$) in the culture medium serum supplement [2, 4]. Higher concentrations of NGF (50 ng/mL), however, produce much larger increases in ChAT (Figs 1 and 2; NGF treated cultures = approx 100–400 % untreated control reaggregate ChAT activity). Co-treatment with NGF plus GM1 ganglioside did not potentiate this effect (Fig. 1). The absolute level of control ChAT activity varied between culture batches as expected and shown previously [2] but was always of the same order of magnitude.

NGF at 50 ng/mL similarly increased the level of mAChR (approx. 200% of control binding; Fig. 3) with GM1 ganglioside having no additional effect. ECMA lesioning at 8 DIV reduced the reaggregate ChAT activity to around 50% of control activity as previously reported [2] and conversely doubled the level of mAChR (Figs 1 and 3). Addition of 50 ng/mL NGF three times at 48 hr intervals (with or without 100 μg/mL GM1 ganglioside) at the same time as ECMA by protocol A did not produce elevations of ChAT at 15–16 DIV as seen in non-ECMA treated control cultures at this timepoint (Fig. 1) and produced no further enhancement in the level of mAChR (Fig. 3).

However, using protocol B where the first of the three *in vitro* NGF treatments was delayed until 10 DIV (2 DIV after initial ECMA treatment) and cholinergic function similarly investigated at 15–16 DIV, there was a complete reversal of the lesion-induced ChAT reduction produced by the cholinotoxin (Fig. 2). (The combined results as shown were derived from two separate culture runs where statistically significant reductions and reversals were demonstrated in each case.)

Discussion

We have now shown that high concentrations of NGF (50 ng/mL) can produce large elevations in ChAT activity in normal, developing reaggregate cultures of rat whole

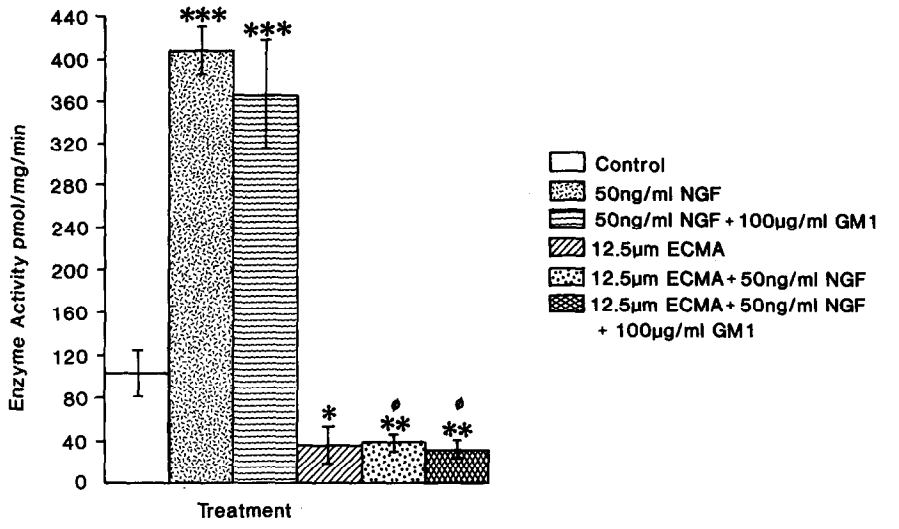

Fig. 1. Effect of NGF and GMl ganglioside on ChAT activity in untreated and ECMA-treated brain reaggregate cultures. ECMA (12.5 μM) was added to the cultures at 8 DIV. B-NGF (50 ng/mL) was added at 8, 10 and 12 DIV when partial culture medium changes were also performed (5/8.5 mL). In some cases 100 μg/M GMl ganglioside was also present. At 15–16 DIV cultures were harvested, washed and assay for protein and ChAT activity. Each histogram represents N = 8–12 individual culture flasks from two separate culture runs. Statistics were analysed by the Student's t-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ significantly different from control, untreated cultures. Ø = not significantly different from cultures treated with ECMA alone.

