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Development of markers for cholinergic neurones in re-aggregate cultures of foetal rat whole brain in serum-containing and serum-free media: effects of triiodothyronine (T_3)

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1 Development has been studied in re-aggregate cultures derived from the 16 day foetal rat brain and the effects of triiodothyronine (T_3) investigated. Cultures were maintained in either a medium containing 10% serum (S^+), or in serum-free culture medium (S^-) or in serum-free medium containing 30nM T_3.

2 The muscarinic cholinoceptor, measured by specific binding of [^{3}H]-quinuclidinyl benzitate ([^{3}H]-QNB) at 9 and 14 days *in vitro*, was at a lower level in the serum-free cultured cells compared with those in serum-containing culture medium (S^+). In cultures in the latter medium, receptor concentration at day 14 was of a similar magnitude to that in rat brain at an equivalent postnatal age. Binding increased with development from 9 to 14 days *in vitro* in the S^+ medium but not in the S^- medium. T_3 treatment caused an 85% increase in [^{3}H]-QNB binding compared with the cultures in S^- medium at day 14 to a level equivalent to that found in the cells grown in S^+ medium. This increase was reflected in the B_{max} but not in the K_D (approx. 0.1nM).

3 Choline acetyltransferase (ChAT) activity developed more slowly in the S^- medium than in the S^+ medium where the specific activity approximated values obtained *in vivo*. T_3 treatment of cultures grow in S^- medium significantly enhanced the developmental rate of increase of ChAT activity.

4 The characteristics of [^{3}H]-choline uptake and metabolism in the cultures was examined. Uptake was strictly Na^+-independent but was energy-dependent, and inhibited by 2, 4′-dinitrophenol (2, 4′-DNP) and cooling (0–4°C). Neither iodoacetate nor ouabain had any effect on the amount of uptake. Hemicholinium (HC3) was a potent inhibitor of uptake (70% inhibition at 10 μM HC3). Metabolism studies showed virtually no conversion to [^{3}H]-acetylcholine ([^{3}H]-ACH) in re-aggregates grown in either the S^+, S^- or T_3 containing media. However, a small amount of [^{3}H]-choline was incorporated into phosphorylcholine. T_3 treatment had no effect on this metabolic profile.

5 The kinetics of [^{3}H]-choline uptake by the re-aggregates was also studied in the re-aggregate cultures (after 12 and 22 days *in vitro*) using [^{3}H]-choline at 0.05–100 μM. Both Eadie-Hofstee transformation and least-squares analysis of the data showed that the uptake comprised only a single low-affinity component with an apparent K_t = approx. 50 μM. Unlike ChAT and [^{3}H]-QNB binding, there appeared to be no difference between the uptake in the different culture conditions.

6 It is concluded that the differentiation of cholinergic neurones and muscarinic receptors in serum-free cultured re-aggregates from foetal rat brain is enhanced by thyroid hormone treatment. The development of [^{3}H]-choline uptake does not seem to be associated with cholinergic cells under these culture conditions, and is unaffected by thyroid hormone treatment.

Introduction

Thyroid hormone has been shown to influence the development of cholinergic neurones and their synaptic components in several *in vivo* studies. Thus, thyroid deficiency induced in neonatal rats causes a brain region-dependent retardation of the development of choline acetyltransferase (ChAT) activity and choline uptake (Ladinsky *et al.*, 1972; Kalaria *et*

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al., 1981), and the muscarinic cholinoceptor (Patel et al., 1980). However, in areas such as striatum it is still not certain whether such treatment results in the development of fewer cholinergic terminals (and synapses) or in intraterminal deficiencies of (ChAT) and choline uptake capability (see Kalaria et al., 1981). Triiodothyronine (T_3)-induced hyperthyroidism in developing rats is similarly connected with changes in cholinergic function. There is a marked developmental increase in rat brain-stem acetylcholine (ACh) synthesis, acetylcholinesterase (AChE) and ChAT activity which is not seen in cerebral cortex (Rastogi et al., 1977). A similar finding for thyroxine-treated rat heart ACh content was described earlier by Khanna & Pandhi (1972).

There are also changes in adult rat brain following perturbation of thyroid function. Thus, in striatum of thyroid-deficient adult rats both ChAT and [^{3}H]-choline uptake activity remained unchanged whilst [^{3}H]-spiroperidol binding was impaired suggesting that the number of dendritic-spines on the cholinergic interneurones which bear dopamine receptors is probably reduced (Kalaria & Prince, 1983).

More precise information on the effects of hormones on developing nerve cells has been derived from the use of *in vitro* tissue culture systems where cell growth can be supported in chemically-defined media. Many studies have used primary, monolayer cultures of CNS tissue (both pure neurones and glia) to characterize the development of various components of the cholinergic system (see Massarelli et al., 1976; Louis et al., 1981; Massarelli et al., 1974 a, b; Betz, 1981; Balazs & Kingsbury, 1982).

Furthermore, Honegger & Lenoir (1980) have shown that repeated addition of thyroid hormone (T_3) to serum-free cultures of foetal rat brain re-aggregates (mixed cultures of neurones and glia) greatly enhances the differentiation of the cholinergic neurones as measured by changes in ChAT activity. Cellular proliferation was not affected by the hormone treatment (Honegger & Lenoir, 1980). Another finding was that the addition of nerve growth factor (NGF) or high potassium concentrations to serum-free re-aggregate cultures also greatly stimulated the developmental increase in ChAT activity, this phenomenon being dependent on the presence of T_3 in the culture medium (Honegger & Lenoir, 1982; Honegger, 1983). This 'permissive' influence of T_3 also appears to be necessary for the action of other trophic factors on cholinergic neuronal differentiation in the aggregates (Guntert & Honegger, 1982).

We have set out in this paper to provide an extended and detailed study of the effects of thyroid

