2 Materials and Methods

2.1 Materials

2.1.1 Equipment for subcellular fractionation

- glass-teflon homogenizer, clearance 200 µm tissue grind pestle (Brann Biotech International, Melsungen, Germany)
- motor-driven homogenizer (Janke & Kunkel KG, Staufen i. Breisgau, Germany)
- L8-55 ultracentrifuge (Beckman Instruments, Palo Alto, California, USA)
- swinging bucket rotor SW 41 Ti (Beckman Instruments, Palo Alto, California, USA)
- ultracentrifuge tubes polyallomer (Beckman Instruments, Palo Alto, California, USA)

2.1.2 Equipment for radiolabeling iodothyronines

- microflex vials (Radiochemical Centre, Amersham, UK)
- HPLC operating in an isocratic system (Knauer GmbH, Berlin, Germany)
- 5-µm Eurospher 100-C18, 4x250-mm column (Knauer GmbH, Berlin, Germany)
- plates coated with 60 F254 silica gel (Merck, Darmstadt, Germany)
- Sep-Pak C_{18} cartridges (Waters Associates, Milford, Massachusetts, USA)

2.1.3 Equipment for extracting, purifying, and separating iodothyronines

- Polytron Ultra-Turrax (Janke & Kunkel IKA-Labortechnik; Staufen, Germany)
- 12-ml disposable polypropylene tubes with push-in stoppers (Sarstedt, Nürmbrecht, Germany)
- Minisart SRP 15, PTFE-membrane 0.2 µm filters (Sartorius AG, Göttingen, Germany)
- micro test tubes (2-ml, safe-lock; Eppendorf, Hamburg, Germany)

- HPLC operating in a gradient system, composed of:

  - gradient pump LKB 2249 (Pharmacia, Freiburg, Germany)
  - Degasys LKB DG 1310 (Pharmacia, Freiburg, Germany)
  - LKB low pressure mixer (Pharmacia, Freiburg, Germany)
  - 5-µm Eurospher 100-C18, 4x250-mm column (Knauer GmbH, Berlin, Germany)
  - advanced LC sampler processor ISS 200 (Bodenseewerk Perkin-Elmer GmbH, Überlingen, Germany)
  - fraction sampler LKB 2211 Superrac (Pharmacia, Freiburg, Germany)

- vacuum pump concentrator (Speed-Vac concentrator, Sauer, Reutlingen, Germany) incorporating a refrigerating condensation trap (Savant) connected to a sliding vane rotary vacuum pump (Vacuumbrand, Wertheim, Germany)
- ultrasonic bath (Nuclear Products, 3M Center, St. Paul, Minnesota, USA)
- centrifuge 5413 (Eppendorf, Hamburg, Germany)

2.1.4 Equipment for RIA quantification

- assay racks Rack LKB 5x10 (Pharmacia, Freiburg, Germany)
- 10x55 mm disposable conical tubes (Sarstedt, Nürmbrecht, Germany)
- 1470 Wizard multi-detector gamma counter for ^{125}I (Wallac, Turku, Finland)
- centrifuge Cryofuge 5000 (Heraeus, Germany)
- DYNOtest TSH, DYNOtest T_4, DYNOtest T_3, DYNOtest fT_4 (B.R.A.H.M.S. Diagnostica GmbH, Berlin Germany)
- Reverse T_3 (Serono Diagnostica GmbH, Freiburg, Germany)
- antibodies for TSH RIA (National Institute of Arthritis, Diabetes & Digestive and Kidney Disease, National Institutes of Health, Bethesda, Maryland, USA)

2.1.5 Chemical and reagents for subcellular fractionation

- sucrose for biochemistry (Merck, Darmstadt, Germany)
- iopanic acid (IOP; Sigma Chemical Co., St Louis, Missouri, USA)
- magnesium chloride hexahydrate (Sigma Chemical Co., St Louis, Missouri, USA)
- potassium dihydrogen phosphate (Merck, Darmstadt, Germany)
- dipotassium hydrogen phosphate (Merck, Darmstadt, Germany)
- DNA quantification (Wizard Genomic DNA Purification Kit, Promega, Madison, Wisconsin, USA)
- E. coli alkaline phosphatase (Sigma, Steinheim, Germany)
- ECL Western blot system (Amersham Pharmacia Biotech., Freiburg, Germany)
- syntaxine (Calbiochem-Novabiochem GmbH, Bad Soden, Germany)
- Na-adenosin-2',3'-cyclic-phosphate (Sigma, Steinheim, Germany)
- ammonium molybdat (Sigma, Steinheim, Germany)
- Fiske and Subbarow reducer (Sigma, Steinheim, Germany)
- Na-deoxycholate (Sigma, Steinheim, Germany)
- ß-NaDH (reduced form, Sigma, Steinheim, Germany)

2.1.6 Chemicals and reagents for radiolabeling iodothyronines

- sodium ^{125}iodine (Radiochemical Centre, Amersham, UK)
- chloramine-T (Merck, Darmstadt, Germany)
- sodium hydroxide (Merck, Darmstadt, Germany)
- sodium disulfite (Merck, Darmstadt, Germany);
- anhydrous ammonium acetate (Fluka, Deisenhofen, Germany)
- ethyl acetate (Merck, Darmstadt, Germany)
- orthophosphoric acid (Merck, Darmstadt, Germany)
- ammonia (NH_3); 32% solution (Merck, Darmstadt, Germany)

2.1.7 Chemicals and reagents for extracting, purifying, and separating iodothyronines

- methanol (HPLC grade; Merck, Darmstadt, Germany)
- acetonitrile (HPLC grade; Merck, Darmstadt, Germany)
- H_2O (HPLC grade; Fluka, Deisenhofen, Germany)
- 100% glacial acetic acid (Merck, Darmstadt, Germany)
- dehydrated alcohol (Merck, Darmstadt, Germany)

2.1.8 Chemicals and reagents for RIA quantification

- 3,3',5,5'-tetraiodothyroacetic acid (Tetrac), 3,3',5-triiodothyroacetic acid (Triac), 3,5-diiodothyroacetic acid (Diac), 3,3',5,5'-tetraiodo-L-thyronine (thyroxin, T_4), 3,3',5-triiodo-L-thyronine (T_3), 3,3',5'-triiodo-L-thyronine (rT_3), 3,5-diiodo-L-thyronine (3,5-T_2), 3,3'-diiodo-L-thyronine (3,3'-T_2), 3',5'-diiodo-L-thyronine (3',5'-T_2), and thyronine (Henning Berlin GmbH, Berlin, Germany)
- T_4 and T_3 conjugated to bovine serum albumin (Henning Berlin GmbH, Berlin, Germany)
- 3-Br-[5-^{125}I]-T_1 (Formula-GmbH, Berlin, Germany)
- polyethylene glycol 6000 (Serva, Heidelberg, Germany)
- bovine γ-globulin from plasma (Sigma, Steinheim, Germany)
- sodium dihydrogen phosphate monohydrate GR (Merck, Darmstadt, Germany)
- disodium hydrogen phosphate dehydrate GR (Merck, Darmstadt, Germany)
- bovine albumin (BSA; Sigma, Steinheim, Germany)
- L-cysteine (Sigma, Steinheim, Germany)
- mercury-[o-(carboxyphenyl)thio]ethyl sodium salt (merthiolate; Sigma, Steinheim, Germany)
- EDTA (Sigma, St. Louis, Missouri, USA)
- acid coomassie brilliant solution (coloring reagent for protein quantification; Biorad, Munich, Germany)

2.1.9 Drugs

- desipramine hydrochloride (Sigma, St. Louis, Missouri, USA)

2.2 Studies in humans

2.2.1 Hormone determination in human serum

Iodothyronines were measured in the serum of the following patients and control samples.

