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Detection of thyroid hormone receptors in the olfactory system and brain of wild masu salmon, *Oncorhynchus masou* (Brevoort), during smolting by *in vitro* autoradiography

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Abstract.

Thyroid hormone regulates a number of physiological functions during smolting in salmonids. However, the target sites and roles of thyroid hormone in the central nervous system (CNS) are not known in detail. We detected thyroid hormone-specific binding sites (i.e. thyroid hormone receptors) in the olfactory epithelium and the brain (the olfactory bulb, the telencephalon, the mid-brain and the cerebellum) of wild masu salmon, *Oncorhynchus masou* (Brevoort), during smolting by means of *in vitro* autoradiography with frozen sections. A saturation experiment with the brain indicated the presence of a single class of binding sites of high affinity. T_3-specific binding was detected in the olfactory epithelium and in all regions of the brain except the olfactory bulb. The T_3-specific binding value in the olfactory epithelium was higher than in all other regions of the brain. This binding value in the olfactory epithelium increased at the full-smolt stage. The presence of thyroid hormone receptors in various regions of the CNS suggests that thyroid hormone plays an important role in the functional change in the brain and the olfactory epithelium during smolting.

Introduction

Smolting (parr-smolt transformation) in salmonids is a complex phenomenon on comprising various morphological, physiological and behavioural changes that are under endocrine control (Hoar 1976). These events appear to be regulated by various hormones such as thyroid hormone, growth hormone and cortisol (Hoar1988; Young; Björnsson; Prunet, Lin & Bern 1989). Thyroid hormone is one of the most important hormones during smolting. It is suggested that thyroid-hormones are related to body silvering (Ura, Hara & Yamauchi 1994), seawater adaptation (Folmar & Dickhoff 1980), and downstream migration behaviour (Hoar1976). However, the mechanisms of thyroid hormone action remain largely unexplored. In particular, little is known about the relationship between thyroid hormone and downstream migration.

Serum levels of thyroid hormone gradually increase as smolting progresses and peak at the full-smolt stage, declining thereafter (Dickhoff, Folmar, Mighell & Mahnken 1982; Yamauchi, Koide, Adachi & Nagahama 1984). These studies suggest that the peak in thyroid hormone production affects the behavioural change in downstream migration by controlling the central nervous system (CNS). The existence of a thyroid hormone receptor was indicated in the brain of rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Bres & Eales1988). Specific binding of triiodothyronine (T_3) in the nuclei of the brain and the olfactory epithelium increased during thyroid-stimulating hormone-induced smolting (Scholz, White, Muzi & Smith 1985). In these studies, however, homogenates of whole-brain tissues were used. Therefore, the localization of the thyroid hormone receptor in various portions of the brain remains unclear. In the present study, We searched for thyroid hormone-specific binding sites (i.e. thyroid hormone receptors) in the olfactory epithelium and the brain (the olfactory bulb, the telencephalon, the mid-brain and the cerebellum)of wild masu salmon during smolting by means of in vitro autoradiography with frozen sections.

Materials and methods

Fish

Yearling wild masu salmon, *Oncorhynchus masou* (Brevoort), were collected monthly from the Shakotan River, Hokkaido, Japan by either a cast net or an electric shocker (Model12 Electrofisher, Smith-Root, Washington, USA) from November 1991 to May 1992 and from

November 1992 to May 1993. Fork length (FL) and body weight (BW) gradually increased from November 1991 to April 1992 (FL, 10.2-11.8 cm; BW, 10.4-11.8 g), and reached maximum values in May (FL, 14.1 cm; BW, 30.7 g; Fig. 1A). Parr marks were evident without any body silvering in the parr (November) samples and were obscured in the pre-smolt (April) samples. In the full-smolt (May) samples, parr marks were completely absent, while the fins were clear with intense black pigment at the outer extremities of the dorsal and caudal fin lobes. Water temperature of the river reached a minimum value of 0°C and the river surface was covered with ice in February 1992. The water temperature of the river increased from March to May 1992 (5.0-10.8°C; Fig. 1B).

