Thyroid hormone regulates Na^+ currents in cultured hippocampal neurons from postnatal rats

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SUMMARY

The causes for mental retardation due to perinatal hypothyroidism are not fully understood. Here we show that the most potent component of thyroid hormone, 3,5,3′-triiodo-L-thyronine (T3), selectively increases the density of voltage-activated Na^+ currents in hippocampal neurons from newborn rats. Thus, the well known effects of thyroid hormone on energy expenditure and Na^+/K^+ ATPase activity could to some extent result from the enhanced Na^+ influx through voltage-activated Na^+ channels. In addition, a down-regulation of the Na^+ current density in neurons could contribute to some of the neurological symptoms accompanying hypothyroidism, including slowing of mentation, of neuronal conduction velocities, the alpha rhythm of the electroencephalogram, and increased latencies of evoked potentials and reflexes.

1. INTRODUCTION

Severe perinatal hypothyroidism leads to cretinism. In areas of most pronounced iodine deficiency in some developing countries, as many as one in ten newborns is at risk of permanent brain damage (Dunn 1993), and even in some European cities periods of transient postnatal hypothyroidism are common (Delange *et al.* 1986).

It is generally accepted that thyroid hormones exert their effects predominantly via binding to nuclear receptors and regulation of the transcription of several genes (e.g. Brent *et al.* 1991; Mangelsdorf & Evans 1995). Although there is evidence that thyroid hormones selectively regulate a limited set of genes, the direct targets of thyroid hormone action have not unambiguously been identified (Oppenheimer *et al.* 1994). Thyroid hormones have been shown to influence the expression of a variety of transporters, enzymes and receptors involved in transmitter uptake, metabolism and detection (Honegger & Lenoir 1980; Patel *et al.* 1980; Atterwill *et al.* 1984; Dussault & Ruel 1987; Tejani-Butt *et al.* 1993) of some cytoskeletal proteins (e.g. Aniello *et al.* 1991) and neurotrophins (e.g. Lindholm *et al.* 1993; Alvarez-Dolado *et al.* 1994; Hashimoto *et al.* 1994) as well as Na^+/K^+ ATPases (e.g. Lindholm 1984; Atterwill *et al.* 1985).

Of the iodinated substances released from the thyroid gland thyroxine (T4) and 3,5,3′-triiodothyronine (T3) are thought to have significant biologic activity. T3 has been found to be several times more potent than T4 in eliciting the metabolic effects of thyroid hormone (e.g. Chopra 1991 for review). Recent investigations have demonstrated effects of T3 on the synthesis of Na^+ channels in skeletal myotubes (Brodie & Sampson 1989), and direct effects on the gating of Na^+ channels in cardiac myocytes have been described (Harris *et al.* 1991; Dudley & Baumgarten 1993). Likewise, effects of T3 on Ca^{2+} channels have been demonstrated in heart and skeletal muscle (e.g. Kim *et al.* 1987; Mager *et al.* 1992). Effects on voltage-gated ion channels in nerve cells, which generate neuronal excitability, have, however, so far not been demonstrated. As patterned neuronal activity is required for the fine tuning of appropriate synaptic connections in higher vertebrates (Goodman & Shatz 1993), an influence of thyroid hormone on the expression of Na^+ currents could contribute to the disturbances of mental development caused by thyroid hormone deficiency in the postnatal period.