2.2.1.1 Healthy controls

Sixty-two healthy subjects were studied for comparison with different patient groups. Thirty-two were women, 30 men, and their mean age was 47.3 ± 18 years (range: 22 - 89 years). As an age dependence of 3,5-T_2 had been reported in a previous study (Nishikawa et al., 1981), we took care to include large enough percentages of all relevant age groups in the control group. Ten subjects were between 20 and 30 years of age, 10 between 30 and 40, 9 between 40 and 50, 11 between 50 and 60, 9 between 60 and 70, 9 between 70 and 80, and 4 over 80. The subjects younger than 60 years of age were employees of various departments of the University Hospital Benjamin Franklin (UKBF) who were personally known to the author. The controls older than 60 were healthy retired volunteers who occasionally work for a drug research institute and whose good and stable state of health had been documented in the records of this institute for a period of several years. At the time of the investigation, none of the controls had any apparent clinical illness, any history or current signs of thyroid disorder, was taking thyroid hormones or any other medication known to affect serum concentrations of thyroid hormones, such as oral contraceptives or ß-adrenergic blockers (Wenzel, 1981; Davies and Franklyn, 1991).
All blood samples from the patients and healthy controls were drawn in the morning between 7 and 10 a.m. Since blood samples were routinely drawn from all patients

for diagnostic purposes, this opportunity was used to obtain 5 ml of additional blood to determine thyroid hormones for research purposes.

2.2.1.2 Patients with thyroid disorders

Nine patients with hyperthyroidism (six women and three men with a mean age of 36.3 ± 4.5 years; range: 28 - 44 years) and 12 patients with hypothyroidism (eight women and four men with a mean age of 43.5 ± 6.3 years; range: 34 to 58 years) were also studied before treatment for the thyroid disorder was initiated. Serum samples were kindly provided by Dr. Reinhart Finke from the Department of Endocrinology, UKBF.

2.2.1.3 Patients with different somatic, nonthyroidal diseases

The following studies were performed in cooperation with Dr. Hans Joachim Gramm from the Department of Anesthesiology and Intensive Care Medicine, UKBF (studies 2.2.1.3.1 and 2.2.1.3.2); Dr. Thomas Höll from the Department of Neurosurgery, UKBF (study 2.2.1.3.3); and Prof. Klaus-Jürgen Gräf, Medical Clinic, Rudolf Virchow Klinikum of the Charité, Berlin (study 2.2.1.3.4).

- **2.2.1.3.1 Patients with sepsis**

  Twenty-four patients were studied who were admitted to the intensive care unit either post-operatively or following trauma and whose clinical course was complicated by sepsis. Ten of these patients had peritonitis and 14 pneumonia. Ten were women, 14 men, and their mean age was 56.2 ± 19.0 years (range: 16 - 89 years). The diagnosis of sepsis was established according to criteria previously published (Meinhold et al., 1991).

- **2.2.1.3.2 Patients with head and/or brain injury**

  Fifteen patients admitted to an intensive-care unit with a diagnosis of closed-head injury were also studied. Six of these patients were women, nine men,

and their mean age was 36.2 ± 13.8 years (range: 28 - 44 years). Blood samples were drawn during the first two days following injury when the patients were still in an unconscious state.

- **2.2.1.3.3 Patients with brain tumors and metastases**

Twenty-one patients hospitalized for brain surgery in the Department of Neurosurgery were also investigated. Twelve were women, nine men, and their mean age was 50.1 ± 12.5 years (range: 22 - 70 years). Six of these patients had a glioblastoma, five a meningioma, four an astrocytoma, and five were scheduled for removal of brain metastases, their primary tumors being at a different site. An adenoma was excised from the pituitary gland of one patient.

The general condition of all of these patients was relatively good. None was suffering from a severe concomitant disease. Blood samples were drawn immediately before surgery, while the patient was under full anesthesia. The anesthetics propofol and fentanyl and a muscle relaxant were administered.

- **2.2.1.3.4 Patients with liver diseases**

Serum iodothyronine levels in 22 patients with severe liver diseases were measured prior to liver transplantation. Nine were women, 13 men, and their mean age was 50.4 ± 30.2 years (range: 23 - 82 years). The patients were suffering from various liver diseases, such as chronic hepatitis, Morbus Wilson, hemachromatosis, and alcoholic cirrhosis. They were part of a much larger sample of patients who were scheduled for liver transplantation and in whom serum concentrations of triiodothyronine and thyroxin were routinely measured. These 22 patients were purposely selected from the larger sample because they had particularly low serum levels of T_3.

The critically ill patients admitted to the intensive care unit (groups 2.2.1.3.1 and 2.2.1.3.2) were treated with appropriate antibiotics and various drugs to stabilize vital

functions. In patients with sepsis and head injuries, hemodynamic stabilization was achieved by controlled volume load, dobutamine and, where necessary, norepinephrine. Four patients with sepsis were also given dopamine. None of these patients were receiving glucocorticoids. Patients with known thyroid disorder or thyroid hormone supplementation were not included in the study.
All patients received 30 - 35 cal/kg body weight (BW) daily either enterally or parenterally (70% in carbohydrate form and 30% in lipid form). 1.5 g/kg BW amino acids were also administered.

2.2.1.4 Acute stress

In the stress experiment, blood samples were drawn from 10 physicians before and after delivering a lecture at a clinical conference. This type of stress has previously been shown to induce pronounced increases in serum cortisol levels (Baumgartner et al., 1985). The conference took place between 3 and 5 p.m. Thus four blood samples were collected: one at 3 p.m. and one at 5 p.m. on the day of the conference and one sample each at 3 and 5 p.m. on a control day not less than one week later.

2.2.1.5 Sleep deprivation

Six healthy male subjects, all of whom were physicians working at the University Hospital Benjamin Franklin, were subjected to a whole night's sleep deprivation. Their mean age was 32 ± 3 (range: 27 - 37) years. An indwelling catheter was inserted into the cubital vein in all subjects at 8 p.m. Blood was drawn at 20-minute intervals between 10 p.m. and 6 a.m. During this period the subjects were not permitted to sleep. They spent the night together watching television, reading, or playing games. All of them also spent three control nights of sleep in a sleep laboratory. The first two nights served for adaptation. On the third night, an intravenous catheter was placed in the forearm of each subject. The catheter was attached to a tube that passed through a hole in the wall into the next room. Each volunteer retired to bed at 9 p.m. and the lights were switched off at 10 p.m. Blood

samples were drawn every 20 minutes between 10 p.m. and 6 a.m. EEG recordings were performed during the same period.

2.2.2 Hormone determination in human brain tissue

Human tissue samples were taken from tissue collections in which deiodinase activities had already been measured previously (Campos-Barros et al., 1996).

- #### 2.2.2.1 Healthy donors

  Samples of normal human tissue were collected by the Departments of Neurosurgery and Neuropathology of the University Hospital Benjamin Franklin, respectively. They had been excised during neurosurgical operations (n = 5) for therapeutic purposes or at autopsy (n = 6) for histopathological investigation. Samples from the anterior pituitary and from various regions of the brain (cortex, hippocampus, pons, and cerebellum) were obtained from donors at autopsy, which was performed between 36 and 72 hours after death. Between death and autopsy the bodies were stored at 4 – 6 °C. Five of the donors were male and one female and their mean age was 58.4 ± 12.5 years (range: 36 - 70 years). They had died of disorders not primarily affecting the brain, such as lymphoma, myocardial infarction, diabetes mellitus, primary pulmonary hypertension, and dilatative cardiomyopathy.