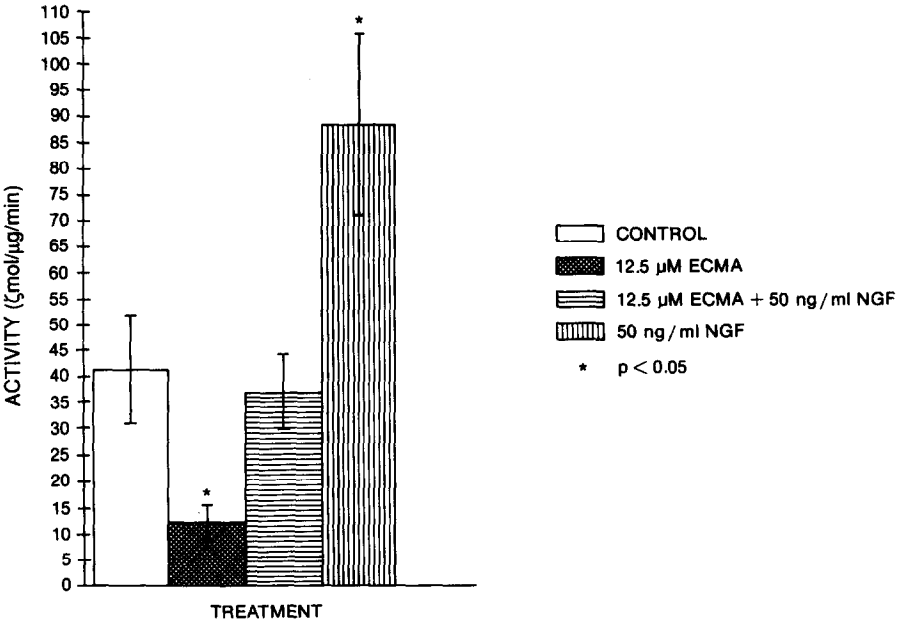

Fig. 2. Effect of delayed NGF addition on ChAT activity in untreated and ECMA-lesioned brain reaggregate cultures. ECMA (12.5 μM) was added to the cultures at 8 DIV. B-NGF (50 ng/mL) was added subsequently at 10, 12 and 14 DIV when partial medium changes were performed. At 15–16 DIV the cultures were harvested, washed and assayed for protein and ChAT activity. Each histogram represents the mean result from N = 8–14 flasks from two separate culture batches. Statistics were performed by the Student's t-test where *significantly different from control, untreated reaggregates or aggregates treated with ECMA + NGF.

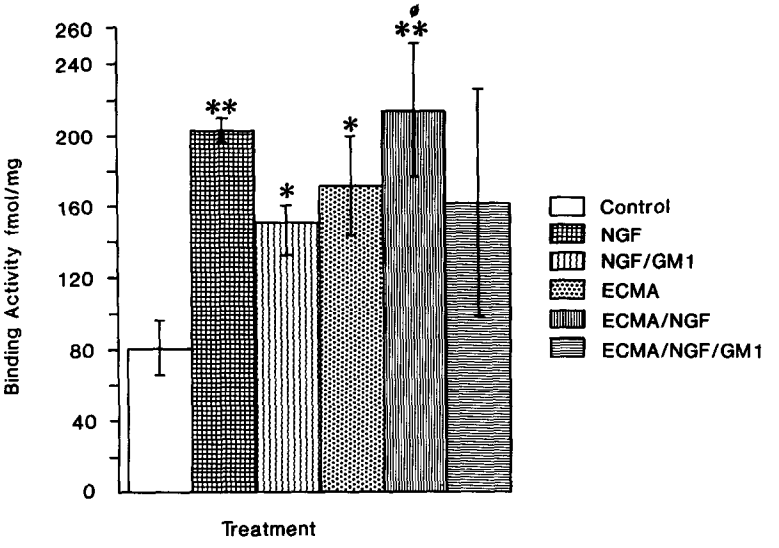

Fig. 3. Effect of NGF and GM1 ganglioside on muscarinic receptor (mAChR) binding in untreated and ECMA-treated brain reaggregate cultures. ECMA (12.5 μM) was added to the cultures at 8 DIV. B-NGF (50 ng/mL) was added 8, 10 and 12 DIV when partial culture medium changes were also performed (5/8.5 mL). In some cases 100 μg/M GM1 ganglioside was also present. At 15–16 DIV cultures were harvested, washed and assay for protein and specific [^{3}H]QNB binding. Each histogram represents N = 8–12 individual culture flasks from two separate culture runs. Statistics were analysed by the Student's *t*-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ significantly different from control, untreated cultures. Ø = not significantly different from cultures treated with ECMA alone.