2. MATERIALS AND METHODS

Cell cultures were prepared using the protocol described in Leßmann *et al.* (1994). Hippocampi removed from postnatal wistar rats (P0–P5) were cut into 1 mm thick tissue blocks. The CA3 regions were collected in ice-cold phosphate-buffered saline (PBS) supplemented with 10 mM HEPES, 1 mM pyruvate, 10 mM glucose, 2 mM glutamine. A 100 ml volume of this solution contained in addition: 0.1 ml of 6 mg ml^{-1} DNAse stock, 100 mg bovine serum albumin, 250 µl of a stock solution of 10^4 units ml^{-1} penicillin and 10 mg ml^{-1} streptomycin, 5 mg phenol red; pH was adjusted to 7.3 with NaOH (MPBS). MPBS+ contained, in addition 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$. After washing two times in ice-cold MPBS+ and once in MPBS the tissue was incubated for 8–15 min at 37 °C in MPBS containing 0.25 %

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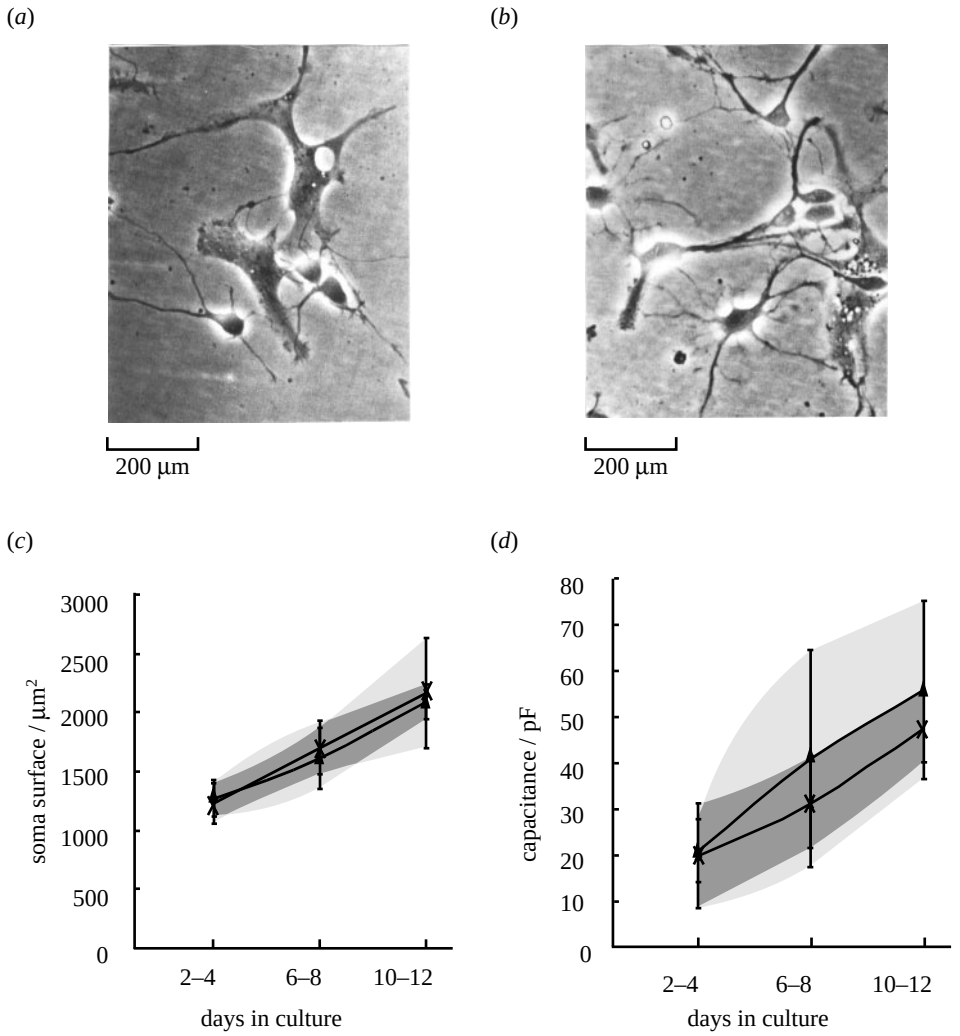