  Five tissue samples from the tempo-cortical lobe were obtained during temporal lobe resection in patients with temporal lobe epilepsy. The effects of storage, of temperature, and of the time interval between death and autopsy on thyroid hormone concentrations were investigated in the striata of male Sprague Dawley rats (n = 12). After decapitation, the brain regions of one control group (n = 4) were immediately frozen on dry ice and stored at -70 °C until assay. The tissues of the two other groups (each n = 4) were kept at room temperature for 4 hours, then stored at 4 °C for 24 and 96 h, respectively, before freezing. This design was chosen to simulate post-mortem events in humans.

- **2.2.2.2 Samples of human brain tumors and metastases**

  Samples of tumor tissue were obtained during surgery from the patients who had previously donated serum samples (see above). The tissues were immediately frozen in liquid nitrogen and stored at -70 °C until assay.

2.3 Studies in experimental animals

2.3.1 Animals

Euthyroid adult male Sprague Dawley rats weighing approximately 250-300 g were employed throughout. They were housed in pairs with a 12 h light-dark cycle (6 a.m.-6 p.m.). Food and water were available ad libitum. Following an adjustment period of at least one week in the new environment, the following studies were performed.

2.3.2 Group 1: Control animals

The first experimental study was designed for the quantification of physiological concentrations of iodothyronines in the following two groups of naive animals.

- **2.3.2.1 Homogenates of different brain areas, pituitary glands, and liver of the rat**

  A group of eight rats served for the quantification of iodothyronines in homogenates of 12 areas of the brain (olfactory bulb, frontal cortex, parieto-occipital cortex, hippocampus, amygdala, septum, limbic forebrain, striatum, midbrain, hypothalamus, cerebellum, and medulla), in the pituitary glands, and in liver. Blood from the decapitation wound was taken, centrifuged, and aliquots of sera were collected. All rats were decapitated without anesthesia between 11 a.m. and 1 p.m. one week after their arrival.

- **2.3.2.2 Subcellular fractions of rat brain regions**

A group of eight rats served for the determination of iodothyronines in several subcellular fractions, namely nuclei, mitochondria, synaptosomes, myelin, and microsomes. To determine concentrations of iodothyronines in the homogenate of the respective areas used for subcellular fractionation, iodothyronines were extracted and measured from an aliquot of 1/10 of the whole homogenate. As no 3,5-T_2 was measured in any subcellular fraction in control animals, an additional group of 48 rats was sacrificed in order to further investigate concentrations of 3,5-T_2 in pools of 12 subcellular fractions for one hormone determination. This experiement was designed to evaluate whether the 3,5-T_2 RIA employed was sensitive enough to measure 3,5-T_2 in subcellular fractions or whether the reason not to measure it in the subcellular fractions was simply due to the fact that this hormone is, indeed, not bound to any of the different cellular compartments under investigation.
Housing and sacrifice conditions are reported above (2.3.2.1).

2.3.3 Group 2: Antidepressant treatment with desipramine

Four groups of ten rats each were employed to study the effects of the antidepressant desipramine (a nonepinephrine re-uptake inhibitor) on subcellular iodothyronine concentrations. Three groups received daily doses of 5, 20, and 40 mg/kg desipramine, respectively. The drug was dissolved in NaCl and administered by gavage once daily for 14 days. Ten further rats received NaCl alone by gavage for 14 days. The rats were decapitated without anesthesia between 11 a.m. and 1 p.m.

2.3.4 Group 3: Circadian variations of iodothyronines

One group of 36 male euthyroid rats were kept on a 12:12 h light-dark schedule (lights on at 6 a.m.), and under standard laboratory conditions were killed by decapitation at 4 h intervals (six animals each) over a 24 h period. During the dark

phase of the 24 h cycle, the animals were killed under a dim red light (3-5 lux). Brains were removed and dissected as usual (see 2.5.1).

2.4 Hormone determination

2.4.1 RIA buffers

Phosphate buffer (0.04 M, pH 8.0) containing 243 mg/l merthiolate and 2 g/l BSA (for the T_3, rT_3, 3,5-T_2, and 3,3'-T_2 RIAs) or 0.5 g/l BSA (for the T_4 RIA) served as experimental buffer. The iodoamino acids were dissolved in 0.1 M sodium hydroxide and diluted to final assay concentrations using this buffer. The [^{125}I]-T_4, [^{125}I]-T_3, [^{125}I]-rT_3, 3-Br-[5-^{125}I]-T_1, and [^{125}I]-3,3'-T_2 tracers were each dissolved in the respective experimental buffer containing 100 mg/l L-cysteine. The stop solution formed by the phosphate buffer, 243 mg/l merthiolate, 30% (w/v) polyethylene glycol (PEG), and 1,3 mg/ml bovine γ-globulin was pipetted (3 ml/tube) to precipitate the antibody-bound radioactivity in the T_4, rT_3, T_3, 3,3'-T_2, and 3,5-T_2 RIAs.

2.4.2 Synthesis of 3,5-T_2 tracer

The tracer 3-bromo-[5-^{125}iodo]-thyronine (3-Br-[5-^{125}I]-T_1) was kindly provided by Rudi Thoma (Formula GmbH). It was synthesized as a carrier-free labeled product with a specific radioactivity of 74 Mbq/nmol by an interhalogen exchange from 3,5-dibromo-L-thyronine, separated from the reaction products, and purified by HPLC.

2.4.3 Synthesis of 3,3'-T_2 tracer

Labeled 3,3'-T_2 of maximum specific radioactivity was obtained by radioiodination of 3-iodo-L-thyronine (3-T_1) with chloramine T (Nakamura et al., 1977). 3-T_1 was dissolved in 0.01 mol/l sodium hydroxide and further diluted in 0.1 mol/l phosphate buffer (pH 7.0). Chloramine T (Merck, Darmstadt) and sodium disulfite were also

diluted in the phosphate buffer. For labeling, 10 µl of the following reagents were pipetted into a microflex vial (Radiochemical Centre, Amersham) containing 40 µl 0.5 mol/l phosphate buffer (pH 7.5): 37 Mbq (1 mCi) ^{125}Iodine (approximately 0.5 nmol iodine, Radiochemical Centre, Amersham), 2 nmol 3-T_1, 0.2 µmol chloramine T. After an incubation period of 30 s, 1.5 µmol sodium disulfite were added. The reaction mixture was injected onto the HPLC. HPLC separation of 3,3'-[^{125}I]-T_2 was carried out using a 5 µm Eurospher 100-C18, 4x250 mm column (Knauer GmbH). The column was equilibrated with a gradient of 55% methanol and 45% 0.02 M ammonium acetate buffer pH 4.0 at a flow rate of 1 ml/min. After running the gradient, the column was washed with 100% methanol at a flow rate of 1 ml/min for 30 min in order to remove contaminants that elute from the column. 3-T_1 and 3,3'-[^{125}I]-T_2 eluted from the column after 5 and 12 min respectively, whereas rT_3 (formed as reaction product for double-labeling the 3-T_1 molecule) eluted from the column after 22 min (fig 2). The fraction eluting from the HPLC corresponding to the 3,3'-[^{125}I]-T_2 peak (about 75% of the total radioactivity used for the labeling) was collected and diluted with 100% methanol containing 0.25% concentrated ammonia. This solution was stored at -20 °C.

