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EFFECT OF NERVE GROWTH FACTOR AND THYROTROPIN RELEASING HORMONE ON CHOLINERGIC NEURONES IN DEVELOPING RAT BRAIN REAGGREGATE CULTURES LESIONED WITH ETHYLCHOLINE MUSTARD AZIRIDINIUM

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Abstract—Foetal rat whole brain reaggregate cultures were prepared in a serum-supplemented (S+) or serum-free medium (S−). Ethylcholine mustard aziridinium (ECMA) was added to the cultures at 9 days *in vitro* (DIV) at concentrations of 12.5, 25 or 50 μM. Choline acetyltransferase (ChAT) activity was measured at +2, +48 and +96 hr following treatment. In certain experiments the neurotrophic factors, thyrotropin releasing hormone (TRH: 50 μg/ml, daily from 9 DIV) or nerve growth factor (NGF: 7S subunit, 5 ng/ml, 0 and +48 hr following ECMA) were added during ECMA treatment.

In both types of reaggregate cultured in S+ and S− media there was a 40–80% loss of ChAT activity following ECMA exposure (final concentration = 12.5 μM), presumed to reflect cholinergic cell loss.

In both S+ and S− brain reaggregates NGF produced increased ChAT activity with more marked effects in S+ (45–55% increase, +48–96 hr) than in S− medium (20–25% increase, 2–96 hr). No effect on cholinergic muscarinic receptors (specific ^{3}H-QNB binding) was evident after treatment with NGF. TRH had no effect on ChAT activity in the S+ cultures but produced small increases in the S− culture condition (approx 20%, + 2–48 hr). Despite a residual "ECMA-resistant" pool of ChAT in the cultures, neither neurotrophic agent was found to cause a reversal of the lesion.

In conclusion, the cholinotoxin ECMA appears to produce a cholinergic deficit in both developing S+ and S− reaggregates. This was not reversible by NGF or TRH at the concentrations and under the conditions tested. NGF had marked effects on ChAT activity without affecting muscarinic receptors in untreated developing brain reaggregates cultured in an S+ medium.

Ethylcholine mustard aziridinium (ECMA) has been proposed as a selective cholinergic neurotoxin *in vivo* [1, 2]. We have shown that *in vitro* low concentrations of ECMA (12.5 μM) produce selective cholinergic neuronal lesions in developing rat brain reaggregate cultures grown in a serum-supplemented medium [3, 4]. This occurs in two phases with initial inhibition of choline acetyltransferase (ChAT) and later, the apparent loss of cholinergic neurones. Using synaptosomes *in vitro*, Pittel *et al.* [5] have recently suggested that ECMA binds to the high affinity choline carrier better than to the low affinity carrier and that at low local concentrations *in vivo* similar to those effective *in vitro* (up to 11 μM), where uptake inhibition is reversible, it thus accumulates in the neurones causing selective cholinotoxicity. Indeed, in primary brain monolayer cultures it has recently been reported that ECMA has similar specific cholinotoxic actions to those in reaggregates up to 22.5 μM and that these effects are prevented by choline [6].

One of the major abnormalities in Alzheimer-type dementia in man is a degenerative change in the cholinergic system with loss and/or hypoplasia of cholinergic neurones in the basal forebrain region. In aged rodents learning and memory impairments have similarly been associated with atrophy of cholinergic neurones of the nucleus basalis magnocellularis, the septal-diagonal band area and corpus striatum (see Ref. 7). The cause of these degenerative changes is not known, although it is suspected that one contributory factor could be abnormalities in the action or production of target-derived neurotrophic factors (see Refs 8 and 9). However, we have shown that both normal and Alzheimer brain extracts contain equivalent amounts of uncharacterized neurotrophic factors (NTF) capable of elevating ChAT in rat brain cultures [10], and no evidence was found of deficient nerve growth factor (NGF) messenger RNA production in senile dementia of the Alzheimer type or in lesioned rat brain [11]. However, other studies have demonstrated that injured or denervated brain *in vivo* does secrete increased concentrations of NTFs [12] and conditioned media from an injured lower vertebrate CNS promote neurite out growth from mammalian cultured brain neurones *in vitro* [13]. Thus, "reactive synaptogenesis" in both the developing and ageing brain may still involve NTFs and it may, therefore, be possible to "reverse" central cholinergic deficiencies by administering exogenous neurotrophic factors.