Figure 1. Influence of T3 on the morphological appearance of hippocampal neurons. (*a*) Phase contrast micrograph of cells dissociated at P3 and kept in B18 medium for three days. (*b*) Parallel culture from same preparation as shown in (*a*), but cultured for three days in the presence of 5.2 nM T3 (photographed with a 32× objective, same magnification for (*a*) and (*b*)). Comparison of soma surface areas (*c*) and membrane capacitances (*d*) versus time in culture for cells cultured in the presence (triangles) or absence (crosses) of 5.2 nM T3. Each data point calculated from at least six cells from the same preparation. Error bars and grey shadings indicate standard deviations. Mean values showed no significant differences at any time of measurement for soma surface or capacitance ($p > 0.05$, Student's *t*-test). Membrane capacitances were calculated from the areas under the capacitive artefacts, obtained by applying square test pulses of 10 mV after rupturing the membranes, to obtain the whole cell configuration of the patch-clamp recording technique. Soma surfaces were calculated under the assumption of an elliptical cell geometry with two small axes and one large main axis after estimation of the axes under a measuring ocular.

trypsin. Cells were dissociated by trituration using plastic pipette tips and suspended in 5 ml DMEM (Gibco) supplemented with 10% foetal calf serum (FCS). The suspension was centrifuged at 900 rpm for 5–8 min at 4 °C. After resuspension of the pellet in 2 ml ice-cold DMEM supplemented with 10% FCS, cells were plated at densities of 50 000 cells per dish in D, L-polyornithine-coated (1 mg ml^{-1} in borate buffer) 3.5 cm diameter plastic culture dishes in 1 ml DMEM plus 10% FCS and incubated at 37 °C in humidified atmosphere containing 10% CO_2. After 24 h, when cells had attached to the dishes, the medium was exchanged to serum-free B18 medium (Brewer & Cotman 1989) containing either no T3 or 5.2 nM T3.

Cell culture dishes were mounted on the stage of an inverted microscope (Zeiss IM 35), equipped with a long-distance condenser and an Achromat 32 LD objective. Patch-electrodes were pulled with a Narishige PP83 puller from thick-walled 1.5 mm diameter borosilicate glass tubing (Science Trading, GB 1508P) to tip diameters of about 1.5 µM. Resistances using the filling solutions described as follows ranged from 3 to 10 MΩ. For recording of Na^+ currents patch-pipettes contained (in mM) 140 CsCl, 5 NaCl, 0.2 $CaCl_2$, 2 EGTA, 1 $MgCl_2$, 15 HEPES, pH adjusted with CsOH to 7.3, and bath solutions contained (in mM) 120 NaCl, 4 4-AP, 10 TEA, 0.2 $CaCl_2$, 10 $MgCl_2$, 0.5 Cd^{2+},10 glucose, pH adjusted to 7.3 with CsOH. K^+ currents were recorded using pipette solutions of (in mM): 140 K^+gluconate, 5 NaCl, 0.2 $CaCl_2$, 2 EGTA, 1 $MgCl_2$, 15 HEPES, pH adjusted with KOH to 7.3 in bath solutions containing (in mM): 146 N-methyl-D-glucamine (NMDG), 4 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 0.5 Cd^{2+}, 10^{-3} TTX, 10 glucose, pH adjusted to 7.3 with HCl. Data were recorded with a List EPC7 patch-clamp amplifier filtered with a corner frequency of 3 kHz, digitized on a Digidata 1200 AD-converter board (Axon Instruments) with PClamp 6 software and stored on hard disk of an IBM-compatible personal computer. Data

were further analysed using the universal data evaluation program AUTESP (Garching Instruments).