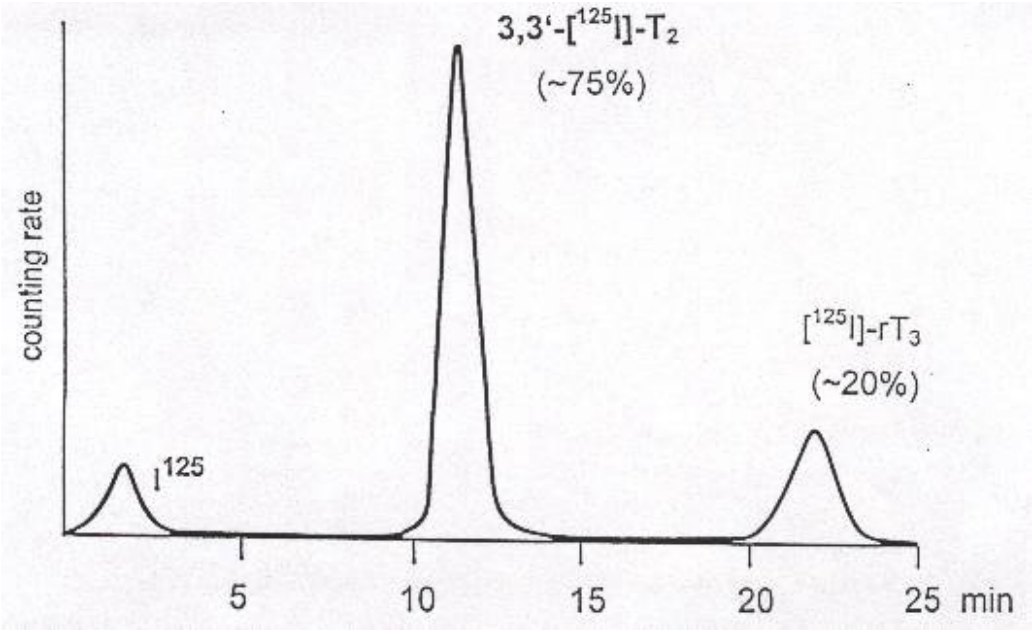

Fig 2. HPLC chromatogram resulting from the separation of the reaction mixture after chloramine T radioiodination with 3-T_1 as substrate to produce 3,3'-[^{125}I]-T_2.

The radiochemical purity was tested by thin-layer chromatography (silica gel 60 F254 plates, Merck) in ethyl acetate: methanol: 3 mol/l ammonia (volumes, 50 ml + 20 ml + 10 ml). The Rf values were 0.15 and 0.30 for 3,3'-T_2 and 3-T_1, respectively.

An additional purification of the tracer was required immediately before its use for RIA measurements. This was done by consecutively pipetting the following reagents into a disposable Sep-Pak C_{18} cartridge (Waters Associates):

- **A** 1 ml of 100% methanol to prepare the Sep-Pak C^{18} cartridges to receive the solution containing the tracer
- **B** the amount of tracer needed for the RIA measurement dissolved in 1 ml of 50% methanol and 0.03% orthophosphoric acid
- **C** 1 ml of 50% methanol and 0.03 orthophosphoric acid to wash out the impurities contaminating the tracer solution
- **D** repeat point C twice

The purified tracer was eluted with 1 ml 100% methanol, and the eluate containing the tracer was dried out under a stream of nitrogen. Further dilution and calibration of the tracer to the desired concentration for RIA quantification (i.e. 4000 cpm/100 µl) was performed by adding RIA experimental buffer containing 100 mg L-cystein/L, as described above (2.4.1).

2.4.4 Preparation of 3,5-T_2-binding antibody

Antiserum to 3,5-T_2 was provided by Prof. Harald Meinhold from the Department of Radiology and Nuclear Medicine, UKBF. It was produced in three rabbits immunized by serial injections of 3,5-T_2 conjugated to bovine serum albumin in complete Freund's adjuvant as described previously (Meinhold, 1986).

All immunized rabbits produced antisera suitable for RIA of 3,5-T_2. The antiserum used in the experiments was obtained after three injections of the immunogen, eight weeks after starting immunization. The final dilution was 1:250,000, which bound about 40% of tracer in an incubation volume of 250 µl.

2.4.5 Preparation of 3,3'-T_2 -binding antibody

Antiserum to 3,3'-T_2·was also provided by Prof. Harald Meinhold. It was produced in three rabbits immunized by serial injections of a conjugate of 3,3'-T_2 and bovine serum albumin in complete Freund's adjuvant as described previously (Meinhold, 1986). All immunized rabbits produced antisera satisfactory for RIA of 3,3'-T_2· Analogous to the production of 3,5-T_2 antibody (2.4.4), the antiserum selected for RIA of 3,3'-T_2 was obtained eight weeks after starting immunization, after three injections of the immunogen. The final dilution was 1:150,000, which bound about 40% of tracer in an incubation volume of 250 µl.

2.4.6 RIA procedure for 3,5-T_2 in serum and tissue samples

The RIA of 3,5-T_2 in serum and excised tissue was carried out in 10x55 mm plastic tubes with the addition of various reagents as follows: a) 50 µl 3,5-T_2 standard at concentrations ranging from 0.48 to 20 fmol/tube. For the serum measurements, standards were diluted in experimental buffer containing the same volume of ethanol-extracted iodothyronine-free serum as the unknown samples; b) 100 µl of antibody against 3,5-T_2; c) 100 µl tracer solution containing about 4000 cpm of 3-Br-5-[^{125}I]-T_1; d) 50 µl of the tissue or serum sample dissolved in experimental buffer; e) experimental buffer to yield a final volume of the standards of 250 µl/tube.

After 24 h incubation at room temperature, the antibody-bound iodothyronine was precipitated by adding 3 ml PEG stop solution to each tube, then centrifuged at 3,000 rpm and 4 °C for 45 min. The supernatant was decanted and discarded. The tubes were inverted for 10 min on blotting paper to absorb the remaining liquid. Finally, the tubes were placed in a γ-counter and the precipitated, bound radioactivity was counted.

To ensure that subjecting the serum samples to two freeze-thaw cycles had no effect on serum concentrations of iodothyronines, three different samples of serum from healthy controls were thawed and refrozen five times. The concentrations of

iodothyronines were measured each time after thawing. The results showed that repeated freeze-thaw cycles had no effect on the concentrations of iodothyronines in serum.

2.4.7 RIA procedure for 3,3'-T_2 in serum and tissue samples

The RIA of 3,3'-T_2 in serum and excised tissue was carried out in 10x55 mm plastic tubes using a similar procedure as described above for RIA of 3,5-T_2 (2.4.6). Various reagents were added as follows: a) 50 µl 3,3'-T_2 standard at concentrations ranging from 0.48 - 60 fmol/tube. For the serum measurements standards were diluted in experimental buffer containing the same volume of ethanol-extracted iodothyronine-free serum as the unknown samples; b) 100 µl of antibody against 3,3'-T_2; c) 100 µl of tracer solution containing about 4000 cpm of 3,3'-[^{125}I]-T_2; d) 50µl of the tissue or serum sample dissolved in experimental buffer; e) experimental buffer to yield a final volume of the standards of 250 µl/tube.

After 24 h incubation at room temperature, the antibody-bound iodothyronine was precipitated as described above for the 3,5-T_2 RIA (2.4.6).