Much interest has centred recently on NGF, since in addition to its clearly demonstrated role in the

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survival and in the regulation of specific properties of sympathetic and sensory neurones in the periphery [14], it does influence developing cholinergic neurones in the CNS [15]. There are significant increases of ChAT activity after NGF treatment of developing rat brain reaggregates [15], of explant or mixed cultures of striatal or septal regions of embryonic rat brain [16, 17], and in several brain areas *in vivo* [8]. In normal adult animals *in vivo*, however, this does not occur, but interestingly does so when there are lesions of the septo-hippocampal axonal pathway [19]. In addition, NGF has recently been reported to reverse cholinergic cell body atrophy in aged rats [7].

Like NGF, thyrotropin releasing hormone (TRH) appears to have beneficial clinical effects in patients with amyotrophic lateral sclerosis (ALS) and has been reported to have a trophic effect on lower motor neurones and enhances ChAT activity in cultured rat embryonic spinal cord ventral horn neurones [20]. With the final objective, therefore, of defining both the production and action of NGF, TRH and other NTFs in a cholinergically-lesioned brain model *in vitro*, respectively, we have firstly, investigated whether ECMA can lesion cholinergic neurones in reaggregate cultures of developing rat brain cultured in a serum-free medium. Secondly, we have examined the action of NGF and TRH in these cultures and in those grown in a serum-supplemented medium.

MATERIALS AND METHODS

Materials. All chemicals used were of the highest purity available and obtained from either Sigma Chemical Co. (Surrey, U.K.) or BDH Ltd. (Poole, Dorset, U.K.). Soluene-350 was obtained from Packard Instrument Co. (Berks), [1-^{14}C]acetyl CoA (solid) and [^{3}H]quinuclidinyl benzilate (QNB; 33 Ci mmol^{-1}) were obtained from Amersham International PLC (Amersham, Bucks, U.K.). Tissue culture materials were as follows: Dulbecco's Modified Eagles Medium (DMEM), and Hams F12 medium were obtained from Gibco Bio-Cult, Europe. The foetal calf serum was also purchased from Gibco Europe, and stored frozen at −20° before incorporation in the media. The ECMA precursor was obtained from Salford Ultrafine Chemical Company Ltd. Nerve growth factor (7S NGF) from mouse submaxillary glands was obtained from Sigma (Cat No. N8384). TRH (pGlu–His–Pro amide) was also purchased from Sigma (Cat No. P9012).

Cell culture. Foetal brain reaggregate cultures were grown essentially by the method of Honegger and Lenoir [15], and as described by Atterwill *et al.* [21]. Whole brains from 80–100 16-day-old rat foetuses (yielding approximately 20 culture flasks) were dissected aseptically in a sterile solution of ice-cold isotonic Hanks D2 solution and then washed extensively in a nylon gauze bag (Nybolt—pore size 205 μm). The tissue was then dissociated by extrusion through the gauze by stroking with a glass rod in 30 ml Hanks D1 solution (Ca^{2+}, Mg^{2+}-free). The suspension was next refiltered through Nybolt mesh (103 μm pore size) and centrifuged. This procedure was repeated twice. The final cell pellet was resuspended in culture medium S+ = DMEM, plus 10% foetal calf serum plus extra glutamine (see below); S− medium was based on the N2 medium of Bottenstein and Sato (see Ref. 15) comprising a 3:1 mixture of DMEM and Hams F12 medium containing insulin (5 μg/ml^{-1}), transferrin (100 μg ml^{-1}), putrescine (10 nM), selenium (30 nM) and progesterone (20 nM). Gentamicin (25 μg ml^{-1}) and extra glutamine (final concn, 300 μg ml^{-1}) were also added to both S+ and S− culture media.