3. RESULTS

(*a*) *Effects of T3 on the morphological appearance of hippocampal cells*

As previous studies had shown T3 concentrations close to the total, not the free T3 concentration found in serum to effect neurons in cell culture (Honegger & Lenoir 1980; Atterwill *et al*. 1984; Brodie & Sampson 1989; Harris *et al*. 1991; Dudley & Baumgarten 1993), we used a similar concentration to obtain pronounced effects. Hippocampal neurons grown in either the absence or presence of 5.2 nM T3 for 2–12 days in culture showed no major differences in morphological appearance (figure 1). As shown in figure 1*c*, the surface areas of neuronal somata increased with time in culture in both T3-deficient and T3-supplemented cultures. Likewise, cell capacitances showed similar increases under both culture conditions (figure 1*d*). Numbers of neurites and neurite lengths of cells cultured in the presence or absence of 5.2 nM T3 showed no obvious differences. Neurite lengths after 8 days in culture amounted to 226 ± 86 μm in T3-free medium (10 cells counted) and 211 ± 64 μm, in 5.2 nM T3-containing medium (10 cells counted). For instance, the number of neurites per cell in the same preparation were 5 ± 2 in both T3-free and T3-containing medium ($n = 10$). Rami *et al*. (1986) observed reduced pyramidal cell body volumes and reduced numbers of branching points in hippocampi of hypothyroid rats. The effects were largest at area CA4 at postnatal day 10 (P10) and less prominent at P6 and the other hippocampal areas. The observation of no obvious morphological effects of T3 in our cultures, which confirms observations of Filipčik *et al*. (1994), may be explained by the relatively younger age at which most of our experiments were performed. Alternatively, T3 could exert additional effects by, for instance, an upregulation of the production of neuro-