2.4.8 Serum determination of other iodothyronines and thyrotropin (TSH)

In order to confirm the "low T_3 syndrome" in the four patient groups with NTI, serum concentrations of T_3, T_4, and rT_3 were measured in the serum samples of these patients and in healthy controls. In patients with hyper- and hypothyroidism, serum concentrations of T_4, free-T_4 (fT_4), and TSH were also measured to confirm thyroidal status. The hormones were determined in duplicate using the following commercial radioassay kits: DYNOtest TSH, DYNOtest T_4, DYNOtest T_3, DYNOtest fT_4 (all from B.R.A.H.M.S. Diagnostica GmbH); Reverse T_3 (Serono Diagnostica GmbH). Hormone determination was performed on samples of serum from the patient groups

and from some of the healthy controls in a single assay. We took care to ensure that both control group and patient group were matched for age and sex.

Serum concentrations of T_4 and T_3 were determined in experimental animals by a slightly modified double-antibody RIA as previously described for human serum (Meinhold, 1986). For assaying total T_4 and T_3 in the rat sera, standards were established in iodothyronine-free rat serum. The serum concentrations of TSH were measured by a specific RIA developed for the rat, using immunoreactants kindly supplied by the National Institute of Arthritis, Diabetes & Digestive and Kidney Disease of the National Institutes of Health (Bethesda, Maryland, USA).

2.4.9 Tissue determination of other iodothyronines

The tissue concentrations of T_4, T_3, and rT_3 were determined after extraction, purification, and separation of the iodothyronines from the tissue samples, as described below (2.5.3). T_4, T_3, and rT_3 were purified and separated by HPLC, evaporated to dryness, and taken up in the experimental buffer prior to RIA measurement. T_4 and T_3 RIAs were performed as reported previously (Pinna et al., 1999), whereas the rT_3 RIA was recently established in the laboratory of the Department of Radiology and Nuclear Medicine, UKBF (Pinna et al., in preparation).

In brief, labeled T_4, rT_3, and T_3 with high radioactivity were obtained by radioiodination of T_3, 3,3'-T_2, and 3,5-T_2, respectively, with chloramine T (Pinna et al., 1999). Antisera to T_4, rT_3, and T_3 were kindly provided by Prof. Harald Meinhold from the Department of Radiology and Nuclear Medicine, UKBF. They had been produced in rabbits by serial injections of a conjugate of T_4, rT_3, or T_3 and bovine serum albumin in complete Freund's adjuvant (Meinhold, 1986). Antisera for RIA measurement were used in the final dilutions of 1:250,000 (T_4), 1:180,000 (rT_3), and 1:2,400,000 (T_3), which bound about 40% of the tracer (T_4, rT_3, or T_3) in an incubation volume of 250 µl. RIA of T_4, rT_3, or T_3 was performed in 10x55 mm polypropylene conical tubes. Standards were pipetted in aliquots of 50 µl at dilutions ranging from 0.4 to 50 fmol/tube for T_4 and T_3 RIAs, respectively, and 0.96-125

fmol/tube for rT_3 RIA. A total of 100 µl tracer solution containing approximately 4,000 cpm/tube of [^{125}I]-T_4, [^{125}I]-rT_3, or [^{125}I]-T_3 was added.

The intra and inter-assay coefficients of variation (CVs) for tissue samples were determined in five tests. The intra-assay CVs ranged from 4.8% to 6.3% and the inter-assay CVs from 6.4% to 7.8%. Cross-reactions of the antibodies of T_4, T_3 (for details, see Pinna et al., 1999), and rT_3 (Pinna et al., in preparation) with the majority of iodothyronines were negligible.

The RIAs were incubated overnight at room temperature (17-25 °C) on an orbital shaker (170-250 rpm). All the samples of one experiment were processed individually and assayed together within the same run. Each sample was determined in duplicate. The results were corrected on the basis of individual recovery data obtained after addition of tracer during the initial extraction process, in amounts that did not affect the RIA measurements (approximately 150-200 cpm).

2.5 Preparation of animal and human tissue samples

2.5.1 Brain dissection in the rat

Various regions of the brain were dissected according to Glowinski and Iversen (1966).

In brief, brains were carefully removed, blotted, and chilled. Dissection was performed on an ice-cooled glass plate. Twelve regions were separated, and these will be referred to in the text and figures by the following simplified names: 1) cerebellum; 2) medulla; 3) hypothalamus; 4) midbrain; 5) striatum; 6) hippocampus; 7) frontal cortex; 8) parieto-occipital cortex; 9) olfactory bulb; 10) septum; 11) amygdala; 12) limbic forebrain. Five of these regions include more than one distinct anatomical structure: the medulla corresponds to the pons and medulla. The midbrain corresponds to the thalamus and subthalamus; the striatum contains the putamen nucleus and caudate nucleus, and the globus pallidus nucleus (i.e., the basal ganglia of the telencephalon without the amygdala). The cortex corresponds to

the telencephalon without the striatum: It includes white and gray matter of the cerebral cortex.

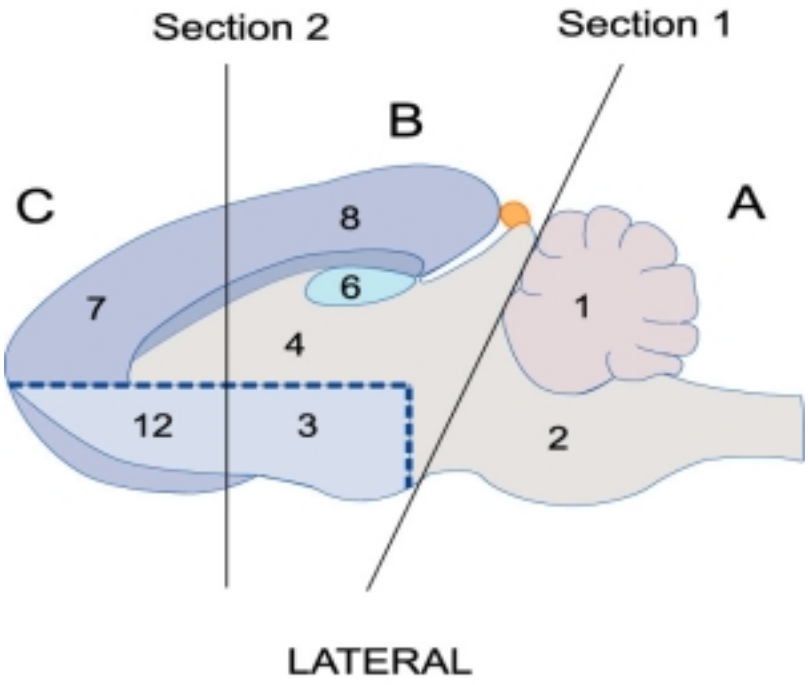

Fig 3. Sagittal section of the rat brain.

The dissection of the brain was performed as follows: First the rhombencephalon (A) is separated from the rest of the brain by a transverse section (section 1; fig 3) and dissected into two parts, the cerebellum (1) and the medulla (2). At the level of the optic chiasma, a transverse section is made that delimits the anterior part of the hypothalamus and passes through the anterior commissure (section 2). This section separates the cerebrum into two parts, B and C (fig 4). The olfactory bulbs (9) are dissected as the frontal part on the top of part C. Part B is divided into seven portions. First the hypothalamus (3) is dissected by taking the anterior commissure as a horizontal reference and the line between the posterior hypothalamus and the mammillary bodies as internal limit (fig 4). The septum (10) is dissected as the anterior part remaining in front of the midbrain. The striatum (5) is dissected with the external walls of the lateral ventricles serving as internal limits and the corpus callosum as external limits (fig 4).