Next 3.5 ml of the cell suspension (10^{7} cells ml^{-1}) was inoculated into 25 ml Delong conical culture flasks and cultured for up to 28–30 days in a 9% CO_2/humidified air mixture (37°) with constant rotation at 70 rpm on a Luckham Rotatest orbital shaker (orbit diameter = approx. 1.0 in.). The speed was gradually increased to 80 rpm. After 3 days *in vitro* the "mini aggregates" were transferred to 50 ml Delong vessels and 5 ml fresh culture medium added. This was repeated on alternate days. Aggregates were harvested at the stated times (see "Drug Treatment" section) for enzyme, protein and receptor binding measurements by allowing them to sediment under gravity and then washing three times with Dulbecco's phosphate buffered saline (PBS) at 25°. The final cells were "flash-frozen" in liquid N_2 and stored (−70°).

Choline acetyltransferase assay. Each separate aggregate sample was thawed and homogenized in sodium phosphate buffer (pH 7.4, 4°), and then treated with Triton X-100 (final concentration 0.5% v/v; 0°, 2 hr) and assayed for ChAT as described previously [21] (Fonnum method: 2 nM choline chloride; 25 μM [^{14}C]acetylCoA; 150 mM NaCl; pH 7.0; at 37°).

Approximately 5–10 μg aggregate protein was used for each enzyme determination and cell incubations were carried out for 10 min in triplicate under these conditions. It has been shown previously [21] that in mature and immature rat brain, partially purified ChAT has similar apparent K_m values for choline and, therefore, validates the use of the present assay conditions for estimating ChAT in the developing brain culture samples.

Muscarinic receptor binding assays. Muscarinic receptor (mAChR) binding was defined as the specific binding of [^{3}H]quinuclidinyl benzilate (QNB: specific radioactivity = 33 Ci mmol^{-1}) in the presence of 100 μM atropine. Aggregate cell pellets were thawed and homogenized in 50 mM Tris–HCl buffer (pH 7.4, 0°), and centrifuged at 14,000 *g* for 10 min in a Burkard Koolspin microcentrifuge. This was repeated twice and the final high-speed pellet resuspended in buffer and used for the binding determination. Specific [^{3}H]QNB binding was measured on 50 μg protein aliquots (approx) as described by Atterwill *et al.* [22] using a single, saturating ligand concentration of 2 nM.

Lactate dehydrogenate. At culture medium changes 5 ml portions of medium were collected and stored frozen at −40°. On thawing the fractions were centrifuged (15,000 *g* × 5 min at 0–4°) and 10 μl samples assayed for total lactate dehydrogenate (LDH) activity using a BCL assay kit in conjunction with a Hitachi 705 autoanalyser (NADH to NAD conversion monitored).

Protein. In all cases protein was measured by the method of Lowry *et al.* [23] using bovine serum albumin as a standard.

Drug treatment of cultures. ECMA was freshly prepared prior to use from the precursor *O*-acetylethylcholine mustard by cyclization and hydrolysis. Conversion to the aziridinium was found by thiosulphate titration to be consistently 76%.

ECMA was added directly to the S+ and S− cultures on the ninth day *in vitro* (9-DIV), in a concentration range similar to that estimated to occur in CSF (12.5–50 μM) after i.c.v. injection [1, 2].

A concentration of 12.5 μM was used in the experiments with S+ cultures as a previously established and effective, selective cholinotoxic concentration [4], whereas in the cultures in a serum-free medium 12.5, 25 and 50 μM concentrations of ECMA were tested.

NGF (7S subunit; final concentration 5 ng/ml; added at 9 and 11 DIV) or TRH (50 μg/ml; daily from 9-DIV) were added from concentrated stock solutions (stored −40°) in PBS during the culture period as shown to both control untreated and ECMA-treated reaggregates.

RESULTS

Effect of ECMA treatment on ChAT activity and LDH release from reaggregates cultured in an S− medium. In order to establish the sensitivity of developing rat whole brain reaggregates to ECMA for comparison with its neurotoxic profile in S+ cultivated cells [4], reaggregates in S− medium were treated at 9-DIV with 12.5 μM, 25 μM and 50 μM ECMA. At +2, +48 and +96 hr later cells were harvested and ChAT activity measured. (Results represent the mean of N = approx. 19 culture flasks from three different culture batches.) Figure 1 shows that after 2–48 hr exposure all concentrations of ECMA caused slight reductions in cellular ChAT activity. Although the reduction at 12.5 μM was not quite significant, in other experiments (see Fig. 3) significance was achieved. In serum-containing cultures (see Fig. 4) 12.5 μM did not appear to cause an inhibition of ChAT at 2 hr although in previous work [4] we have shown that there is an early, but variable, phase of ChAT inhibition (12.5–50 μM ECMA) representing direct enzyme inhibition following intracellular ECMA accumulation [4].