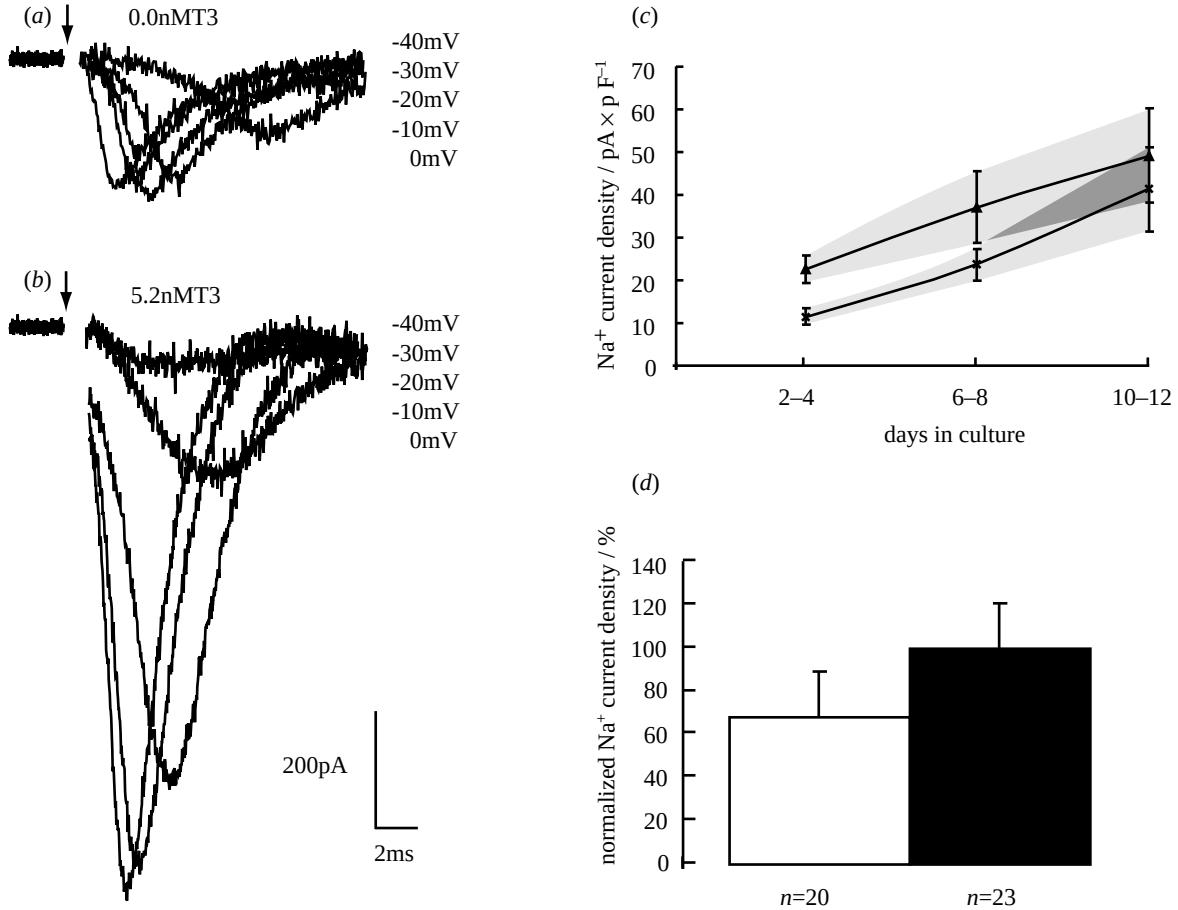

Figure 2. Effects of T3 on voltage-activated Na^+ currents. (*a*) Family of Na^+ currents measured after seven days in T3-free B18 medium. (*b*) Family of Na^+ currents recorded from a sister culture from the same preparation maintained for seven days in B18 medium supplemented with 5.2 nM T3. Holding potentials for (*a*) and (*b*): −70 mV. At time points indicated by arrows cells were depolarized with voltage steps to the potentials indicated at the right of the current traces. (*c*) Average Na^+ current densities versus time in culture for cells from the same preparation (P3) cultured in the presence (triangles) or absence (crosses) of T3. Standard deviations are indicated by error bars and grey shadings. Each mean value calculated from 3–5 measurements. (*d*) Mean values of relative current densities of cells cultured in the presence (black bar) or absence of T3 (white bar). In cultures where more than one current was recorded, currents recorded in T3-free dishes were normalized to the mean values of the currents recorded in T3-supplemented dishes. Error bars indicate standard deviations. Data obtained from 23 cells in T3-free cultures and 20 cells in T3 containing cultures from four preparations, 1–5 recordings per dish.

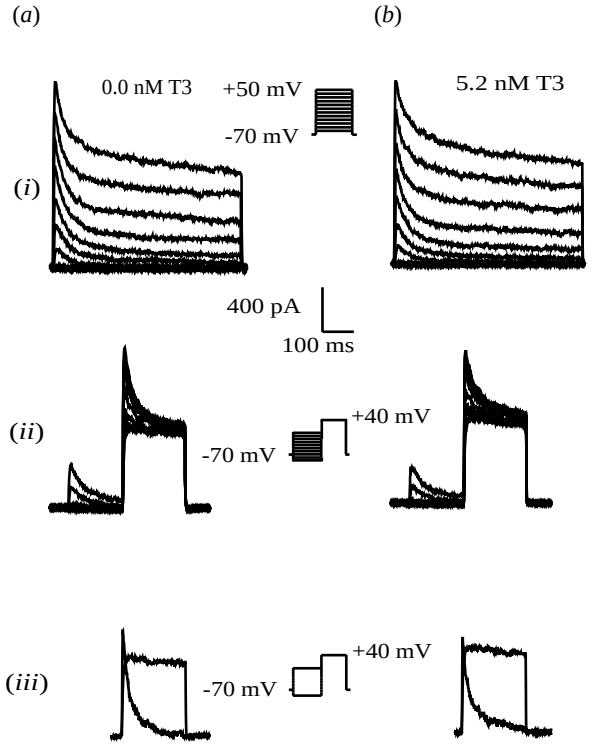

Figure 3. Effects of T3 on voltage-activated K^+ currents. Families of K^+ currents evoked in cells cultured in T3-free B18 medium (*a*) (*i*), or in a parallel culture kept in B18 supplemented with 5.2 nM T3 (*b*) (*i*). The voltage pulse protocol is shown in the inset between (*a*) and (*b*). Separation of I_A and I_K in cells from a T3-free culture (*a*) (*ii*, *iii*) and a T3-supplemented culture (*b*) (*ii*, *iii*) using prepulse and test pulse protocols shown in insets of (*ii*) and (*iii*). I_A calculated as difference between I_{total}, determined for a prepulse to −90 mV and I_K, determined with a prepulse to 0 mV. All recordings in (*a*) from the same cell and in (*b*) from another cell of the same preparation (P5, six days in culture).