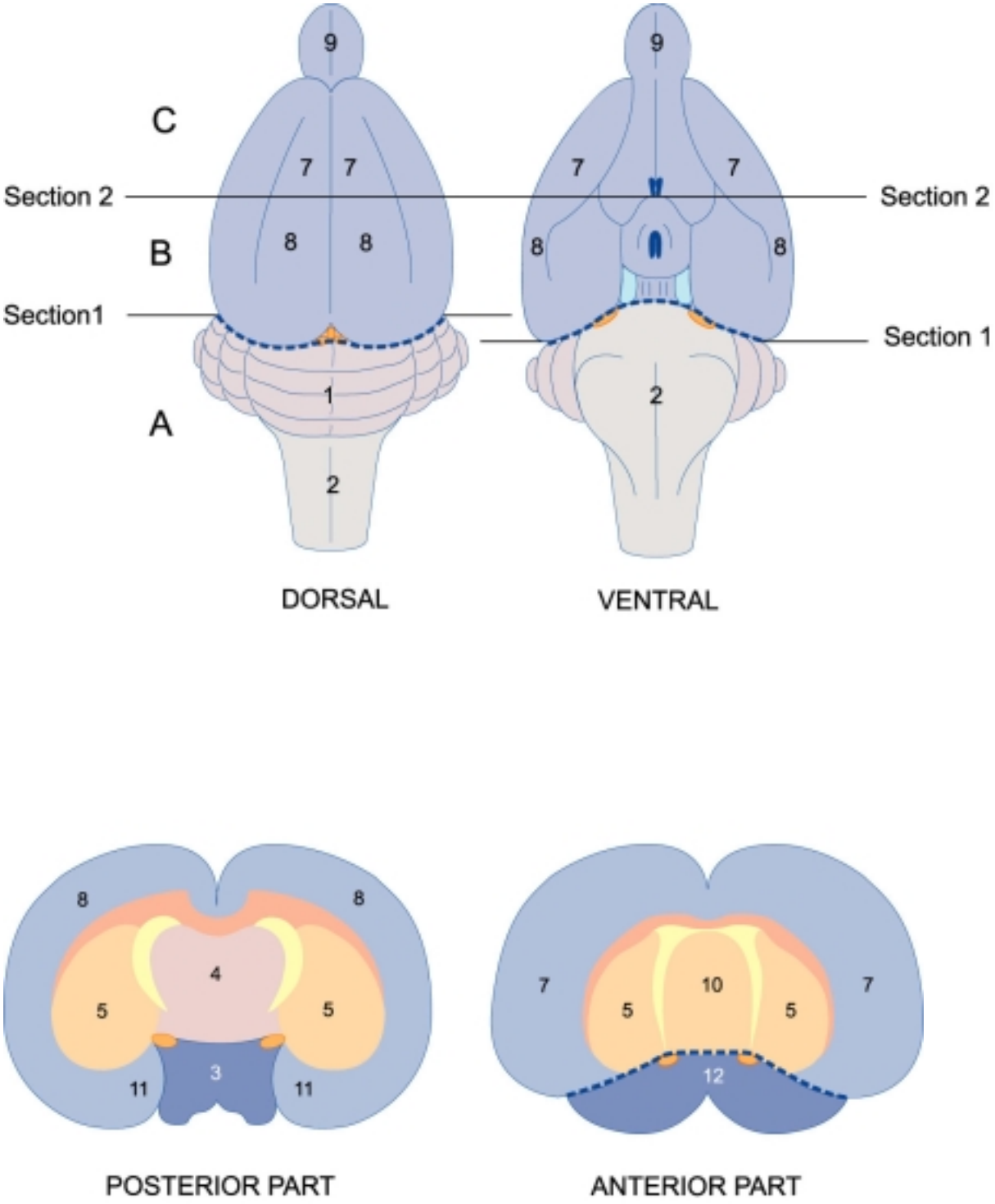

Fig 4. The following areas of the rat brain were dissected: **1**) cerebellum (**Crb**); **2**) medulla (**Med**); **3**) hypothalamus (**Hyp**); **4**) midbrain (**Mid**); **5**) striatum (**Str**); **6**) hippocampus (**Hip**); **7**) frontal cortex (**Cf**); **8**) parieto-occipital cortex (**Cp**); **9**) olfactory bulb (**Ob**); **10**) septum (**Sep**); **11**) amygdala (**Amy**); **12**) limbic forebrain (**Lf**).

The frontal part of the striatum, which is in portion C, is dissected separately and then combined with the posterior parts from portion B. The midbrain (4) is gently separated from the remaining part of the brain. The hippocampus (6) is then dissected. The amygdala (11), forming the external frontal wings extending from the parieto-occipital cortex, is separated from the remainder of part B. The remaining part of the cortex of part B forms the parieto-occipital cortex (8). The lower frontal part from the remainder of part C is also separated. It extends from the olfactory bulbs through the whole of portion C: the limbic forebrain (12). The part of the cortex of part C forms the frontal cortex (7).

The twelve regions of the rat brain were stored immediately at –85 °C until use. Blood was drawn from the decapitation wound and centrifuged, and the serum was stored at –20 °C. The pituitary glands as well as the livers were excised and processed.

2.5.2 Subcellular fractionation

The subcellular fraction method for the isolation of nuclei, mitochondria, synaptosomes, myelin, and microsomes (obtained from selected brain areas of the rat) was established by Oliver Brödel from the Department of Radiology and Nuclear Medicine, UKBF. Various methods (Lovtrup-Rein and McEwen, 1966; Dodd et al., 1981) were combined and slightly modified as described below.

All procedures were carried out at 4 °C room temperature. In order to inhibit deiodination activity, 25 µM iopanic acid (IOP) were added to all unbuffered sucrose solutions. After subcellular fractionation, the isolated fractions were washed for 1 min in a vortex mixer with a solution of 0.32 M sucrose containing 25 µM IOP. They were then subjected to centrifugation at 14,000 rpm for 15 min at 4 °C. The supernatants were discarded and the pellets, which consisted of the subcellular fraction of interest, were stored at –85 °C until use. For each subcellular fraction the tissue of only one rat was used.

- **2.5.2.1 Homogenization of the single areas of the rat brain**

Frozen tissue was placed in ice-cold 0.32 M sucrose at a final dilution of 1:10 (W/v). The tissue was homogenized mechanically with a motor-driven glass-teflon homogenizer (Brann Biotech. Internat.), mortar-cooled in an ice-water mixture throughout. The clearance between mortar and pestle was 0.2 mm, the motor speed 750 rpm, and 20 strokes of the pestle were necessary for a full homogenization of the tissue sample.

- **2.5.2.2 Centrifugation of the homogenates**

The centrifugation steps were carried out in an L8-55 ultracentrifuge (Beckman Instruments) using a swinging bucket rotor SW 41 Ti (Beckman Instruments). The tissue homogenate was centrifuged at 2600 rpm for 15 min to obtain the low-speed supernatant (S1) and the crude nuclear pellet (P1). P1 was further resuspended with 2 ml of 0.32 M sucrose and centrifuged for 10 min at 2200 rpm. The washed P1 was collected and the resultant supernatant (S2) was added to S1.

- **2.5.2.3 Isolation of the nuclear fraction**

In order to isolate the nuclei, the washed crude nuclear pellet (P1) was resuspended with 14 ml of 1.3 M sucrose containing 1 mM magnesium chloride and 1 mM potassium phosphate buffer (pH 6.5), and centrifuged at 19,700 rpm for 45 min. The resultant supernatant was discarded. The purified nuclear pellet was washed as described above (2.5.2), then collected.

- **2.5.2.4 Isolation of the mitochondrial fraction**

The supernatant S1 was diluted with 0.32 M sucrose to yield a final volume of 9 ml, layered directly onto 4 ml of 1.2 M sucrose, and centrifuged at 200,000 x

g for 30 min. The resultant high-speed pellet composed of mitochondria was further washed as described above (2.5.2). Synaptosomes, myelin, and microsomes were retained at the 1.2/0.32 M sucrose suspension interphase (I1). Following careful suctioning with a syringe, about 1-1.5 ml of this material was collected and further fractionated.