Blood and tissue samples

Fish were anaesthetized with ethyl p-aminobenzoate and blood samples were collected from their caudal vessels. Blood was allowed to clot (overnight, 4°C) and the serum was collected by centrifugation and stored at -40°C. The olfactory rosette and the brain were carefully isolated from the head. Tissues were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH7.2) for 30 min on ice. After rinsing with 0.1M PB containing 10% sucrose and 0.1M PB containing 20% sucrose, tissues were infiltrated with Tissue-Tek OCT compound (OCT; Miles Laboratories, Elkhart, Indiana, USA) in polyethylene capsules (Beem Capsule; Ernest F Fullam, Latham, New York, USA). Subsequently, the tissues were embedded in OCT and snap frozen in liquid nitrogen or dry ice acetone and stored at -85°C.

Radioimmunoassay

Serum thyroxine (T_4) and triiodothyronine (T_3) concentrations were measured by radioimmunoassay according to the modified method of Suzuki & Suzuki (1981).

In vitro autoradiography

Serial frontal sections were cut at 10 μm on a cryostat (Cryocut 1800; Reichert-Jung, Wien, Austria) and identified to belong to one of six brain regions (the olfactory epithelium, the olfactory bulb, the telencephalon, the front of the mid-brain, the rear of the mid-brain and the cerebellum including the medulla oblongata), as shown in Fig. 2. To locate these regions, sections adjacent to those for the receptor assay were stained with Delafield's hematoxylin

and eosin (the olfactory epithelium) or cresyl violet. Brain regions were identified using the following:

1. olfactory epithelium - no description given;
2. olfactory bulb - presence of concentric laminae, consisting of (from the periphery inwards): the primary olfactory nerve fibers, the glomerular layer, the external cellular layer, the secondary olfactory fibers, the internal cellular layer;
3. telencephalon - presence of the area ventralis telencephali in the caudal third of the telencephalon;
4. front of mid-brain - presence of the hypothalamus and the ventriculus tertius;
5. rear of mid-brain - presence of the saccus vasculosus;
6. cerebellum including the medulla oblongata-presence of the stratum moleculare, the stratum ganglionare and the stratum granulare.

For terminology, we followed Northcutt & Davis (1983) for the olfactory bulb and the telencephalon and Nieuwenhuys & Pouwels (1983) for the mid-brain and the cerebellum. Frozen sections were mounted on a gelatin or poly-L-lysine-coated Superfrost slide glass (Matsunami, Osaka, Japan) and air dried overnight at 40°C. After hydration in phosphate-buffered saline (0.14 M NaCl, 2.68 mM KCl, 8.04 mM $Na_2HPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4, pH 7.4, PBS), the parts of non-specific binding sites were blocked with 5% bovine serum albumin in PBS for 20min, and incubated with 0.1 nM ^{125}I-T_3 (New England Nuclear, Boston, Massachusetts, USA) (total binding) or 0.1 nM ^{125}I-T_3 + 1000 nM T_3 (non-specific binding) for 24h at 40°C. After incubation, sections were rinsed with PBS containing 0.1% Tween-20, PBS and distilled water. The sections were air dried and analysed with an X-ray imaging analyser (Fuji BAS2000; Fujifilm, Tokyo, Japan). For quantitative analysis, specific binding was calculated by subtracting the non-specific binding from the total binding.

Saturation experiment

In this experiment, the cerebellum including the medulla oblongata was used because the celebellum is a well-known target for thyroid hormone during neural development in mammals (Dussault & Ruel1987). Sections were prepared as described above. After

blocking, the sections were incubated with various concentrations (0.01, 0.1, 0.25, 0.5, 1.0 nM) of ^{125}I-T_3 (total binding) or ^{125}I-T_3 + 1000 0-fold excess of unlabelled T_3 (i.e. 0.1, 1.0, 2.5, 5.0, 10 μM) (non-specific binding) for 24h at 40°C. Maximum binding capacity (MBC) and equilibrium dissociation constant (K_d) were calculated by Scatchard plot analysis.

Statistics

The data presented are expressed as means±SEM. One-way analysis of variance followed by Duncan's multiple range test were conducted on the data.

Results

Changes in serum T_4 and T_3 concentrations

Serum thyroid hormone levels were measured in masu salmon captured during the first sampling season (November 1991 to May 1992). Serum T_4 concentrations which remained low (1.45-5.82 ng/ml) from November to March, increased markedly from April to May and peaked (15.63±1.36 ng/ml) in May. Serum T_3 concentrations were maintained at low levels (0.12-0.47 ng/ml) from November to March and increased gradually thereafter, reaching a peak in April (0.47±0.04 ng/ml) (Fig. 3).