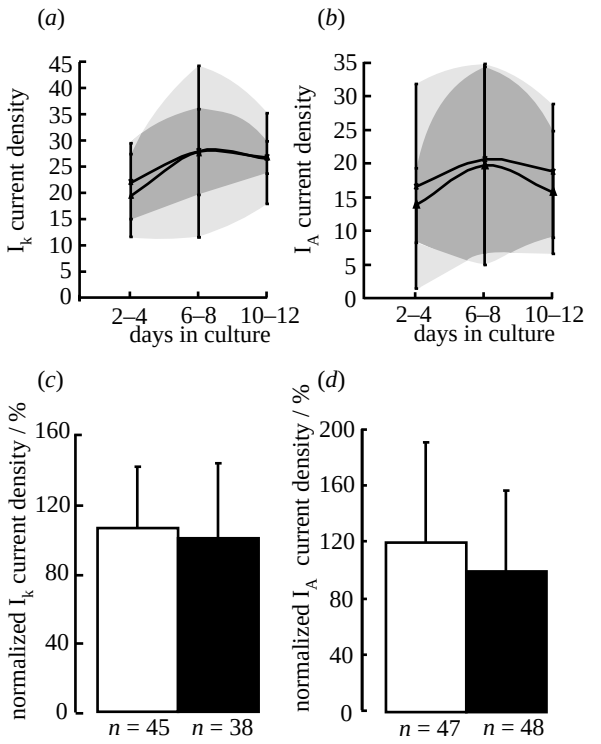

Figure 4. Effects of T3 on I_K and I_A current densities. (*a*) Average densities of I_K measured in cells treated with 5.2 nM T3 (triangles) or kept in T3-free B18 medium (crosses). (*b*) Average densities of I_A measured in cells treated with 5.2 nM T3 (triangles) or kept in T3-free B18 medium (crosses). Each mean value in (*a*) and (*b*) calculated from 4–6 measurements. Error bars and grey shading indicate standard deviations. All recordings in (*a*) and (*b*) from the same preparation. (*c*) I_K densities obtained in T3-free cultures (white bars) normalized to the respective I_K densities obtained in T3-supplemented sister cultures (black bars). A total of 83 currents were evaluated from four preparations. (*d*) I_K densities in T3-free cultures (white bars) normalized to the peak currents obtained for cells cultured in T3-supplemented B18 (black bars). A total of 95 currents from four preparations were evaluated.

trophic factors in glial cells, which contact and influence neurons more tightly *in vivo* than in culture.

(*b*) *Effects of T3 on Na^+ currents*

Voltage-activated ion currents were measured using the patch-clamp technique in the whole cell configuration in pairs of culture dishes from the same preparation grown in either presence or absence of 5.2 nM T3. Recordings were performed on cells of about equal size of a pyramidal-like or bipolar morphology alternately in the two types of dishes. Starting from holding potentials of −70 mV, cells were depolarized with voltage steps of 10 mV increments. Peak amplitudes of Na^+ currents, determined for voltage steps to −10 mV, increased with time in culture. At all time points investigated (2–12 days) the peak amplitudes of Na^+ currents were, however, always larger in cells from T3-treated cultures compared to Na^+ currents recorded from cells of the same preparation cultured in T3-free medium (figure 2*a*, *b*).