- **2.5.2.5 Isolation of the synaptosomal fraction**

The interphase I1 was diluted with 0.32 M sucrose to yield a final volume of 9 ml, layered directly onto 4 ml of 0.8 M sucrose, and centrifuged at 200,000 x g for 15 min. The resultant high-speed pellet was composed of synaptosomes, whereas the myelin was retained at the 0.8/0.32 interphase (I2). The 0.32 M sucrose supernatant (S3) containing the microsomes was collected.

- **2.5.2.6 Isolation of the myelin**

After careful suctioning with a syringe, the interphase (I2), making up about 1 ml, was hypotonically treated in order to remove axonal material from myelin vesicles that had formed during homogenization: The I2 was diluted up to 5 ml with a solution of distilled H_2O and 25 µM IOP, vortex mixed for 1 min, and incubated for 15 min at 4 °C room temperature. After centrifugation at 10,000 rpm for 15 min, the resultant pellet composed of purified myelin was washed using the same solution as for hypotonic treatment and collected.

- **2.5.2.7 Isolation of the microsomal fraction**

The supernatant (S3) containing microsomes was diluted up to 14 ml using 0.32 M sucrose and centrifuged at 200,000 x g for 30 min. The resultant pellet containing purified microsomes was washed as described above (2.5.2) and collected, whereas the supernatant was discarded.

- **2.5.2.8 Electron microscopic characterization of the subcellular fractions**

The purity and morphological quality of the subcellular fractions were characterized by electron microscopy by Prof. Gisela Stoltenburg (Department of Neuropathology, UKBF). The subcellular fractions were prepared for electron microscopy (fixing, embedding, and sectioning) by Hanna Plückhahn from the Department of Neuropathology, UKBF.
All fractions showed a high degree of purity and only minor contamination with each of the other fractions (electron microscopic photographs are available from the author upon request).

- **2.5.2.9 Characterization of the subcellular fractions by biochemical markers**

The purity of the subcellular fractions was further characterized by the following biochemical markers (the methods were established and the analyses performed by Oliver Brödel):

1 DNA quantification for the nuclear fraction (Wizard Genomic DNA Purification Kit®, Promega)

In brief, DNA concentrations in all subcellular fractions were determined as follows. First, the subcellular fractions were incubated with a specific nuclear lysing solution. Then the RNA was enzymatically removed and the protein precipitated. Later, the DNA was precipitated and washed by adding isopropanol. The DNA pellet was solved in 8 mM NaOH and the concentrations measured in a photometer at a wavelength of 260 nm.
The DNA quantification test showed a 10-fold enrichment of DNA in the nuclear fraction (70.4 µg/mg protein) compared with the homogenate (6.7 µg/mg protein). DNA concentrations were between 2- and 4-fold lower in synaptosomes and

microsomes than in the homogenate and were undetectable in the myelin and mitochondria.

2 Succinate dehydrogenase for determining the purity of mitochondria (Pennington, 1961)

In brief, the subcellular fractions were resuspended in a solution of 15 mM potassium phosphate buffer and added to a solution containing sucrose, natrium-succinate, and INT [2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazoliumchlorid]. Incubation was performed until color development (about 15 min). Finally, the extracted colored probes were measured using a photometer at a wavelength of 492 nm.
Results showed a selective 8-fold enrichment of the mitochondrial fraction (79.6 µM/h/mg protein) compared with homogenate (10.3 µM/h/mg protein).

3 CNPase for determining the purity of myelin (2',3'-cyclic nucleotide 3'-phosphohydrolase; Kurihara and Takahashi, 1973)

In brief, a three-step procedure was followed to activate the enzymes. Cyclic adenosine phosphate (cAP) was first incubated with CNPase, which opens the ring of the cAP. A second incubation was required with E. coli alkaline phosphatase to remove the PO_4 groups. The concentration of the phosphate groups was spectrophotometrically measured by the extinction (δE) of the color reaction at the wavelength of 720 nm.
Results showed a selective 10-fold enrichment of the myelin fraction (0.61 δE/h/mg protein) compared with homogenate (0.059 δE/h/mg protein).

4 Measurement of syntaxine by Western blott for determining the purity of the synaptosomal fraction (De Camilli and Takei, 1996). These experiments were performed by Dr. Murat Eravci from the Department of Radiology and Nuclear Medicine, UKBF.

The purity of the synaptosomal fraction was confirmed by using the ECL Western blot system (Amersham Pharmacia Biotech.). The antibody against syntaxine was purchased from Calbiochem-Novabiochem GmbH (Bad Soden).
A 4-fold enrichment of syntaxine was found in the synaptosomal fraction compared with the homogenate.

5 NADPH cytochrome c reductase for determining the purity of microsomes (Sottocasa et al., 1967)

In brief, NADPH cytochrome c reductase activities were measured spectrophotometrically at 30 °C by following the reduction of cytochrome c at 550 nm. The assay mixture of 0.8 ml contained 0.1 mM NADPH, 0.1 mM cytochrome c, 0.3 mM KCN, 50 mM phosphate buffer (pH 7.5). The reaction was started by the addition of the substrate.
The microsomal fraction appeared to be approximately 18-fold enriched (60.7 δE/min/mg protein) compared with the homogenate (3.4 δE/min/mg protein).

6 Lactate dehydrogenase for quantifying the cytosolic contamination of the subcellular fractions (Johnson MK et al., 1963)

In brief, after resuspension of the probes, a 50 µl aliquot of each subcellular fraction was combined with a 0.95 ml aliquot of a solution (pH 7.4) containing 50 mM triethanolamine, 0.33 mM Na-pyruvate, and 80 µM ß-NaDH (reduced form, Sigma). The decay of the reaction was measured every 3 min using a photometer at a wavelength of 340 nm.
This test showed a low cytosolic contamination of the subcellular fractions: nuclei (0.5%), mitochondria (1.2%), myelin (1.0%), synaptosomes (6.6%), microsomes (0.8%).

2.5.3 Extraction of iodothyronines from the subcellular fractions

- #### 2.5.3.1 Suspension of the subcellular fractions

Each subcellular fraction was resuspended by adding 1.25 ml of 100% methanol. In order to measure each sample's iodothyronine recovery rate and monitor the separation of the iodothyronines by HPLC, approximately 200 cpm of tracer for each iodothyronine (T_4, rT_3, T_3, 3,3'-T_2, and 3,5-T_2; 200 cpm/100 µl) were added to each subcellular fraction. The samples were briefly mixed in a vortex and also in an ultrasonic bath until the pellet was dissolved (usually between 15 sec and 1 min).

- #### 2.5.3.2 Extraction of the subcellular fractions

After their suspension, the individual subcellular fractions were vortex mixed for 1 min and centrifuged at 14,000 rpm for 30 min at room temperature using a Eppendorf centrifuge 5413 (Eppendorf). Following centrifugation, the supernatant was collected and further processed as described below (2.5.4.3), and the organelle debris-containing pellet was stored for protein measurements (see 2.5.6).

A minimum of 5 blanks were always included in the extraction protocol. They were extracted together with the subcellular fraction samples using the same experimental model. Each blank was made up of an aliquot of 25 µl 0.32 M sucrose containing 25 µM IOP, equivalent to the mean volume of the subcellular fraction of the complementary probes.