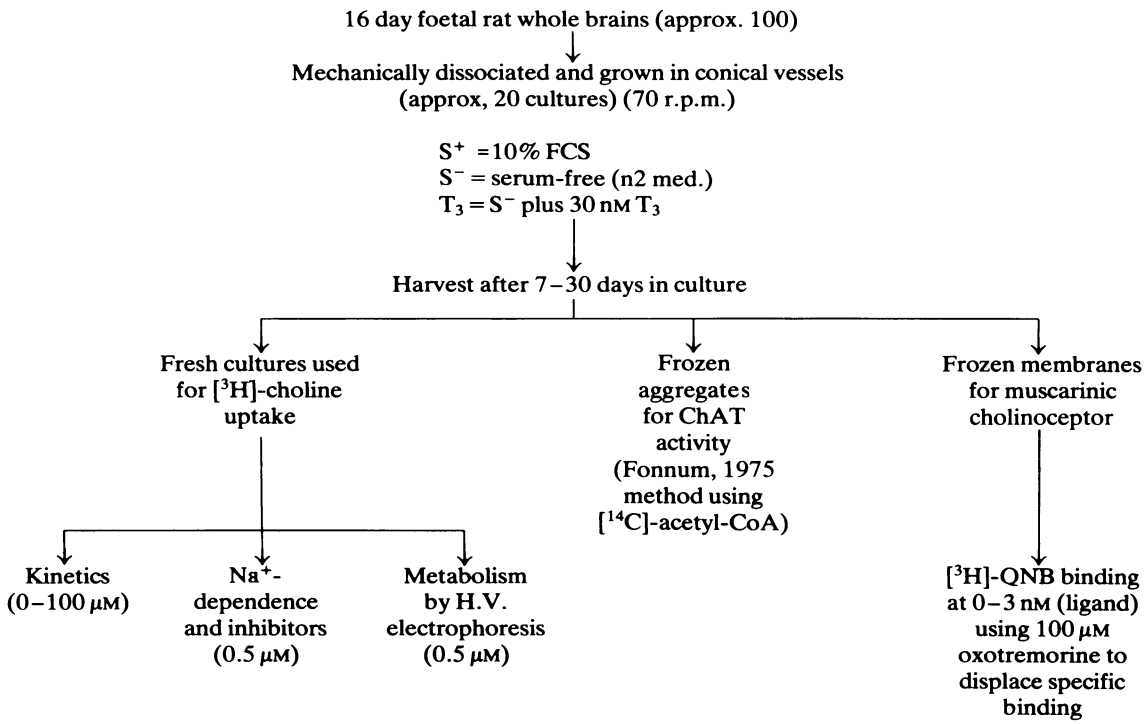

Figure 1 Methods for study of cholinergic system in aggregate cultures

hormone (T_3) on several parameters of the developing cholinergic system in foetal rat whole-brain re-aggregates grown in serum-free medium. ChAT activity, the muscarinic cholinoceptor (^{3}H-quinuclidinyl benzitate binding), and [^{3}H]-choline uptake were all examined and the values compared with those for re-aggregates cultivated in the presence of foetal calf serum (FCS).

Methods

A summary of the culture procedure and neurochemical assays performed is shown in Figure 1.

Cell culture

Foetal brain re-aggregating cultures were grown essentially by the method of Honegger & Lenoir (1980), and as described by Atterwill *et al.*, (1983). Whole brains from 80–100 16 day old rat foetuses (giving 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 gently stroking with a glass rod in 30 ml Hanks D1 solution (Ca^{2+}, Mg^{2+}-free). The suspension was now refiltered through Nybolt 130 μm pore size and centrifuged at 1000 *g* for 5 min (0–5°C). Following mechanical trituration of the tissue (10 passes through 5 ml pipette) in 10 ml D1 solution, the cells were re-centrifuged as above. This procedure was repeated twice. The final cell pellet was resuspended in culture medium (S^+ =Dulbecco's Modified Eagle's Medium DMEM plus 10% foetal calf serum plus extra glutamine, see below; S^- is based on the N2 medium of Bottenstein & Sato (1979) comprising a 3: 1 mixture of DMEM and Hams D12 medium containing insulin (5 mg 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 conc. 300 μg ml^{-1}) were also added to both S^+ and S^- culture media.

Next 3.5 ml of the cell suspension (10^5 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°C) at a constant rotation of 70 r.p.m. on a Luckham Rota test orbital shaker (orbit diameter = 1.0 in). The speed was gradually increased to 80 r.p.m. 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. T_3 (30 nM) was added to the cultures after 2 days *in vitro* and on alternate days thereafter. Aggregates were harvested for enzyme and receptor binding measurements by allowing them to sediment under gravity and washing 3 times with Dulbecco's phosphate buffered saline (PBS) at 25°C. The final cells were frozen in liquid N_2 and stored (−70°C).

Choline acetyltransferase (ChAT)

Each separate aggregate sample (1 flask) was thawed and then treated with Triton X-100 (final concentration 0.5% v/v; 0°C, 2h) and assayed for ChAT as described by Fonnum (1975) (2 nM choline chloride; 110 μM [^{14}C]- acetylCoA; 150 mM NaCl; pH 7.0; 37°C). Approximately 5–10 μg aggregate protein was used for each enzyme determination and cell incubations were carried out for 20 min in duplicate under these conditions. It has been shown previously (see Atterwill & Prince, 1979) 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 culture samples.

Muscarinic receptor binding

Muscarinic receptor 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 oxotremorine. Aggregate cell pellets were thawed and homogenized in 50 mM Tris-HCl buffer (pH 7.4, 0°C), 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 Patel *et al.* (1980) using either a single, saturating ligand concentration of 2 nM, or 0.1–3 nM QNB for kinetic determinations.

[^{3}H]-choline metabolism

This was carried out essentially as described previously for brain slices (see Atterwill & Prince, 1979; Kalaria *et al.*, 1981). In these experiments aliquots of resuspended, washed aggregates were incubated under O_2 in the Krebs-Tris medium (37°C, 15 min) with 12.5 μCi of neat [^{3}H]-choline (final concentration 0.6 μM) in a final volume of 2.5 ml. At the end of the incubation the tissue was transferred to 15 ml conical centrifuge tubes and placed on ice. The tissue was then recovered and washed twice with ice-cold Krebs-Tris medium (pH 7.4, 0–4°C) by brief (1 min) centrifugation at 1000 *g*. Any excess supernatant was decanted and blotted off with filter-paper strips and each sample resuspended in 400 μl of cold extraction medium comprising 15% v/v formic acid in acetone (Toru & Aprison, 1966) with 100 μM physostigmine

salicylate, acetylcholine chloride (2 mg ml^{-1}), choline chloride (2 mg ml^{-1}) and phosphoryl choline chloride (1 mg ml^{-1}) present. Following extensive homogenization the extracts were frozen at −70°C and then samples of the extracts were both counted for tritium and analysed by high voltage, paper electrophoresis as previously described (see Atterwill & Prince, 1979).

[^{3}H]-choline uptake

Aggregates from 3–4 culture flasks (S^+, S^- or S^- plus 30 nM T_3) were pooled in a large tube, allowed to settle and the culture medium aspirated. Unless otherwise stated (see Results) the pooled material was washed twice by resuspension in either room temperature Krebs-Tris buffer containing sodium or sodium-free, Krebs-Tris buffer (made iso-osmolar with sucrose) depending on the type of incubation conditions required. The composition of these buffers is given below.