Longer ECMA exposure (+96 hr) of the reaggregates in serum-free media produced a greater, consistent and more significant reduction in ChAT activity (Fig. 1, 40% reduction) at 12.5 μM. One clear difference between the two culture conditions was the apparent dose-dependent inhibition of ChAT activity in the reaggregates in S− medium, which was not seen in cells cultivated in serum [4].

Lactate dehydrogenase. LDH release into the culture medium was measured as an indicator of cytotoxicity due to the ECMA addition. From Fig. 2 it can be seen that at +48 hr there was significant LDH activity present in the culture medium from control cultures. Treatment with 50 μM caused a large release of LDH activity into the culture medium (approx. 2-fold), whereas at this time the effect of 12.5 μM was less and coincided with minimal effects

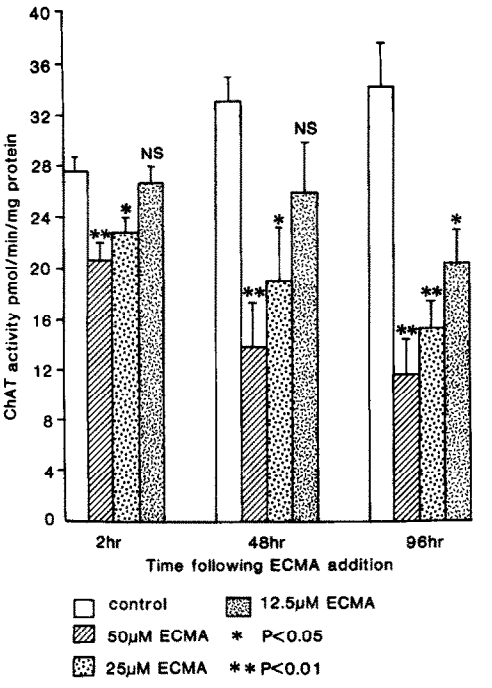

Fig. 1. Effects of ECMA on the ChAT activity of whole brain reaggregates cultivated in a serum-free (S−) medium. Cultures were prepared in an S− medium as described and ECMA added at 9 DIV in final concentrations of 12.5, 25 or 50 μM. At 2, 48 and 96 hr following treatment aggregate samples were removed and assay for ChAT activity as described. Data is shown as the mean enzyme activity ± SEM calculated from assays on N = approx. 20 culture flasks, representing three separate culture batches (to minimize inter-culture variation). Statistical analysis was performed using the Student's *t*-test as shown (NS = not statistically different from untreated, control cultures).

on ChAT activity (see Fig. 1). However, by +96 hr 50 μM ECMA had no further effect possibly due to depletion of most of the releasable enzyme whereas 12.5 μM ECMA still slightly increased LDH release coinciding with significant effects on ChAT activity (see Fig. 1).

Effects of NGF and TRH on ChAT activity in control and ECMA-treated reaggregates cultivated in S− medium. To establish whether NGF or TRH was able to reverse ECMA-induced reductions in ChAT activity, or influence ChAT activity in untreated cultures, control and ECMA-treated reaggregates (12.5 μM neurotoxin) were also treated with NGF 5 ng/ml; 0 and 48 hr following ECMA addition) or TRH (50 μg/ml; added daily). These results are depicted in Fig. 3. ECMA produced similar reductions in ChAT activity to those described above (Fig. 1), except that in this set of cultures ECMA produced a significant loss of ChAT activity +48 hr after exposure to 12.5 μM neurotoxin. Neither NGF nor TRH caused a reversal of this enzyme reduction, except at +48 hr where TRH had a very minimal but statistically significant effect. In contrast, in the control cultures both neurotrophic agents elevated ChAT activity with 10–20% increases apparent as early as +2 hr following treatment. However, the