Out of 30 Na^+ currents recorded in T3-supplemented medium and 30 Na^+ currents recorded in T3-free cultures in 30 pairs of dishes from ten different animals, the average Na^+ currents recorded in T3-free cultures amounted to only $58 \pm 18\,\%$ of the amplitudes of the Na^+ currents measured in T3-treated cultures ($p < 0.05$, Student's *t*-test). In 20 pairs of current recordings from five different preparations, membrane capacitances were determined and current densities calculated. The Na^+ current densities in T3-free cultures amounted to $68 \pm 21\,\%$ of the currents recorded in cells cultured in the presence of 5.2 nM T3 ($p < 0.05$, figure 2*d*). In one preparation, the development of the Na^+ current with time in culture was followed under both conditions for 12 days (figure 2*c*). Up to eight days in culture Na^+ currents in T3-free cultures were significantly smaller than after treatment with T3 ($p < 0.01$, figure 2*c*).

(*c*) *Effects of T3 on K^+ currents*

As the ratio of the Na^+ to the K^+ conductances influences the shape of the action potentials, we investigated whether K^+ currents are similarly increased by the addition of T3 to the culture medium. K^+ currents were recorded, applying depolarizing voltage steps of 600 ms duration, incrementing in steps of 10 mV, starting from holding potentials of −70 mV.

To separate transiently activating K^+ currents (I_A) from delayed rectifier K^+ currents (I_K), prepulses of incrementing amplitudes were applied for 200 ms, starting with a hyperpolarization to -90 mV. K^+ currents were then recorded during test pulses to $+40$ mV applied for 150 ms.

Most cells cultured in the presence or absence of T3 showed I_K as well as I_A (figure 3). From 72 cells recorded in T3-treated cultures three cells showed only I_K; all 72 cells recorded in T3-free solution displayed both types of K^+ current. The K^+ current densities showed relatively smaller increases with time in culture compared to the Na^+ current densities (figure 4*a*, *b*). In contrast to the peak Na^+ current densities, the amplitudes of both types of K^+ currents showed no significant differences in cultures maintained in the presence or absence of T3 (figure 4). On average, the amplitudes of I_K measured in T3-free cultures amounted to $117 \pm 40\,\%$ of I_K measured in T3-supplemented cultures investigated on the same day from the same preparation ($n = 72$ pairs of cells from nine preparations, 4–10 cells recorded per dish). Likewise, on average, the amplitudes of I_A, measured in T3-free cultures amounted to $122 \pm 67\,\%$ of I_A determined in T3-supplemented sister cultures (45 pairs of cells from four preparations investigated, 3–7 recordings per dish). Current densities of I_K recorded in T3-free cultures amounted to $106 \pm 35\,\%$ of I_K densities determined in T3-supplemented cultures (45 cells in T3-free culture, 38 cells in T3-containing cultures from five preparations, 4–6 recordings per dish; figure 4*c*). Current densities of I_A in T3-free cultures were $121 \pm 71\,\%$ of the densities of I_A determined in T3-supplemented cultures (47 cells in T3-free cultures and 48 cells in T3-treated cultures, four preparations, figure 4*d*).

4. DISCUSSION

Our experiments show a selective upregulation of Na^+ current density, but not of K^+ current densities, by 3,5,3′-triiodo-L-thyronine in cultured postnatal hippocampal neurons. The lack of effect on K^+ currents suggests that voltage-activated ion currents are not effected as a set, but regulated independently.

The observed increases in Na^+ currents by T3 could be explained by an upregulation of Na^+ channel protein synthesis, as in skeletal myocytes (Brodie & Sampson 1989). Thus increases in different Na^+/K^+ ATPase subunits known to be upregulated by T3 in many tissues, including postnatal cortical neurons, (e.g. Lindholm 1984; Atterwill *et al*. 1985) could be elicited by an enhanced Na^+ influx through voltage-activated Na^+ currents. This could contribute to the well-known effects of thyroid hormone on the basic metabolic rate. An investigation of the temporal sequence of upregulation of Na^+ channels and ATPase subunits could clarify whether both are coregulated or whether the expression of the Na^+/K^+ATPase subunits is regulated by an enhanced Na^+ influx.