The aggregates were then resuspended in 10–12 ml of the appropriate buffer (ice-cold pH 7.4 0–4°C). When required 0.5 ml aliquots of the suspension were transferred to cooled 25 ml Erlenmeyer flasks containing 4.5 ml of the incubation medium (pH 7.4 at 37°C), and gassed with 100% O_2 and sealed with 'subaseals'. After a preincubation period of 10 min, 50 µl of radiolabelled choline solution was injected through the seal (2.5 µCi [^{3}H]-choline plus unlabelled choline chloride at final concentrations ranging from 0.025–95 µM) and incubations continued at 37°C for 10 min in a shaking water bath. To terminate uptake, the contents of each flask were poured into a 15 ml conical centrifuge tube, 5 ml of ice-cold Krebs-Tris buffer added and the tubes placed on ice. (In control experiments it was established that addition of a large concentration of cold choline to the aggregates at this and subsequent stages did not improve or alter results and thus was of no use in preventing exit of [^{3}H]-choline from the cells). Each tube was then briefly centrifuged (1000 *g* for 1 min) to sediment the aggregates and the medium carefully aspirated. This procedure was repeated twice with ice-cold buffer. After complete removal of the final supernatant, 0.5 ml of distilled water was added to the tissue pellet and homogenized with 15 passes of a glass-glass homogenizer; 200 µl portions were transferred to counting vials and 1 ml of a 1: 1 soluene-350 (Packard Ltd.) -isopropanol

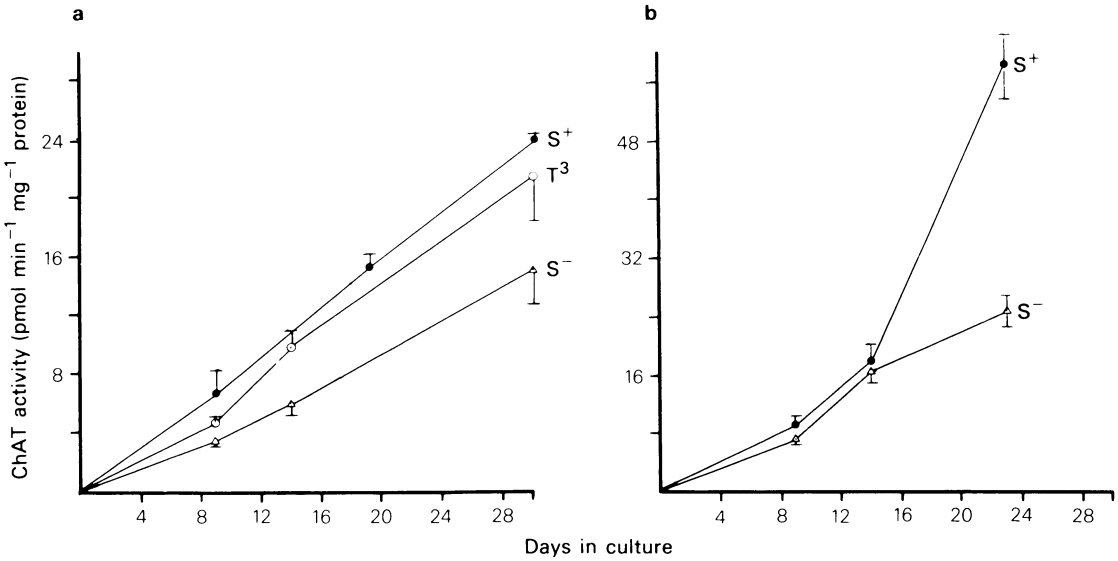

Figure 2 Choline acetyltransferase (ChAT) activity in aggregate cultures grown in the presence of 10% foetal calf serum (S^+; ●——●), in chemically-defined, serum-free medium (S^-; △——△), in S^- medium with triiodothyronine (T_3) treatment (T_3; ○——○). For the T_3 treatment 30 nM T_3 was added to the cultures after 3 days in culture and every other day thereafter. Following harvesting of the cultures at different developmental stages, they were washed in PBS and assayed for ChAT and protein. The enzyme activity curves for different culture experiments are shown. In (a) the S^+ culture was from a separate experiment to the S^- and T_3 conditions. Results are given as mean and vertical lines show s.e.mean. T_3 curve was significantly different from S^- curve after 9 days in culture ($P<0.05$) and 14 days ($P<0.02$), Student's 2-tailed *t* test.

mixture added and left overnight. Then 10 ml of Scintran scintillant (BDH) containing 0.15% HCl was added and the samples counted for tritium on a liquid scintillation spectrometer. At the same time, 200 µl portions of the lysate were diluted 1/50 in 0.1N NaOH and the protein content determined as described below.

Aggregate morphology

For morphological assessment of the aggregates they were fixed for 30 min in 4% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 at 4°C. After washing in phosphate buffer they were osmicated for 1 h in 1% OsO_4 and dehydrated in ethanol before embedding in Spurr's resin. Semithin (0.5 µM) sections were cut and stained with toluidine blue.

Protein

In all cases protein was measured by the method of Lowry *et al.*, (1951) using bovine serum albumin as a standard, and allowing for interference by Tris-containing buffers where appropriate.

Solutions

The sodium-containing, Tris-buffered Krebs medium was of the following composition (mM): Tris-hydrochloride 50, KCl 4.75, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, NaCl 118, D-glucose 11.1, gassed with 100% oxygen, pH 7.4 at 0–4°C or 37°C as required. The sodium-free Krebs-Tris medium was as described above except that the NaCl was replaced with iso-osmolar sucrose.

Materials

All chemicals used were of the highest purity available and obtained from either Sigma Chemical Co., Surrey or BDH Ltd. Soluene-350 was obtained from Packard Instrument Co., Berks. [Methyl-^{3}H]-choline chloride (approx. 10 Ci mmol^{-1}; 1 m Ci ml^{-1}) and [1-^{14}C]-acetyl CoA(solid) were obtained from Amersham International PLC. [^{3}H]-quinuclidinyl benzilate (QNB; 33 Cimmol^{-1}) was purchased from New England Nuclear. Tissue culture materials were as follows: DMEM = Dulbecco's Modified Eagle's Medium, 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°C before incorporation in the media. T_3(L-triiodothyronine, Na^+ salt) was purchased from the Sigma Chemical Co. Ltd and dissolved freshly each run, first in 0.05 M NaOH with subsequent dilutions in culture medium.

Results

Development of choline acetyltransferase (ChAT) activity in foetal rat whole brain re-aggregate cultures: effects of triiodothyronine (T_3)

The differentiation of cholinergic neurones in the whole brain re-aggregates and the dependence on thyroid hormone were first assessed by examining the development of ChAT activity in aggregates cultivated in serum-free conditions with and without 30 nM T_3 present throughout the growth period. For comparison, ChAT activity in cells grown in the presence of 10% foetal calf serum (FCS) was examined.

Three separate experiments are depicted in Figure 2 where either T_3 vs S^-, S^+ alone (batch grown very close to T_3 vs S^- batch), or S^+ vs S^- conditions were investigated in separate culture batches. As this parameter was assessed at a number of 'age points' it was not possible to grow all three conditions simultaneously.

It can be seen from Figure 2a that the specific activity of ChAT increased gradually during development of the re-aggregates in serum-free medium. Addition of 30 nM T_3 after 2 days in culture significantly enhanced the developmental rate of increase in ChAT, with approximately a 40–60% increase over control activity at any age point (Figure 2a).

The developmental rise in ChAT activity in cultures grown in the presence of 10% FCS was greater that in serum-free medium starting around day 14 (Figure 2b). Thus, serum enhances the differentiation of the cholinergic neurones. It can be seen that there is some variability in the level of ChAT activity in the different culture batches, this has also been noted by other groups (Honegger *et al.*, personal communication). Therefore, it is important to perform paired comparisons as we have done here.