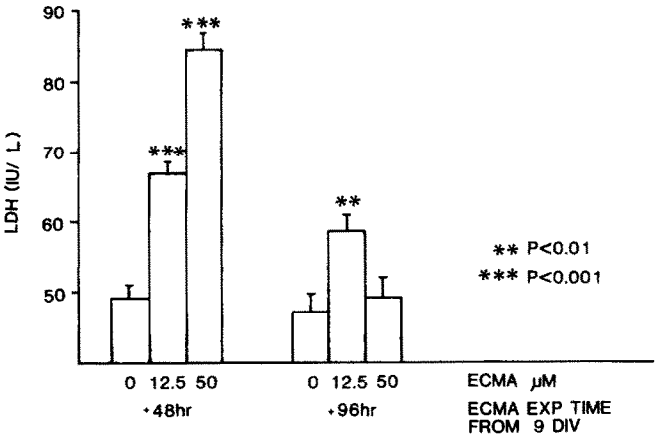

Fig. 2. Effect of ECMA treatment (12.5 and 50 μM final concentration) of rat brain reaggregate cultures in an S− medium on the activity of LDH in the culture medium. Culture medium was taken from the cultures described in Fig. 1 at medium change times after the 9 DIV ECMA-treatment point (i.e. +48 and +96 hr) and assayed for LDH activity on an Hitachi 705 autoanalyser. Results are expressed as mean enzyme activity (in IU/ml) ± SEM from approx. N = 20 culture flasks (representing three separate culture batches).

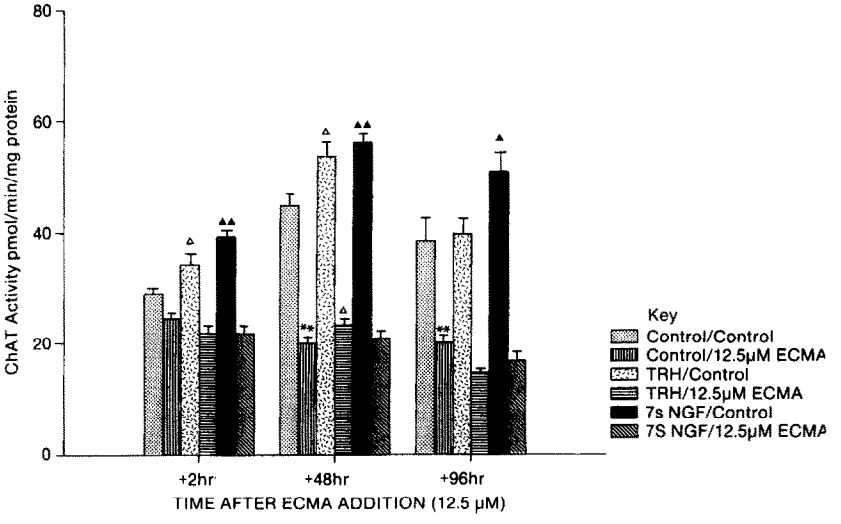

Fig. 3. NGF and TRH actions on ChAT activity in rat brain reaggregate cultures in a serum-free (S−) medium treated with ECMA. ECMA (12.5 μM) was added to the reaggregate cultures on 9 DIV as described previously (see Figs 1 and 2). 7S-NGF (5 ng/ml) was added just before ECMA at 9 DIV and again 48 hr later at the medium change. TRH (50 μg/ml) was added daily from 9 DIV. At +2 hr and +48 hr and +96 hr following treatment aggregate samples were taken and assayed for ChAT. Results are expressed as mean ± SEM for N = 5–8 culture flasks from two separate culture batches. Symbols shown represent statistical difference of treated culture from control untreated culture at that time point (Student's *t*-test); **ECMA-treatment from control, $P < 0.01$; △, TRH treatment from control, $P < 0.05$ at +2 hr or TRH + ECMA treatment from ECMA treatment alone, $P < 0.05$ at +48 hr; ▲, ▲▲, NGF treatment from control, $P < 0.05$ and $P < 0.01$ respectively.