brain. This agrees with previous reports in both reaggregates [4] and in neuronal monolayer cultures where the ED_{50} for NGF on cultured septal cholinergic neurones was found to be around 10 ng/mL [10]. It has now also been shown for the first time that NGF treatment elevates muscarinic receptor binding (mAChR) in developing brain reaggregates in agreement with its previously reported action in cultured rat phaeochromocytoma (PC12) cells [11]. The action on mAChR in the brain reaggregates apparently has a different NGF concentration-dependence to effects on ChAT since no effects on the mAChR were seen at the lower NGF concentrations of 5 ng/mL where significant ChAT increases were demonstrated [2]. It also demonstrates a broader and direct action of NGF on the central cholinergic synapse rather than just on the presynaptic component which cannot be due to transynaptic regulation. Increases in the level of presynaptic cholinergic 'activity' would be expected to down-regulate mAChR by this type of mechanism. The postsynaptic cells bearing muscarinic receptors could be of the cholinergic phenotype, non-cholinergic or glial cells. The 'up-regulation' of the mAChR following cholinergic lesioning with ECMA is similar to that which occurs *in vivo* following lesioning of cholinergic neuronal tracts [12] and supports a predominantly postsynaptic localization for a large proportion of these receptors in the brain reaggregates.

It was originally postulated [7] that the apparent failure of even high concentrations of NGF to reverse the ECMA-induced cholinergic lesion *in vitro* despite a residual 'surviving' pool of ChAT in the ECMA-treated cultures may be due to the apparent extracellular neurotoxic mechanism of action of ECMA [7]. Possible alkylation of NGF receptor proteins by the ECMA during the initial periods of NGF treatment would preclude retrograde NGF transport to, and neurotrophic action on, nuclei in the cholinergic perikarya. Basal forebrain cholinergic neurones are specifically sensitive to the action of NGF, which is transported retrogradely from the neocortical and hippocampal areas. These neurones have specific NGF binding sites [13, 14]. We have now shown that three additions of 50 ng/mL of NGF commencing 2 days following ECMA lesioning when ECMA has fully degraded and new NGF receptor proteins have presumably resynthesized allows a complete reversal of the ECMA-induced cholinergic lesioning (Fig. 2). In both treatment protocols exposure to exogenous NGF occurred similarly for three times at 48 hr intervals over a similar total time period. The inability of NGF in protocol A to reverse the lesion cannot be due to the lag between the last NGF addition and harvesting since ChAT was maximally elevated in control cultures by this protocol. These results imply, therefore, that the failure of high concentrations of NGF to reverse the cholinergic 'lesioning' in reaggregates when initially added simultaneously with the toxin may be due to inactivation of NGF and/or NGF-receptor complexes by ECMA. Indeed, the extracellular mechanism for the cholinotoxicity of ECMA in serum-based culture media [9] could be mediated by a similar phenomenon even in the absence of exogenously added NGF. This, however, would not account for the ability of ECMA to lesion in serum-free media unless NGF secretion by non-neuronal cells was comprised in some way by the neurotoxin [2].

It is not yet known whether this newly demonstrated NGF enhancement of ChAT activity in lesioned brain reaggregates is due to stimulation of new ChAT synthesis in surviving cholinergic neuronal subpopulations or to stimulation of survival or regrowth of neurites from damaged neurones, or both. Interestingly, Hefti *et al.* [10] demonstrated that in normal cultured foetal septal cholinergic neurones stimulation of the synthesis of new ChAT proteins by NGF occurs rather than neurite outgrowth. Along with the induction of ChAT, NGF increases the intracellular storage capacity for Acetylcholine (ACh) on a similar timescale [15]. However, the recent finding that in cultured rat basal forebrain neurones NGF treatment increases the surviving number of morphologically distinct classes of cholinergic neurones (stellate and pyramidal) without effect on the bipolar type demonstrates an ability to differentially affect cholinergic cell survival [16].

This lesioned culture system now provides a valuable model with which to study and evaluate the actions of novel neurotrophic factors which may be able to reverse experimentally-induced lesions and be of use in treating those neurodegenerative disorders where cholinergic cell loss is involved, for example, in Alzheimer's Disease.

In summary: developing rat whole brain reaggregate cultures were treated at 8 days *in vitro* (8 DIV) with the alkylating cholinotoxin, ethylcholine mustard aziridinium (ECMA) at a final concentration of 12.5 μM. Simultaneous addition of NGF (50 ng/mL) at 8 DIV and then at 10 and 12 DIV (with or without GM1 ganglioside 100 μg/mL) did not reverse the ECMA-induced reduction of choline acetyltransferase (ChAT) activity measured at 15–16 DIV. However, delayed NGF treatment at 10, 12 and 14 DIV following ECMA treatment at 8 DIV was able to reverse the cholinotoxin-induced loss of ChAT activity. The failure of high concentrations of NGF to reverse the cholinergic 'lesion' when the first addition occurs simultaneously with ECMA may involve alkylation of NGF receptor complexes. Further observations were that NGF (50 ng/mL) induced 100–400% increases in both the ChAT activity and muscarinic receptor binding of control, untreated reaggregates, respectively.

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