Effect of L-triiodothyronine (T_3: 30 nM) on [^{3}H]-quinuclidinyl benzilate (QNB) binding in foetal rat brain re-aggregate cultures grown in chemically-defined medium

In order to follow the effects of T_3 on the development of the muscarinic cholinoceptors *in vitro*, specific [^{3}H]-QNB binding was examined at a 3 nM saturating ligand concentration in membranes prepared from rat whole brain re-aggregates culture for 9 and 14 days. The results are shown in Table 1. In the presence of 10% foetal calf serum, cholinoceptor binding increased markedly from 9 to 14 days in culture indicating a 65% developmental rise in this parameter in the cells (Table 1), which was similar to that obtained for the ChAT activities (see Figure 2).

Table 1 [^{3}H]-quinuclidinyl benzilate (QNB) binding in developing foetal rat whole brain re-aggregate cultures

Culture Medium	*Number of days in culture* 9		14
S^+	92.2 ± 18.5 (c)	(a)	152.8 ± 19.1 (d)
S^-	79.9 ± 16.8 (e)	(b)	86.2 ± 14.4 (f)
S^- (plus 30 nM T_3)	101.7 ± 19.6		159.6 ± 21.7

Results represent mean specific binding at 3 nM [^{3}H]-QNB in fmol mg^{-1} protein ± s.e.mean. Data were analysed using Student's 2-tailed *t* test as follows: (a) = $P<0.05$, (b) = NS, (c) = NS, (d) = $P<0.05$, (e) = NS, (f) = $P<0.05$; $n = 5-6$ cultures per group.

In serum-free medium the QNB binding activity was lower than in the serum-containing medium, the difference being significant after 14 days in culture only (Table 1). Furthermore, under these conditions there was no developmental increase in the level of cholinoceptor binding from 9 to 14 days in culture. However, the addition of 30 nM T_3, after 2 days in culture, to those aggregates in S^- medium did affect the [^{3}H]-QNB binding. There was an increase in binding in the T_3-containing medium at both age points (Table 1), and this reached statistical significance at 14 days in culture. Moreover, the developmental increase in receptor binding was restored in the presence of the hormone (Table 1), and the values obtained in the presence of T_3 were comparable to those seen in the S^+ aggregate cultures.

In preliminary experiments using 14 day old (in culture) aggregates it was shown by Scatchard analysis (0.1–3 nM [^{3}H]-QNB) that the change in binding produced by T_3 addition was actually an increase in the B_{max} of [^{3}H]-QNB binding (75% increase) and not in the apparent K_D (0.1 nM). This K_D value is similar to that found in adult rat brain tissue (see Patel *et al.*, 1980).

Time-course and Na^+-dependence of [^{3}H]-choline uptake by rat brain re-aggregate cultures

The time-course of [^{3}H]-choline uptake into the brain cell re-aggregate cultures was examined to establish a suitable time-point for future experiments on this parameter and to see if it was taken up in a

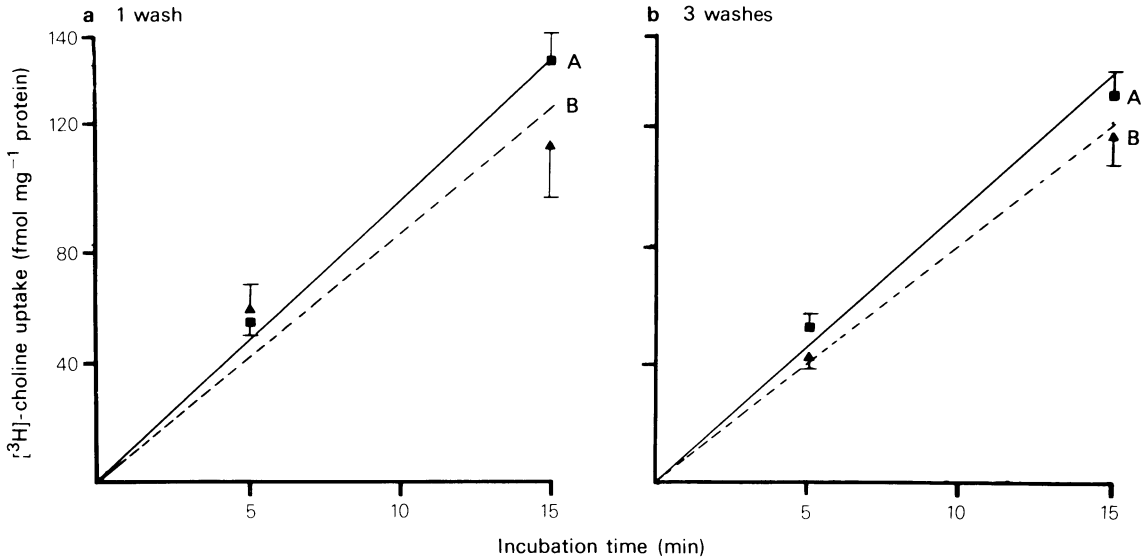

Figure 3 Time course, Na^+-dependency and effect of washing on [^{3}H]-choline uptake (0.5 μM final concentration) by the aggregates. S^+ cultures (after 14–22 days) were removed from their culture medium and washed either (a) once in Na^+-free Krebs-Tris buffer where Na^+ was replaced by sucrose (A;■———■) or once in Krebs-Tris buffer containing 118 mM Na^+ (B;▲– – –▲); or (b) three times as A or B above. They were then preincubated at 37°C in the appropriate buffer for 10 min followed by an incubation with [^{3}H]-choline 0–15 min in a shaking water-bath. Following removal of excess, extracellular [^{3}H]-choline by washing and centrifugation (see Methods) at the end of the uptake period [^{3}H]-choline and protein were determined. Results are expressed as mean uptake in fmol mg^{-1} protein (6–8 determinations) against incubation time (min). Vertical lines show s.e.mean. No significant differences between the A (Na^+-free) and B (Na^+-plus conditions) were seen in either experiments (a) or (b) (Student's *t* test).