Saturation and Scatchard plot analysis

Tissue samples from the parr stage (November) were used in this analysis. Saturability of ^{125}I-T_3 binding to the cerebellum including the medulla oblongata was determined using a range of ligand concentrations from 0.01 to 1.0 nM. Specific binding almost reached a plateau at 0.1 nM (Fig・4A). Scatchard plot analysis indicated a single class of binding sites. The K_d value was 3.86×10^{-10} M. The MBC value was 0.519 fmol/cm^3 tissue (Fig. 4B).

Detection of the T_3-specific binding sites in the olfactory epithelium and the brain

Tissue samples from the parr stage (November) and full smolt stage (May) were used in this experiment. Specific binding of ^{125}I-T_3 in six parts of the brain are as shown in Fig. 5. T_3-specific binding in the olfactory epithelium was higher than in other parts of the brain at both the parr and full-smolt stages. Relatively little binding in the olfactory bulb and the

telencephalon was observed at these stages. The binding in the cerebellum was highest in the brain of fish at the parr stage. However, the binding in the mid-brain was higher than in other brain regions of fish at the full-smolt stage.

Autoradiography

^{125}I-T_3-specific binding in the olfactory epithelium (the olfactory rosette) was mainly observed by radioactive signals in the olfactory epithelial layer. Binding was observed by low radioactive signals in the connective tissue and the olfactory nerve (Fig. 6A, B). Total binding was observed by high radioactive signals in the olfactory epithelium (Fig. 6C), while non-specific binding was observed by very low radioactivity (Fig. 6D).

Changes in T_3-specific binding value in the olfactory epithelium

Tissue samples from the parr stage (November), pre-smolt stage (April), and full-smolt stage (May) were used in this experiment. ^{125}I-T_3-specific binding in the olfactory epithelium indicated low levels (8.0-9.0 dpm/mm^2) at the parr and pre-smolt stage, and increased markedly at the full-smolt stage (16.5-17.2 dpm/mm^2) in May 1992 and 1993 (Fig. 7).

Discussion

In the present study, the patterns in serum T_4 and T_3 fluctuations in wild masu salmon were consistent with those in hatchery-reared fish (Ura et al. 1994). However, the maximum value of serum T_4 concentrations in wild masu salmon at the full-smolt stage was higher (15.6 ng/ml) than that in hatchery reared fish at the same stage (about 7.0 ng/ml; Ura et al. 1993; H. Kudo, unpublished data).

Saturation and Scatchard plot analysis of ^{125}I-T_3-specific binding value to the cerebellum indicated that only one binding site was present and this binding site had a high affinity. The K_d value in the present results was similar to that in the liver of brown trout, *Salmo trutta* L. (3.61×10^{-10} M; Lebel & Leloup1989). The MBC could not be compared with previous studies because the unit of previous studies (fmol/mg DNA or fmol/mg protein) was different from the unit in the present study (fmol/cm^3 tissue). Further work by the present method is needed to examine changes in K_d and MBC values during smolting in the CNS. In previous studies, the thyroid hormone receptor was detected by *in vitro* binding assay with the

homogenate of tissues (Bernal & Pekonen1984; Ruel, Faure & Dussault1985; Bres & Eales1988). In these studies, T_3 binding sites were detected in cell nuclei from brain homogenate, but this method requires many samples. Therefore, a detailed analysis of intricate organs, e.g. CNS, could not be performed. The *in vivo* autoradiography of the thyroid hormone receptor was examined using tissue sections and ^{3}H-sensitive film after injection of ^{125}I-T_3 in rat brains (Dratman, Crutchfield, Futaesaku, Goldberger & Murray1987). By observing the developed ^{3}H-sensitive film of the brain, localization of T_3 binding was clarified. However this method could only semi-quantify the T_3-specific binding value because radioactivity was measured by counting the density of the silver grains in the autoradiogram. In addition, this method could not be used to compare different stages, such as the parr and smolt stages in the present study, owing to the short half-life of ^{125}I-T_3. With the present technique, a light fixation by 4% paraformaldehyde can prevent receptor binding from decreasing and the sample can be stored at -85°C until use. On the other hand, the receptor binding of the intact sample rapidly decreased during storage at -85°C (H. Kudo, unpublished data). The present technique was therefore, very suitable to examine changes in T_3-specific binding values during smolting. Moreover, the quantification and localization of the thyroid hormone receptor could be actualized using the same sample by use of an X-ray imaging analyzer. Adjacent sections could be used for additional analyses, i.e. histochemistry, immunohistochemistry and autoradiography, of other ligands. Thus, the present *in vitro* autoradiography technique using frozen sections is a powerful tool in detecting the thyroid hormone receptors in all regions of a single brain, although the localization of T_3 binding sites could not be detected at the cytological level.