In contrast to cardiac myocytes, where action potential duration is shortened by an upregulation of K^+ currents by T3 (Rubinstein & Binah 1989), and where different K^+ channel subtypes were found to be regulated independently and to a different extent in atrial and ventricular myoctes (Shimoni & Severson 1995), we found no upregulation of K^+ currents by T3 in postnatal hippocampal neurons. As there were no obvious effects on either I_K or I_A, we did not attempt to further subdivide the K^+ currents.

A selective regulation of different ion currents has also been noted in neuroblastoma cells treated with either nerve growth factor (NGF) or ciliary neurotrophic factor (CNTF) (Lesser & Lo 1995). While CNTF upregulated only K^+ currents, NGF was shown to upregulate Na^+, K^+ as well as Ca^{2+} currents. Here, we found a pattern of ion channel regulation different from any effect of neurotrophins so far described. This does, however, not exclude the possibility of an upregulation of Na^+ currents in hippocampal neurons secondary to an increase in neurotrophin expression by T3. To clarify this point further, experiments are needed to investigate whether neurotrophins other than NGF or CNTF could have mediated the observed increase of Na^+ currents. Moreover, NGF and CNTF could regulate distinct sets of ion channels in neuroblastoma cells and hippocampal neurons. A celltype-specific regulation of Na^+ channels by T3, or the role of additional, unidentified cofactors, could perhaps also explain why a previous study failed to show changes of veratridine-stimulated ^{22}Na-uptake in granule neuron-enriched cerebellar cultures (Balázs *et al*. 1985). On the other hand, uptake studies could have failed to show effects of T3 because a compensatory upregulation of the Na^+/K^+ ATPase activity could have balanced the enhanced Na^+ influx into the cells.

During the terminal maturation of neurons, an acceleration of the action potential upstroke velocity (Mori-Okamoto *et al*. 1983; McCormick & Prince 1987), and an increase in conduction velocity, even prior to the onset of myelination (Foster *et al*. 1982; Fulton 1987), have been observed. These findings are consistent with the observation of an upregulation of Na^+ current densities during pre- and postnatal maturation (e.g. Gottmann *et al*. 1988; Huguenard *et al*. 1988; O'Dowd *et al*. 1988). Increases in conduction velocities (e.g. De Vries *et al*. 1986; Beghi *et al*. 1989), decreases in the amplitudes of peripheral compound action potentials (e.g. Fincham & Cape 1968, Quattrini *et al*. 1993), a slowing of the alpha-rhythm of the electroencephalogram (EEG) (e.g. Ross & Schwab 1939; Scarpalezos *et al*. 1973; Pohunková *et al*. 1989) and increased latencies of evoked potentials (e.g. Norcross-Nechay *et al*. 1989; Huang *et al*. 1989) have been known for a long time to occur in neonatal and also in adult hypothyroidism. It will be challenging to find out whether some of these symptoms can be traced back to effects of thyroid hormone on Na^+ expression.

Changes of the relative densities of inward versus K^+ currents can lead to changes in the action potential shape during maturation, as shown for amphibian neurons (Barish 1986). Although changes in action potential waveform caused in hippocampal neurons by treatment with T3 await further investigation, our results suggest that thyroid hormone could exert an

influence on postnatal signalling properties. Even though neurite outgrowth and synapse formation do not necessarily require neuronal activity, appropriate activity is necessary for the fine tuning of neuronal connections (discussed in Goodman & Shatz (1993)). In *Xenopus* spinal neurons Ca^{2+} influx during the time in development when broader action potentials are generated triggers gene expression for enzymes involved in transmitter metabolism (Gu *et al*. 1994). It will be interesting to find out whether decreases in neuronal depolarization due to hypothyroidism might cause some of the known developmental delays of protein expression (Oppenheimer *et al*. 1994).

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