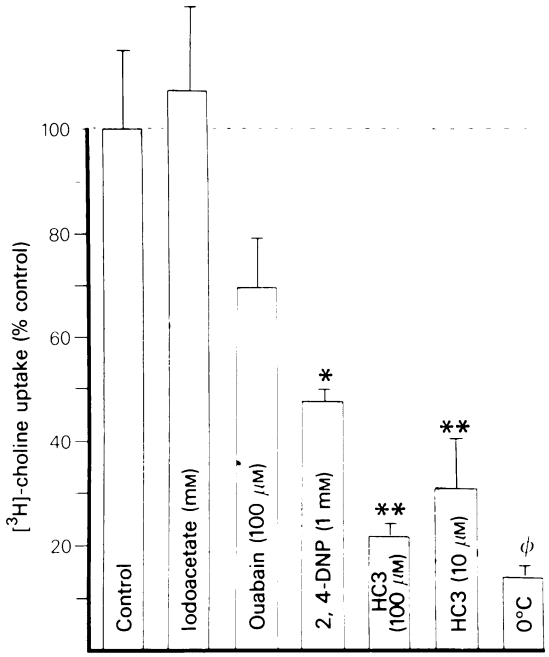

Figure 4 Effect of various drugs and temperature on [^{3}H]-choline uptake by rat whole-brain re-aggregate cultures. Aggregates cultured for 14–21 days in S^+ medium were used for this series of experiments. Cells were prepared as previously described and preincubated at 0°C or at 37°C for 10 min in the presence of the test compound in incubation medium containing Na^+. [^{3}H]-choline was then added at a final concentration of 0.5 μM and uptake measured for 10 min. Following washing of the aggregates, tritium and protein were determined as before. Each histogram represents the mean of 4–8 determinations expressed as % of control, untreated [^{3}H]-choline uptake (100%) in each drug-treated sample. Vertical lines show s.e. mean. $^*P<0.05$, $^{**}P<0.02$, $^{\phi}P<0.01$, significantly different from control (Student's *t* test).

time-dependent fashion. At the same time the Na^+-dependence of uptake was assessed by incubation in a normal Krebs-Tris incubation buffer and in one where Na^+ had been replaced by sucrose. In these experiments the extent to which the cells were washed before the uptake expriment in the sodium-free buffer was considered important and, therefore, examined in detail. Preincubations were carried out in the appropriate buffer, and all experiments were performed at 37°C.

It is apparent from Figure 3 that the total [^{3}H]-choline uptake into the re-aggregates cultivated in S^+ culture medium increased linearly up to 15 min incubation. Therefore, in all future experiments a 10 min time-point was chosen.

In the absence of sodium (Figure 3) no difference in [^{3}H]-choline uptake was seen compared with the samples incubated in the presence of sodium. This was not due to the degree of pre-washing of cells as no change in the result was noted between aggregates washed once (Figure 3a) or three times (Figure 3b). It is thus concluded that the [^{3}H]-choline uptake is Na^+-independent.

Effect of various drugs and temperature on [^{3}H]-choline uptake by rat whole brain re-aggregate cultures

In order to determine whether [^{3}H]-choline uptake into the re-aggregates was an energy-dependent process the sensitivity of the process to inhibition by various metabolic inhibitors and low temperature was investigated. The effects of ouabain (an inhibitor of Na^+, K^+-ATPase) and hemicholinium (HC3 – a potent inhibitor of high-affinity choline uptake) were determined; the aggregates were exposed to the drugs during both the preincubation and incubation stages.

From Figure 4 it is evident that the [^{3}H]-choline uptake process determined in cultures grown for 24 days in the presence of 10% FCS was energy-dependent, as significant inhibition occurred with the uncoupling agent, 2, 4-dinitrophenol (DNP) and at 0°C. However, no inhibition was observed with iodoacetate (glycolysis inhibitor).

Ouabain which completely inhibits Na^+, K^+-ATPase in immature and mature rat brain tissue (Atterwill & Balazs, 1984) did not significantly reduce the [^{3}H]-choline uptake in line with the observations for Na^+-dependency.

HC3 at both 10 and 100 μM inhibited [^{3}H]-choline uptake (Figure 4). This was surprising for a Na^+-independent, low-affinity uptake process as we have demonstrated for these cells (see later). The temperature-dependence was also investigated at [^{3}H]-choline concentrations ranging from 0–100 μM. At all choline concentrations uptake was inhibited by cooling to 0°C.

Kinetics of [^{3}H]-choline uptake and effect of triiodothyronine (T_3) treatment

Kinetic analysis of [^{3}H]-choline uptake in re-aggregates cultured for 22 days was performed using [^{3}H]-choline concentrations between 0.05–100 μM (in the presence of sodium). Neither Eadie-Hofstee transformation of the data nor least squares analysis (by computer) indicated a high affinity uptake component (Figure 5). Data best fitted a single low affinity uptake component plus diffusion giving

$$\left(V = \frac{V_{max}.\,S.}{K_t + S} + K_D.S\right)$$

a K_t(app) = approximately 13–20 μM choline.

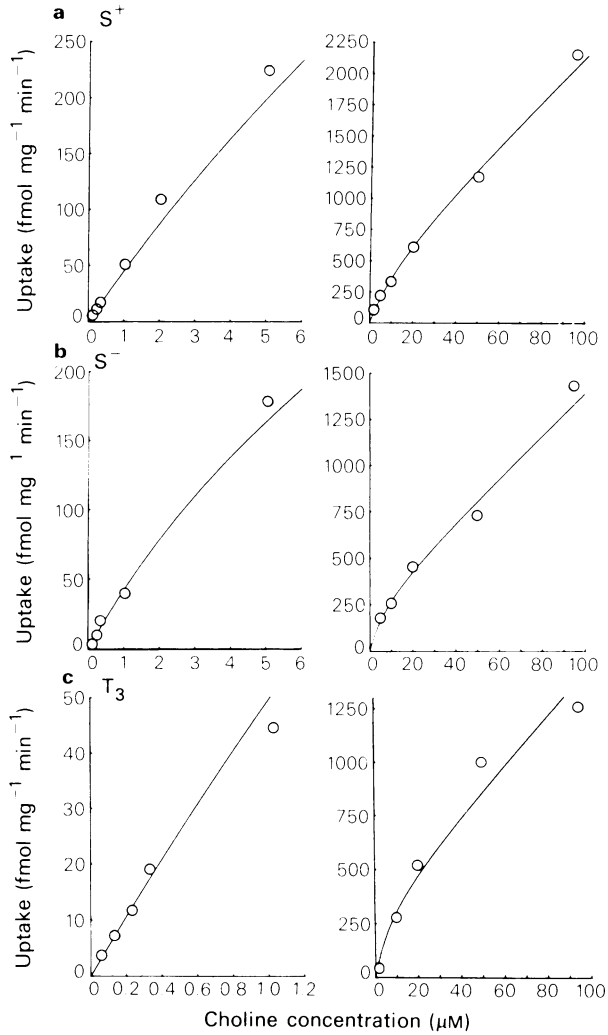

Figure 5 Kinetic analysis of [^{3}H]-choline uptake into foetal brain re-aggregates cultured for 22 days under the 3 different conditions. Uptake was determined as described between 0.05–100 μM [^{3}H]-choline (final concentrations) and the data plotted by least squares fitting. The left-hand graphs show the velocity (fmol mg^{-1} min^{-1}) data in the low choline concentration range (0–5 μM) and the right-hand graphs show the complete concentration range (0.05–100 μM). (a) S^+, (b) S^-, (c) S^- plus treatment with 30 μM triiodothyronine (T_3) as described previously. Computer analysis showed that the data best fitted a single low-affinity uptake-component model with a diffusional component described by the equation $V = \frac{V_{max}S}{K_t + S} + K_D S$ giving the parameters S^+: $K_t = 17\,\mu M$, $V_{max} = 499$ fmol mg^{-1} min^{-1}, $K_D = 17\,\mu M$; S^-: $K_t = 8\mu M$, $V_{max} = 280$ fmol mg^{-1} min^{-1}, $K_D = 11\,\mu M$; S^- (plus T_3); $K_t = 8\,\mu M$, $V_{max} = 343$ fmol mg^{-1} min^{-1}, $K_D = 11\,\mu M$. (The index of fit was improved approximately 2 fold from a single component fit to single component plus a diffusional component fit. This also applied to a 12 day old culture (not shown).) The low concentration range data (left-hand plots) showed no indication of curvature further indicating the lack of second saturable high-affinity uptake component.