T_3-specific binding was detected in the olfactory epithelium and all regions of the brain except the olfactory bulb, indicating the presence of thyroid hormone receptors in the CNS of masu salmon. The present study is the first report to demonstrate the presence of T_3-specific binding in detailed regions of the brain in teleosts. The results indicate that the highest binding was recorded in the cerebellum of fish at the parr stage. The cerebellum of teleost is known to contain many motor neurons (the cerebellomotorius system) (Nieuwenhuys & Pouwels 1983). Further, nuclear T_3 receptors in the brain have been found to increase during neural development in higher vertebrates (Chandrasekhar, Moskovkin & Mitskevich 1979; Dussault & Ruel1987; Ferreiro, Bernal, Morreale de Escobar & Potter1988), suggesting

that the thyroid hormone regulates synaptic formation and arborization of axons and dendrites. Thus, the present results suggest that the cerebellum at the parr stage may play a role in similar processes. The motor function of fish at the parr stage might also be developing. On the other hand, T_3-specific binding in the mid-brain, which includes the hypothalamus, the thalamus and the optic tectum, was higher than in other brain regions in samples of the full-smolt stage. Therefore, the thyroid hormone receptors of the mid-brain at the full-smolt stage may be related to smolt-specific functions, such as downstream behaviour, osmoregulation and visual function. In this study, T_3-specific binding values in the olfactory bulb and the telencephalon were very low. However, a high level of thyroid hormone binding and expression of c-*erb* A (thyroid hormone receptor encoding gene) has been reported in the mitral cell layer of the rat olfactory bulb (Ruel et al. 1985; Mellström, Naranjo, Santos, Gonzalez & Bernal 1991). The low level of T_3-specific binding in the olfactory bulb might be caused by the fact that the number of cells is few.

The T_3-specific binding value in the olfactory epithelium was higher than in any other region of the brain at both the parr and full-smolt stages. In this tissue in mammals, a constant turnover has been demonstrated from basal cells into olfactory receptor cells with axons projecting into the olfactory bulb (Monti Graziadei & Graziadei 1979). Hence, the high T_3-specific binding value in the olfactory epithelium may imply that thyroid hormone receptors in the nuclei of basal cells or olfactory receptor cells may be involved in synaptic formation during turnover, similar to the developing neuron. This interpretation may explain why the T_3-specific binding value in the olfactory epithelium was higher than that in the brain.

TheT_3-specific binding value in the olfactory epithelium clearly increased at the full-smolt stage. In hatchery-reared masu salmon, however, it was maintained at low levels (under 8 dpm/mm^2) even at the full-smolt stage (H. Kudo, unpublished data). This result may relate to the fact that the serum T_4 level in wild fish was higher than that in hatchery-reared fish, which may be a characteristic of wild fish. Further work is needed to examine the details of the difference between wild and hatchery-reared fish under various physiological factors. For instance, downstream migration is associated with various environmental changes (e.g. water, food and physical factors). The increase in the T_3-specific binding value in the olfactory epithelium may be caused by the intense turnover of

the olfactory epithelial cells. Changes in the type of skin, teeth and pelvic fin have also been related to environmental changes, e.g. smolting in coho salmon (Gorbman, Dickhoff, Mighell, Prentice & Waknitz 1982). Alternatively, the olfactory epithelium may be exposed to different types of odorants in the sea as compared to the river, thus bringing about a change in T_3-specific binding.

More recently, we identified an olfactory-specific protein of 24kDa (N24) in kokanee salmon, *Oncorhynchus nerka* (Walbaum) (Shimizu, Kudo, Ueda, Hara, Shimazaki & Yamauchi 1993). The immunoreactivity of N24 in olfactory tissues was stronger at the Parr stage than at the full-smolt stage using Western blot analysis. This result indicates that N24 varies in inverse proportion to thyroid hormone during smolting of wild masu salmon. It is assumed that the thyroid hormone may inhibit the expression of N24 in the olfactory receptor cell. Thus, thyroid hormone receptors and N24 may prove to be useful molecular markers for investigating the olfactory functions during smolting, including imprinting and migration, in salmonids.