NGF effects reached greater levels of significance than those of TRH and persisted until the +96 hr timepoint.

Effects of NGF and TRH on ChAT activity and specific [^{3}H]*QNB binding in ECMA-treated cultures grown in the serum-supplemented (S+) medium.* In control, developing reaggregates cultured in an S+ medium (where essential components for cholinergic differentiation such as T_3 are present) NGF (5 ng/ml), but not TRH, treatment produced a marked elevation (40–55% increase) of ChAT activity (Fig. 4), which was not apparent until 48 hr of exposure and was maintained until +96 hr. However, clearly neither NGF nor TRH were able to reverse the ECMA-induced loss of ChAT activity (Fig. 4) except at one time point (+48 hr) where NGF had a very

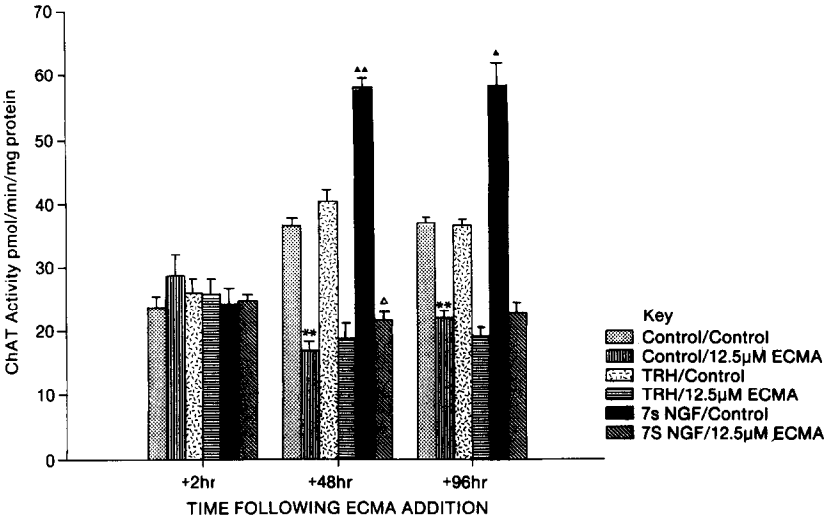

Fig. 4. NGF and TRH effects in untreated and ECMA treated developing rat brain reaggregates cultivated in an S+ medium. ECMA (12.5 μM) was added to cultures at 9 DIV. 7S-NGF (ng/ml at +0 and +48 hr) or TRH (50 μg/ml at daily intervals) were also added into some culture flasks. At the time points shown +2 hr, +48 hr, +96 hr following ECMA ± NGF or TRH aggregate samples were taken and assayed for ChAT activity. Results are expressed as mean ± SEM for N = 5–8 culture flasks from two separate culture batches. Symbols represent significant differences tested by the Student's *t*-test; **ECMA-treatment from controls, $P < 0.01$; △, NGF + ECMA treatment from ECMA treatment alone, $P < 0.05$ at +48 hr only; ▲, ▲▲, 7S-NGF treatment from control cultures at $P < 0.05$ and $P < 0.01$ respectively.

small but statistically significant effect.

The 2.5 S-NGF subunit was also tested in some preliminary experiments, but unlike 7S-NGF did not elevate ChAT activity in control or ECMA-treated reaggregates.

Muscarinic receptors (Fig. 5) were also assessed by measuring specific [^{3}H]QNB binding. ECMA treatment (12.5 μM) produced an initial inhibition of [^{3}H]QNB binding (approx. 25% decrease) probably by direct alkylation as previously described [4]. However, receptor binding activity returned to normal values by 48 and 96 hr following exposure to the neurotoxin. This is assumed to reflect intact postsynaptic and neighbouring neural cells to the cholinergic neurones resynthesizing receptor protein when ECMA is used at selectively cholinotoxic concentrations [4]. In neither the control nor the ECMA-treated cultures did NGF or TRH have any effect on muscarinic receptor density.