2.5.4 Extraction of iodothyronines from the brain homogenates

- #### 2.5.4.1 Homogenization of the tissue samples

Fresh or frozen excised tissue samples were weighed prior to homogenization. Homogenization of each tissue sample was performed in 3 ml of 100% methanol using a Ultra-Turrax polytron (Janke & Kunkel IKA-Labortechnik) and 12 ml plastic tubes placed on ice. The 100% methanol was also kept on ice throughout the homogenization procedure. The polytron was washed with 1 ml of 100% methanol and later with distilled H_2O prior to use in the homogenization of the following probe. Radio-labeled iodothyronines (T_4, rT_3, T_3, 3,3'-T_2, and 3,5-T_2; ~350 cpm/ 100 µl) were added to each homogenate (100 µl to each tube) in order to measure their recovery and identify the iodothyronine of interest after extraction and HPLC separation.

- #### 2.5.4.2 Extraction of the brain homogenates

The homogenized tissue samples containing the relevant labeled iodothyronines were placed in a vortex mixer for 1 min and centrifuged at 3000 rpm for 30 min at 4 °C. Then the supernatants were collected and further processed as described below (2.5.4.3); the pellets were stored for protein measurements as described later (2.5.6).

Homogenates of the tissue samples were always extracted together with blanks. Blanks underwent the same extraction protocol as the tissue samples, the only difference being that a volume of distilled H_2O was added instead of the complementary probes.

- **2.5.4.3 Preparation of the subcellular fractions and homogenate extracts for HPLC**

The supernatants resulting from the extraction of either subcellular fractions or tissue sample homogenates were filtered through 15 mm diameter non-sterile, single-use syringe minisart SRP 15, PTFE-membrane filters (0.2 µm pore size; Sartorius AG) and subsequently placed in a speed vacuum pump to dry out prior to HPLC.

2.5.5 Extraction of diiodothyronines from serum

For the determination of serum 3,3'-T_2 and 3,5-T_2 concentrations, aliquots of serum were extracted with two volumes of dehydrated alcohol, evaporated to dryness, and taken up in the experimental buffer. Extracts from 200 µl of original serum were processed individually in triplicate and assayed within the same run. Standards were set up in extracts from 200 µl of original iodothyronine-free human or rat serum, which served as internal control. They were dissolved in experimental buffer and pipetted onto standard curve assay tubes. Addition of tracer with maximum specific radioactivity, in an amount that did not influence the measurements, allowed the results to be adjusted on the basis of individual recovery data.

2.5.6 Protein quantification

Protein quantification was performed by employing the method of Bradford (1976) with slight modifications. In brief, standards were obtained from a stock solution of 0.25 N NaOH containing 200 µg/ml of protein (100 µg γ-globulin and 100 µg BSA/ml) and ranged from 0.002 to 0.02 mg/tube.

The pellets that resulted from the extraction of either tissue sample homogenates or subcellular fraction probes were handled as follows. First, they were left to dry out under a hood for a time varying between 18 h (subcellular fractions) and 48 h (tissue sample homogenates). An aliquot of 250 µl or 1 ml of 1 N NaOH was then pipetted

onto each dried pellet resulting from a subcellular fraction or a tissue homogenate and stored overnight at 4 °C. Pellets were resuspended by using a shaker for 30 min; the additional use of an ultrasonic bath was sometimes required for a better resuspension of the tissue samples. Each subcellular fraction probe was then diluted further by adding 750 µl of distilled H_2O, whereas each tissue homogenate pellet was diluted with a volume of distilled H_2O ranging from 2 to 4 ml.

To determine the unknown protein amounts, 100 µl aliquots of standards or of the complementary probe were pipetted into 12x55 mm plastic tubes together with 200 µl of coloring reagent for protein quantification (Biorad). The final volume of 1 ml was reached by adding 700 µl of distilled H_2O. Both standards and unknowns were pipetted in duplicate, vortex mixed, and incubated for 30 min. The samples were measured using a photometer at a wavelength of 595 nm.

2.6 HPLC

2.6.1 Preparation of the tissue sample extracts for the autosampler

The dried tissue sample extracts were resuspended in 36% acetonitrile and 64% HPLC water containing 1% acetic acid. Samples were placed in an ultrasonic bath for about 10 min to allow a complete resuspension of the pellet. Automated injection onto the HPLC was performed using an Advanced LC Sampler Processor ISS 200 autosampler (Perkin Elmer GmbH).

2.6.2 Purification and separation of the extracted iodothyronines by HPLC

HPLC purification and separation of the iodothyronines was carried out using a 5 µm Eurospher 100-C18, 4x250 mm column (Knauer GmbH). The column was equilibrated with a solution of 36% acetonitrile containing 1% acetic acid at a flow rate of 1 ml/min and developed with a gradient using a linear increase to 38% in 25 min. After running the gradient the column was washed with 100% acetonitrile for 5

min at a flow rate of 2 ml/min in order to remove contaminants that eluted from the column.

2.6.3 Iodothyronine collection for RIA quantification

The fractions eluting from the HPLC were collected using a Superrac fraction collector (Pharmacia) in 3 ml plastic tubes at 1 min intervals (1 ml/min fractions). The retention time for each individual iodothyronine was determined and the respective radioactive peak for each relevant hormone that eluted from the HPLC was identified by a gamma-counter. These fractions were collected in 3 ml glass tubes (volume of fraction ranging from 1 to 2 ml) and evaporated to dryness using a speed vacuum pump (Savant). The residues were dissolved in RIA experimental buffer prior to RIA measurement.

2.7 Data analysis

In the animal experiments data are given as means ± SEM. As in humans the normal range for 3,5-T_2 and 3,3'-T_2 in serum was established as the means ± 2 SD, the means ± SD are also presented in the text, tables and respective figures. Individual comparisons between the control group and each of the treatment groups were performed using the Mann-Whitney U-test. Linear regression analysis was conducted to evaluate the effects of age and sex on 3,3'-T_2 and 3,5-T_2 serum concentrations in humans.

For the analysis of the sleep deprivation experiments in humans, the mean values for each hormone measured in all blood samples taken between midnight and 6 a.m. were calculated and compared with those obtained during the night of sleep using the Wilcoxon rank test.

The circadian variations were analyzed by applying the following statistics. Tissue T_4, T_3, and 3,5-T_2 concentrations in the different brain regions and in liver at the different time points were compared by one-way ANOVA. When significant differences were found, a periodic regression analysis was performed by Fourier

periodic curve fitting using the software package CoStat (version 5.0; CoHort Software, USA). This analysis yields information on the probability of the data following sinusoidal fluctuations within a pre-established time period (in this case, 24 h) and calculates circadian rhythm parameters. These parameters include the daily mean value or rhythm-adjusted mesor (i.e., the mean level around which the circadian rhythm varies), the amplitude (i.e., the level of the cosine function above or below the daily mean value), and the acrophase (i.e., the peak time of the sinusoid maximum, expressed in hours : minutes). The significance of the calculated theoretical curve was tested by determining the associated 95% confidence interval for the amplitude (amplitude >0).

Altogether we conducted 42 comparisons using the Mann-Whitney U-test. Strict application of Bonferroni's correction of the *p*-value would result in a limit of significance of $p<0.0011$, in which case the results of only 9 statistical calculations would remain significant. However, in our study we found 29 significant results. Thus, if Bonferrroni's correction were strictly applied, several relevant findings would probably be lost. Furthermore, our study marks the first time that diiodothyronine tissue concentrations were measured with appropriate methods. The study is therefore hypothesis generating. Therefore, we also considered results with *p*-values between 0.05 and 0.0011 as significant. However, these results must be confirmed in an independent study.