In conclusion, the analysis of T_3-specific binding by the present technique enabled detection of thyroid hormone receptors in various regions of the CNS, and indicated the high binding capability of thyroid hormone receptors in the olfactory epithelium. The present results suggest that the thyroid hormone plays an important role in the functional changes in the brain and the olfactory epithelium during smolting. Further work is required to investigate whether changes in the expression of thyroid hormone receptor encoding gene or protein occurs along with changes in T_3-specific binding capability during smolting. Studies are now under way regarding the cDNA cloning of thyroid hormone receptors and N24 at our laboratory. These studies will provide information on the role of the thyroid hormone during smolting in the CNS including the olfactory system of salmonids.

Acknowledgments

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Figure Legends

Figure 1. Changes in fork length (FL; ●), body weight (BW; ○) of wild masu salmon, and water temperature (□) in the Shakotan River during smolting from November 1991 to May1992. Values represent the means±SEM (n=10-15).

Figure 2. Schematic illustration of a lateral view of the masu salmon brain and olfactory system showing sites of frozen sections (dashed lines). OE, olfatory epithelium; OB, olfactory bulb; T, telencephalon; MB_l, front of mid-brain; MB_2, rear of mid-brain; C, cerebellum including the medulla oblongata.

Figure 3. Changes in serum thyroxine (T_4; ○) and triiodothyronine (T_3; ●) 1evels of wild masu salmon during smolting in1991-1992. Values represent the means±SEM (n＝8-10).

Figure 4. Saturation of ^{125}I-T_3 binding in the cerebellum. (A) The cerebellum section was incubated for 24h at 40°C with a range of ^{125}I-T_3 concentrations from 0.01 to 0.5 nM. (B) Scatchard plot of the specific binding in (A). K_d=3.86×10^{-10} M, MBC=0.519 fmol/cm^3 tissue.

Figure 5. Compadson of ^{125}I-T_3 specific binding in various parts of the olfactory system and the brain in wild masu salmon parr (A) and full-smolts (B). Values represent the means±SEM (n=4-5). ND, non-detectable.

Figure 6. Autoradiograms of ^{125}I-T_3 binding in the olfactory rosette of masu salmon. (A) total binding; (B) counter stained with hematoxylin and eosin, Small arrowheads indicate the olfactory epithelium. Large arrowheads indicate the olfactory nerve. C, total binding; D, non-specific binding. Bar 1.0 mm.

Figure 7. Changes in T_3-specific binding in the olfactory epithelium of wild masu salmon during smolting. Values represent the means±SEM (n; sections=13-31).
＊Significantly different from parr and pre-smolts (P<0.01).