DISCUSSION

In agreement with previous reports [3, 4] using serum-supplemented (S+) rat brain reaggregate cultures, we have shown that 12.5 μM ECMA produces a substantial reduction in ChAT in developing serum-free (S−) reaggregates (where cholinergic development is already retarded [22]) following 48 and 96 hr exposure. This presumably similarly reflects loss of, or damage to, the cholinergic neurones, although the selectivity of the lesion has not yet been fully delineated in this culture condition. This should, therefore, facilitate future examination of lesion-induced neurotrophic factors *in vitro* as has already been described *in vivo* [12, 13]. Developing rat brain reaggregates have been shown under normal conditions, to release such factors into their medium [24]. In general, ChAT activity in both S− and S+ cultures rose gradually over the developmental period *in vitro* as previously demonstrated [3, 15]. The fact that the rate of increase was not as high as previously reported, that the difference between S+ and S− ChAT activity values were not as great, and that these values were slightly lower than reported may be due to a number of factors. These factors include the removal of "autoconditioning" factors from the culture media [24] by multiple cell sampling and the use of relatively immature cultures when ChAT activity rises more slowly (see Ref. 3).

Treatment of the control reaggregates with NGF produced elevated ChAT activity in both S+ and S− culture conditions supporting previous reports of its positive action on central cholinergic neuronal differentiation both *in vivo* and *in vitro* [15, 16, 18]. The effect in the serum-free cultures was less marked (20–25% increase) than in the serum-supplemented cells and was present as early as 2 hr after addition suggesting non-specific effects on neuronal function not linked to synthesis of new ChAT molecules.

Although it is generally agreed that NGF effects on cholinergic neurones do not appear until around 48 hr after exposure (see below and Ref. 16), short-term effects of NGF on membrane function and phospholipid metabolism have been reported [25].

The ChAT stimulation in S+ cultivated reaggregates was, however, more marked (45–55% increase) and was not detectable until 48 hr after

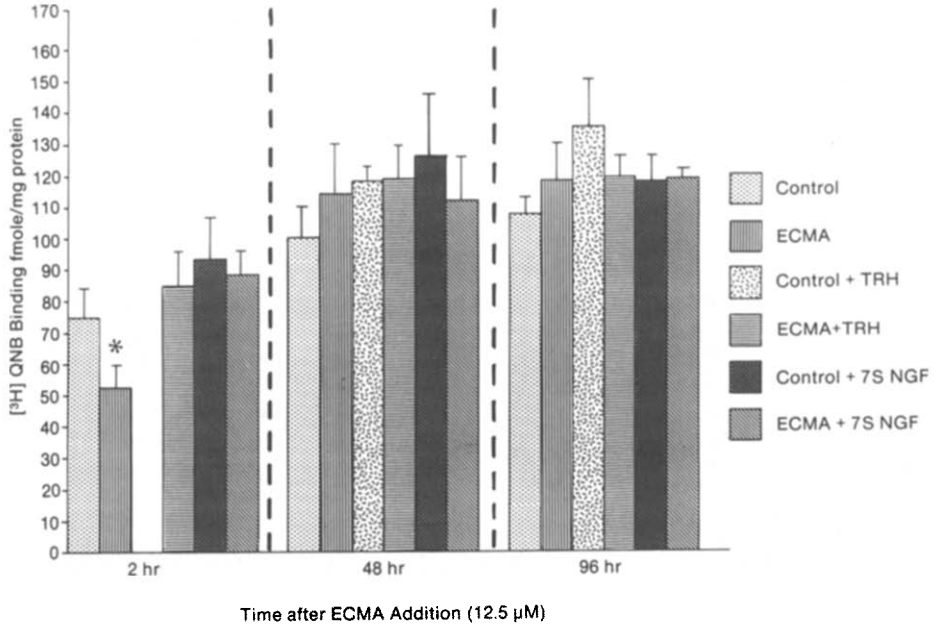

Fig. 5. Effect of ECMA treatment in the presence or absence of either NGF or TRH on specific muscarinic receptor binding in brain reaggregate cultures in an S+ medium. ECMA (12.5 μM) was added to reaggregates in an S+ medium at 9 DIV in the presence or absence of 7S-NGF (5 ng/ml) or TRH (50 μg/ml) as described previously (see Fig. 4). At various times following treatment (+2, 48 and 96 hr) aggregate samples were removed and assayed for specific [^{3}H]QNB binding as described in Materials and Methods. Results are expressed as mean ± SEM and statistics were analysed using the Student's t-test (*ECMA treated culture significantly different from control, untreated culture at $P < 0.05$). No values for TRH addition to control cultures were obtained at the +2 hr timepoint. Results represent mean ± SEM for N = 5–12 culture flasks from three different culture batches.