This was also the case for aggregates cultured for 8 and 12 days. Separate analysis of uptake in the lower concentration range (0.05–2 μM [^{3}H]-choline) displayed linearity in the V/S plot with all three culture conditions (Figure 5), and at all ages confirming the absence of a saturable high-affinity component.

Although there was a high level of between-culture batch variation, the V_{max} data for 22 days old cultures

Table 2 [^{3}H]-choline uptake into re-aggregate cultures after 22 days in culture: effect of triiodothyronine (T_3) treatment

a *Determination at single [^{3}H]-choline conctentrations*

[^{3}H]-choline concentration (μM)	*Culture medium* S^-	T_3	S^+
		$^-$(fmol mg^{-1} min^{-1}) *Uptake*	
50	1887 ± 169.4 (6)	1956 ± 131.2 (4)NS	1596(2)
0.5	35.1 ± 1.25 (6)	40.41 ± 2.81 (4)NS	–

b *Kinetics of [^{3}H]-choline uptake*

Kinetic model	*Culture medium* S^-		T_3		S^+	
	V_{max}*	K_t	V_{max}*	K_t	V_{max}*	K_t
Single uptake component (see Results)	4385 ± 1462	68.1 ± 22.9	3375 ± 856^{NS}	42.5 ± 9.1	3436	66.5
Single component plus diffusion (see Results)	735 ± 300	13.6 ± 5.7	1586 ± 595^{NS}	21.7 ± 8.3	900	21.9

In (b) *fmol mg protein; K_t in μM units. Results given as mean ± s.e. mean. (a) Number of individual cultures indicated in parentheses. (b) Kinetic experiments on 3–4 different culture batches but in the case of S^+ (b) mean results from 2 experiments only. NS, not significantly different from corresponding value in S^- medium.

(either corrected or uncorrected for a diffusional component), failed to show any significant effect of the T_3 (Table 2b). Similarly, [^{3}H]-choline uptake (at 0.5 or 50 μM choline) determined on individual cultures from within a single culture batch did not show an effect of thyroid hormone (Table 2a). The amount of choline uptake by S^+ aggregates was similar to that in the S^- cultivated cells (Table 2).

Metabolism of [^{3}H]-choline by rat whole brain re-aggregate cultures grown under various conditions

In order to discover whether any of the transported [^{3}H]-choline was metabolized to [^{3}H]-ACh by the

Table 3 [^{3}H]-choline metabolism in rat whole-brain re-aggregate cultures

Age of culture (days in vitro*)*	*Culture medium*	*% recovered as [^{3}H]-ACh*	*% recovered as [^{3}H]-choline*	*% recovered as [^{3}H]-phosphoryl-choline*	n
8–9	S^+	0.6	85.7	13.7	4
	S^-	0.8	69.5	29.7	2
23	S^+	1.0	83.7	15.3	4
	S^-	0	86.1	13.9	1
8–9	T_3	0.4	80.9	18.7	4
15		0.3	87.6	12.1	3

Aggregates from either S^+, S^- or T_3-treated conditions at different developmental stages up to 23 days in culture, were washed as described and incubated with 12.5 μCi [^{3}H]-choline (final concentration = 0.6 μM) for 15 min at 37°C in a shaking water bath in a Krebs-Tris medium containing Na^+ (145 mM). The tissue was then recovered, washed twice with ice-cold incubation medium and any excess supernatant removed by blotting. The tissue was then extracted in 15% v/v formic acid in acetone (containing 0.1 mM physostigmine) and subjected to high voltage paper electrophoresis as described in Methods. Each identifiable radioactive spot was counted for tritium and the results expressed as % of total label recovered (over the sum of the individual spots) as each ^{3}H compound. Between 1–4 cultures were examined at each developmental stage.

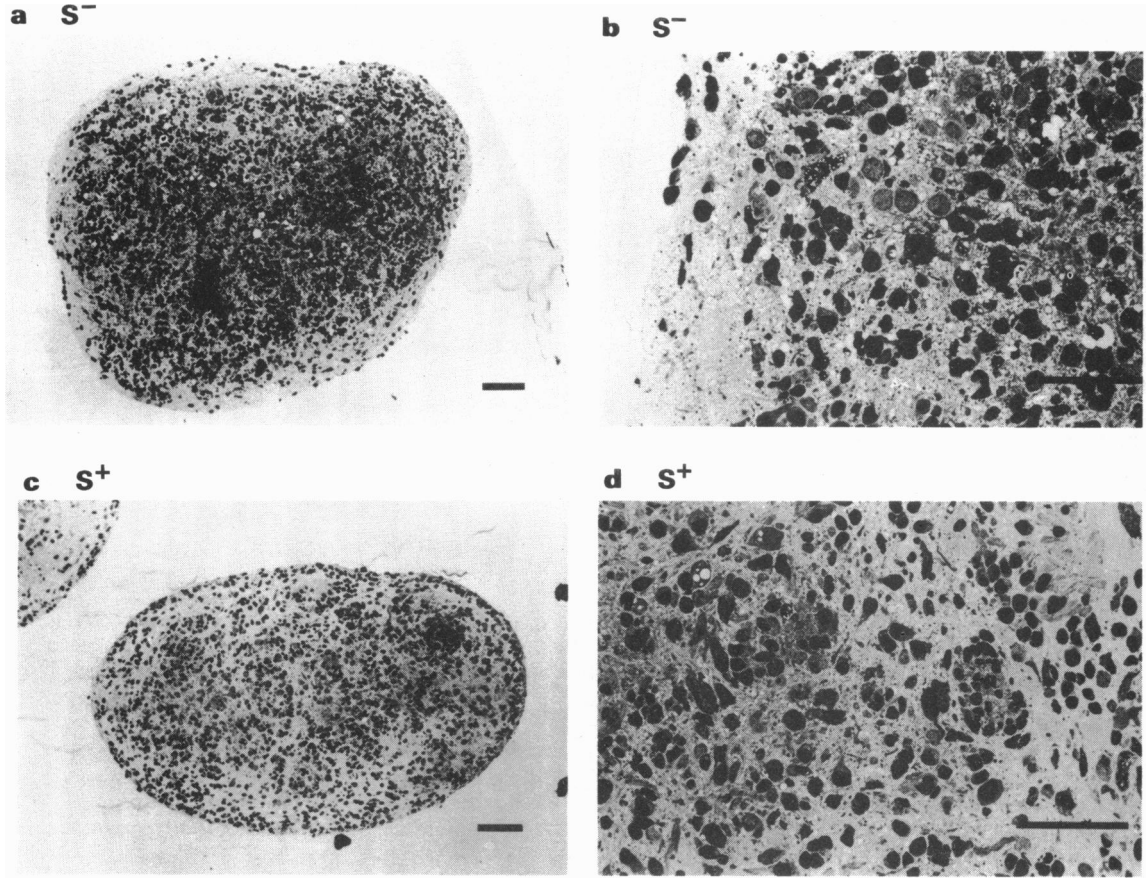

Figure 6 Toluidine blue staining rat whole brain aggregates cultured in either the S^+ (c and d) or S^- (a and b) medium for 10 days. Horizontal scale bars represent 100 μM.

re-aggregates, and whether T_3 influenced the metabolic profile, aggregates grown in either S^+, S^- or S^- plus 30 nM T_3 media were extracted and analysed by high-voltage paper electrophoresis for [^{3}H]-choline metabolites. These experiments were at 0.5 μM [^{3}H]-choline for two reasons. Firstly, a high-specific activity of radiolabel was required for detection purposes, and secondly at lower concentrations any residual high-affinity uptake if present (see kinetics section) would be included. The latter reasoning also applies to the other uptake experiments performed at only a single choline concentration.

Table 3 shows that virtually no[^{3}H]-AChwas recovered from [^{3}H]-choline by the re-aggregates under any three of the culture conditions. Furthermore, there was no age-dependent increase in ACh-synthesizing ability. However, a considerable proportion of phosphorylcholine was produced (15–30% of total radiolabel) in all three culture conditions and at all ages examined. T_3 had no effect on this conversion (Table 3).

Morphology

Figure 6 shows the morphological appearance from toluidine blue staining of 10 day old aggregates cultured in S^+ or S^- media. It is evident that there is a fairly well ordered arrangement of cells within the aggregates with very little pyknosis occurring at this age. Slightly more pyknosis occurs in the S^- medium. Larger cells seem to be in the centre zone of the aggregate with smaller ones nearer the edge and there is a relatively cell-free zone around the outer edge where many fibre bundles occur as shown by immunoreactivity of neurofilament protein (Woodhams, Atterwill & Balazs, unpublished). By 28 days in culture the aggregates start to display considerable pyknosis in the centre and were generally not utilized for neurochemical experiments.

Discussion

The effects of thyroid hormone on neural development have previously been studied using *in vitro* tissue cultures of CNS (see Atterwill, *et al.*, 1983). These studies have primarily been performed with surface cultures in dishes where basic aspects of cholinergic development *in vitro* have already been described in some detail (see Massarelli *et al.*, 1976; Louis *et al.*, 1981; Balazs & Kingsbury, 1982; Burgoyne & Pearce, 1982; Ishida & Deguchi, 1983). Aggregate cultures of rat brain, which are capable of forming organotypic connections and comprise a mixture of cell types have proved a valuable model for the study of hormonal effects on brain cells. They offer reproducibility for neurochemical assays and show extensive morphological and biochemical differentiation in both serum-containing and serum-free, chemically defined media (Honegger & Richelson, 1976; Trapp *et al.*, 1979; Honegger & Lenoir, 1980). Furthermore, T_3 does not affect cell proliferation in aggregate cultures (Honegger & Lenoir, 1980) thus eliminating one variable inherent with *in vivo* studies.

Choline acetyltransferase (ChAT)

The developmental increase in ChAT activity in aggregates cultured in S^- medium is slower than in cells grown in the presence of foetal calf serum (S^+) (Figure 2). In agreement with the work of Honegger & Lenoir (1980) we have also shown that 30 nM T_3 enhances the development of ChAT activity in re-aggregates of whole foetal rat brain cultured in serum-free medium. These authors also found that the accelerated increase in ChAT development in the T_3-treated cultures closely resembles the development of the enzyme *in vivo*, and that the precipitous rise *in vitro* coincides with the intense morphological maturation of the cultured neurones. The degree of increase in ChAT caused by T_3 is actually less than observed previously and may be due to the fact that in the previous study forebrain- rather than whole brain-derived cells were used (Honegger & Lenoir, 1980).

The ChAT activity after 30 days in culture in S^- medium was around 15–25 pmol min^{-1} mg^{-1} protein which is approx 50% of the level found by Honegger & Lenoir (1980) in this medium for forebrain re-aggregates. Another crucial factor determining the final level of enzyme activity in these cultures is the starting age of the original rat brain tissue used for culture (older foetuses leading to much lower ChAT activities in the cultured cells; Honegger & Richelson, 1976). It is also of interest to note that the maximal level of ChAT found in aggregates cultivated in the presence of serum ('physiological' conditions), approx. 60 pmol min^{-1} mg^{-1} protein after 23 days in culture, was lower but of the same order as that seen in adult rat brain cortical tissue *ex vivo* (150–200 pmol min^{-1} mg^{-1} Kalaria *et al.*, 1981).

Muscarinic receptors

Muscarinic cholinoceptors, as measured by [^{3}H]-QNB binding were also studied in the re-aggregate cultures under the different conditions. It has been shown previously that these receptors in culture are pharmacologically responsive, as muscarinic receptor activity was 'down-regulated' by exposure to cholinomimetics (Siman & Klein, 1979). Furthermore, it has recently been demonstrated that in retinal aggregate cultures there is a differential regulation of muscarinic and nicotinic receptors by cholinergic neuronal stimulation *in vitro* (Siman & Klein, 1983). The development of these muscarinic receptors *in vitro* has now been shown to be sensitive to thyroid hormone (Table 1). Specific [^{3}H]-QNB binding increased significantly between 9 day old and 14 day old cultures when the cells were grown in the presence of foetal calf serum, but did not appear to change under serum-free conditions. T_3 increased the number of receptors present in the cultures (but did not alter their K_D values) to levels comparable to those found in the presence of serum. The *in vivo* age-equivalent of a re-aggregate cultured for 14 days derived from 16 day rat foetuses is approximately 9 postnatal days. At this postnatal age muscarinic cholinoceptor density is around 200–400 fmol mg^{-1} protein in forebrain (Patel *et al.*, 1980). In the whole-brain aggregates cultured for 14 days the level was similar to this at around 200 fmol mg^{-1} of specific [^{3}H]-QNB binding.

Because both neurones and glia are present in these cultures it is not possible to specify the exact cellular site of action of the hormone as purified cultured glia derived from chick brain have been shown to possess muscarinic receptors (Repke & Maderspach, 1982). However, the enhanced differentiation of a presynaptic component (ChAT) suggests that these two synaptic changes might be functionally related, but it is not known which parameter is affected first.

The influence of thyroid hormone on muscarinic receptor differentiation in re-aggregates contrasts with the lack of effect in cerebellar monolayer cultures, enriched in granule neurones, where functional cholinergic synapses probably do not exist (Atterwill *et al.*, 1983). This leads one to speculate that the effect of T_3 is probably on the overall maturation of cholinergic synapses rather than on each parameter separately.