treatment. This is consistent with the permissive actions of thyroid hormone (L-T_3) present in serum with NGF in elevating ChAT activity. L-T_3 itself also has marked effects on the differentiation of embryonic whole brain cholinergic neurones *in vitro* in terms of both ChAT activity and muscarinic receptor concentrations [22, 26]. Honegger and Lenoir [15] showed that whereas NGF alone was without effect on cholinergic differentiation in rat brain reaggregates grown in S− media it did produce marked increases in the presence of L-T_3.

One possible extrapolation from the present work is that the apparent inability to reverse an ECMA-induced loss of ChAT activity *in vitro* may be due to a selective loss of a sub-population of cholinergic neurones particularly dependent on a requirement for thyroid hormone and/or NGF (see Ref. 27). In a similar context, both *in vivo* in rat brain [28], and in the reaggregate cultures ECMA never appears to totally eliminate ChAT activity suggesting either the presence of a population of "ECMA-resistant" neurones, partially damaged neurones, or residual perikarya not susceptible to the action of this neurotoxin. Leventer *et al.* [28] have found regional differences in the ECMA-sensitivity of cholinergic neurones between rat hippocampus and striatum. Furthermore, in the chicken and rabbit retina a subpopulation of cholinergic amacrine cells similarly appears to be unaffected by ECMA treatment [29, 30].

It has been shown that NGF increases the synthesis and concentration of ChAT molecules per cholinergic terminal in developing monolayer cultures of rat brain [16, 31] rather than neuronal survival or neurite outgrowth. Thus, if NGF-sensitive, intact cholinergic neurones do remain following ECMA exposure of the reaggregates, NGF should still be able to enhance cholinergic function and increase ChAT activity. The apparent inability to do so may possibly reflect either an additional inhibitory effect of ECMA on new ChAT protein synthesis (although muscarinic receptors can "re-grow" following ECMA [4]), an additional requirement for other trophic factors such as T_3 [15] or GM1 ganglioside [32], or alkylation of the NGF receptor on the cholinergic neurones. Furthermore, the effects of NGF *in vivo* may be dependent on the mechanisms used for inducing lesion formation.

The present work has demonstrated that enhancement of muscarinic receptor binding by NGF does not occur in developing rat brain cultures. This implies that NGF enhances only presynaptic cholinergic function via increased ChAT activity but not the muscarinic receptor binding component, or number of postsynaptic dendrites bearing muscarinic receptor. In contrast, a previous report has shown that in a rat phaeochromocytoma cell line (PC12), which possesses many of the characteristics of adrenal chromaffin cells, NGF can produce a marked elevation of specific [^{3}H]QNB binding [33, 34].

It has been suggested that TRH has a trophic effect on lower motor neurones (LMN) and has been shown

to produce clinical benefit on LMN function in patients with ALS (see Ref. 20). In ventral, but not dorsal, rat spinal cord cultures TRH was shown to markedly elevate ChAT activity and improve morphological appearance [20]. In the present work, however, TRH was not able to reverse the ECMA-induced ChAT deficit in brain reaggregates nor did it have any significant stimulatory effect in normal serum-supplemented cultures despite transient effects in the serum-free cultures. This may represent regional differences in neuronal responsiveness to TRH. However, it has been shown that this peptide can also improve hindlimb function after spinal cord injury in cats, an effect later ascribed to counteraction of the decreased spinal cord blood flow following injury (see Ref. 25).

In conclusion, we have provided several additional findings using developing brain reaggregates which may help to further the understanding of the action of NGF and also its production in normal and lesioned CNS tissues (see Ref. 35). ECMA-induced lesions in serum-free cultivated reaggregates should enable studies of NGF synthesis and release. The effects of exogenous NGF appear to be best studied in lesioned reaggregates grown in an S+ or T_3-supplemented [15] medium.

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