In vivo studies have shown the development of the

muscarinic cholinoceptor to be affected by changes in thyroid status. Forebrain receptor development seems to be unaffected whereas in cerebellum, where no effect is seen in tissue culture (Atterwill *et al.*, 1983), hyperthyroidism accelerates the rate of decrease of receptor density which normally occurs postnatally in this region and hypothyroidism has the opposite effect (Patel *et al.*, 1980).

[^{3}H]-choline uptake

Ex vivo preparations of central nervous tissue such as synaptosomes and brain slices have been shown to possess two uptake systems for choline, a high and a low affinity system. It is generally accepted that the sodium-dependent, high affinity process is regulatory and rate-limiting in providing the substrate for neurotransmitter synthesis in cholinergic nerve terminals, and can be regulated by nervous activity and drugs (Atweh *et al.*, 1976; Burgess *et al.*, 1978). The high-affinity process is attributable to cholinergic nerve terminals rather than to cell bodies or non-neuronal elements (Kuhar *et al.*, 1973; Sorimachi & Kataoka, 1975; Suskiw & Pilar, 1976) although it may not be restricted to central cholinergic terminals as it is present in the superior cervical ganglion (Higgins & Neal, 1982) and in cultured glia (Massarelli *et al.*, 1974a). The Na^+-independent low affinity system occurs in most other tissues, e.g. intesine, kidney, erythrocytes (Sung & Johnstone, 1965; Martin, 1968).

It was surprising to find that even though the levels of muscarinic receptors and ChAT activity in the serum-containing aggregates (Results) approached *in vivo* values virtually no conversion of transported choline to ACh was detected, whilst quite a high percentage (13%) was metabolized to phosphorylcholine. Thus, even though there may be similar concentrations of synapses in the cultures, some uptake system unrelated to cholinergic neurones appears to predominate under our experimental conditions. Honegger & Richelson (1979) have shown that 25–35 day old cultured rat brain aggregates will synthesize ACh from [^{3}H]-choline during 4 h incubations. The uptake system *per se* was not, however, examined, They stressed the necessity for depletion of endogenous choline by prior washing and preincubation in choline-free solutions, but the present procedure included comparable methods. Conversion to [^{3}H]-ACh may not have been detectable in the present work because shorter incubations and no anticholinesterase was used. This seems unlikely, however, because slices of rat cerbral cortex showed 60% conversion under identical conditions (Atterwill & Prince, 1978). Although the neuronal choline carrier must be present in these cultures it may be largely inoperative or altered due to adaptations to either the growth conditions, the inactivity of the neurones *in vitro* (see Higgins & Neal, 1982), or to changes in the endogenous choline concentration. Preincubation of neuronal monolayer cultures in choline-free media, which depletes endogenous choline and leads to 'non steady-state conditions', appears to cause adaptations of the choline carrier *in vitro* (Massarelli *et al.*, 1979; Louis *et al.*, 1983).

In our cultures the uptake of choline was sodium-independent and of a low affinity (K_t approx. 50 μM) with a large diffusional component (70% of total). It was, however, energy-dependent and sensitive to HC3. These observations are all consistent with the predominance of a low affinity uptake in the re-aggregates, directed towards the synthesis of phosphorylcholine. In 22 day old cultured cells T_3 did not appear to affect [^{3}H]-choline uptake by the reaggregates as assayed at low or high choline concentrations. The kinetic parameters of the transport process were also unaltered and serum-containing cultures gave approximately equivalent amounts of uptake. The predominance of a low affinity uptake system with little or no conversion to ACh, together with a relatively high phosphorylcholine synthesis suggests that perhaps we are mostly measuring a glial choline uptake mechanism in the aggregate cultures. The alternative would be uptake into non-cholinergic neurones or cholinergic cell bodies. In support of this hypothesis, Francescangeli *et al.* (1977) have shown that following intraventricular injection of labelled choline in adult rabbits, the levels of synthesized phosphorylcholine per unit protein in isolated glial cells were approximately four times greater than in isolated neurones, even though the rate of choline incorporation into lipids was faster in the neurones. The amount of phospholipid in glia was also greater than in neurones (Francescangeli *et al.*, 1977). Furthermore, high affinity choline uptake into ethanol-exposed glioblast cultures gives rise to phosphorylcholine and not ACh (Massarelli *et al.*, 1977).

There have been a number of studies on choline uptake into glial and 'mixed' neuronal/glial cultures of CNS tissue. Richelson & Thompson (1973) suggested that the K_t for choline transport was of a high-affinity nature in adrenergic and cholinergic clones, and in glial and fibroblast cell lines. Using primary cultures from dissociated chick embryo cerebral hemispheres Massarelli *et al.*, (1974a) demonstrated that the 'younger' mixed neuronal/glial cultures exhibited two uptake systems whereas 'older' cultures comprising mainly mature glial cells (astrocytes) showed only a low affinity choline uptake ($K_t = 5-10\,\mu$M). Also they showed low ($K_t = 20-50\,\mu$M) affinity processes to be present in both immature, undifferentiated glial cell lines and more mature glia from primary cultures of chick brain.

The K_t for the low affinity choline uptake into glia

(20–50 μM and 5–10 μM, Massarelli *et al.*, 1974a and b, respectively) compares fairly well with the K_t obtained for the uptake into our aggregate cultures. It is notable that most low affinity uptake systems determined in tissue preparations are Na^+-independent as was found for the present re-aggregate cultures.

In this study, therefore, we have shown that T_3 promotes the differentiation of cholinergic neurones, as judged by ChAT and muscarinic receptors, in serum-free cultivated re-aggregates from foetal rat whole brain where development is apparently retarded. Which is the primary response as yet remains uncertain. The effect of the hormone on the muscarinic receptor seems to be dependent on the presence of developing functional cholinergic synapses since no effect of the hormone has been found in cultures of neurones from cerebellum (Atterwill *et al.*, 1983). Choline uptake in re-aggregate cultures may reflect uptake into glia, on which thyroid hormone appears to have little or no effect. Thus, using re-aggregating CNS cultures, it may be possible to distinguish between neuronal and glial events in response to hormones and drugs. Studies of the effects on cholinergic neurones in pure neuronal monlayer cultures may be limited since chick brain cultures, comprising a predominance of neurones, display dopaminergic rather than cholinergic characteristics (Louis *et al.*, 1981). However, monolayer cultures of chick brain containing a mixture of neurones and glia appear to develop cholinergic projections after two weeks *in vitro* (Ebel *et al.*, 1974), and rat striatal cultures in serum-free media contain cholinergic interneurones (Messer, 1981). Because of the amenability of the cholinergic system for study in the re-aggregate cultures in chemically-defined media, they may prove of great usefulness for examining the effects of other trophic factors on cholinergic development and function. This has recently been attempted using monolayer cultures from rat brain (see Appell, 1982) to investigate the maturation of hippocampal, septal cholinergic neurones.

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