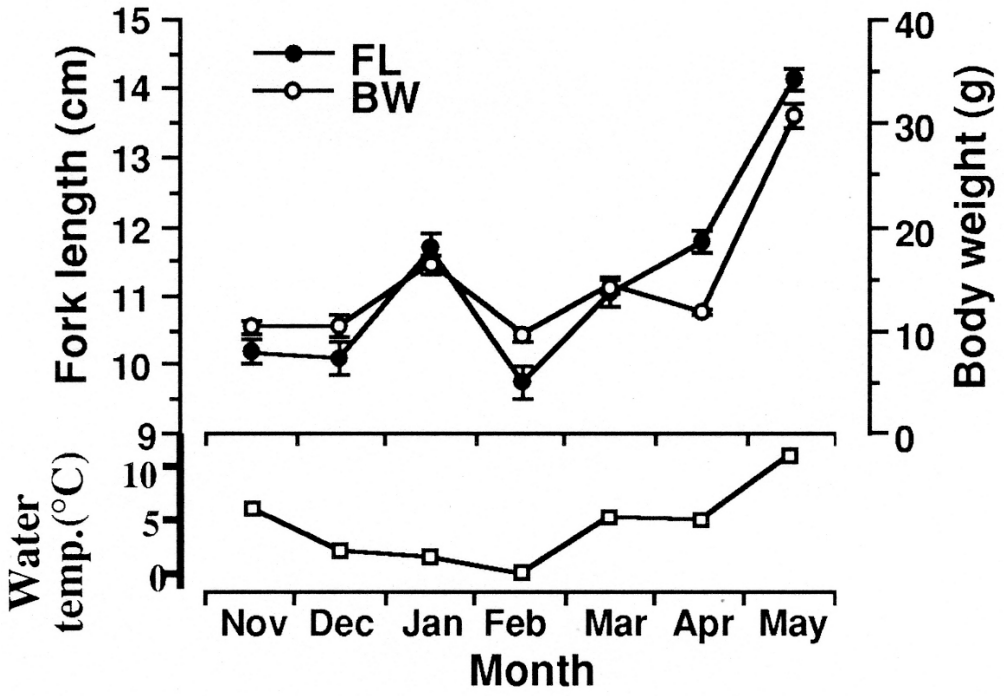

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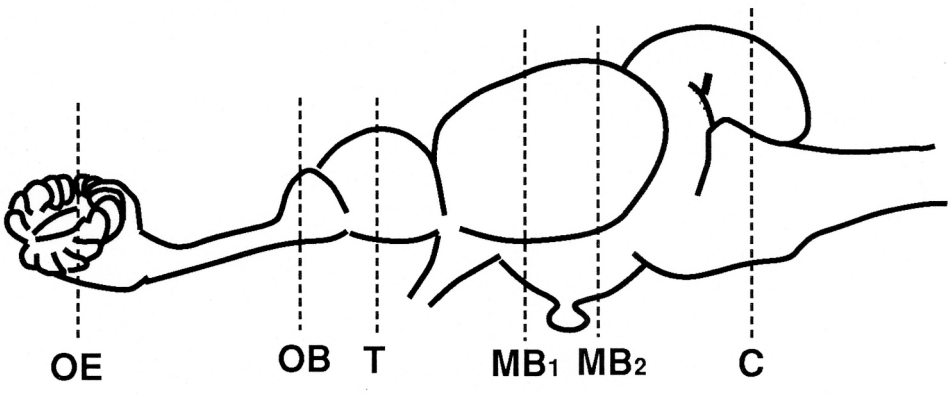
Figure 1



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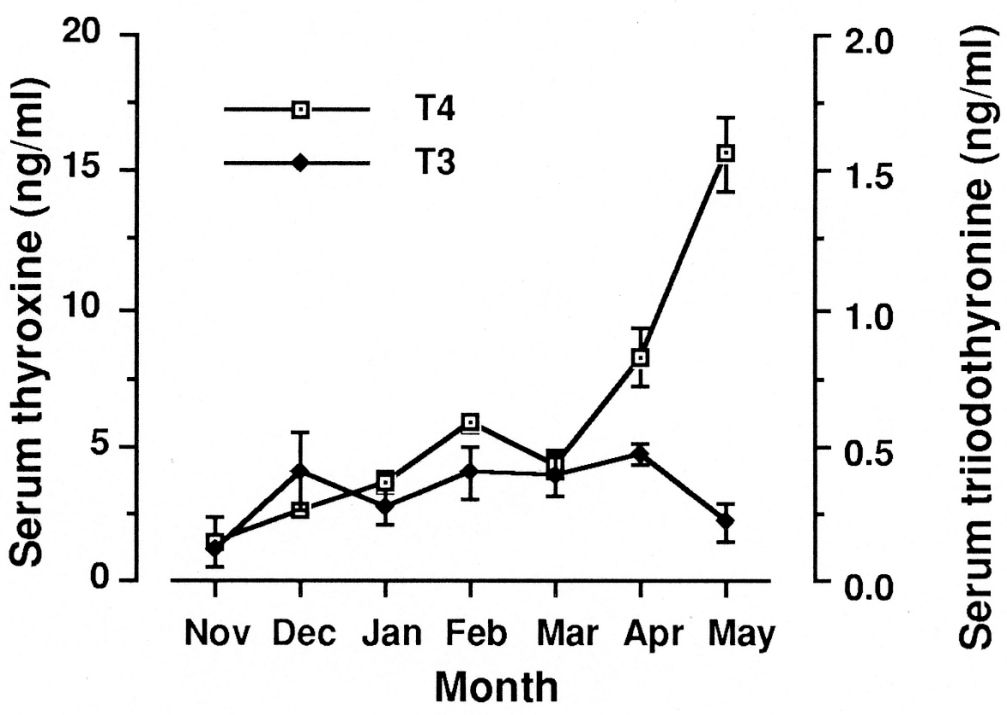

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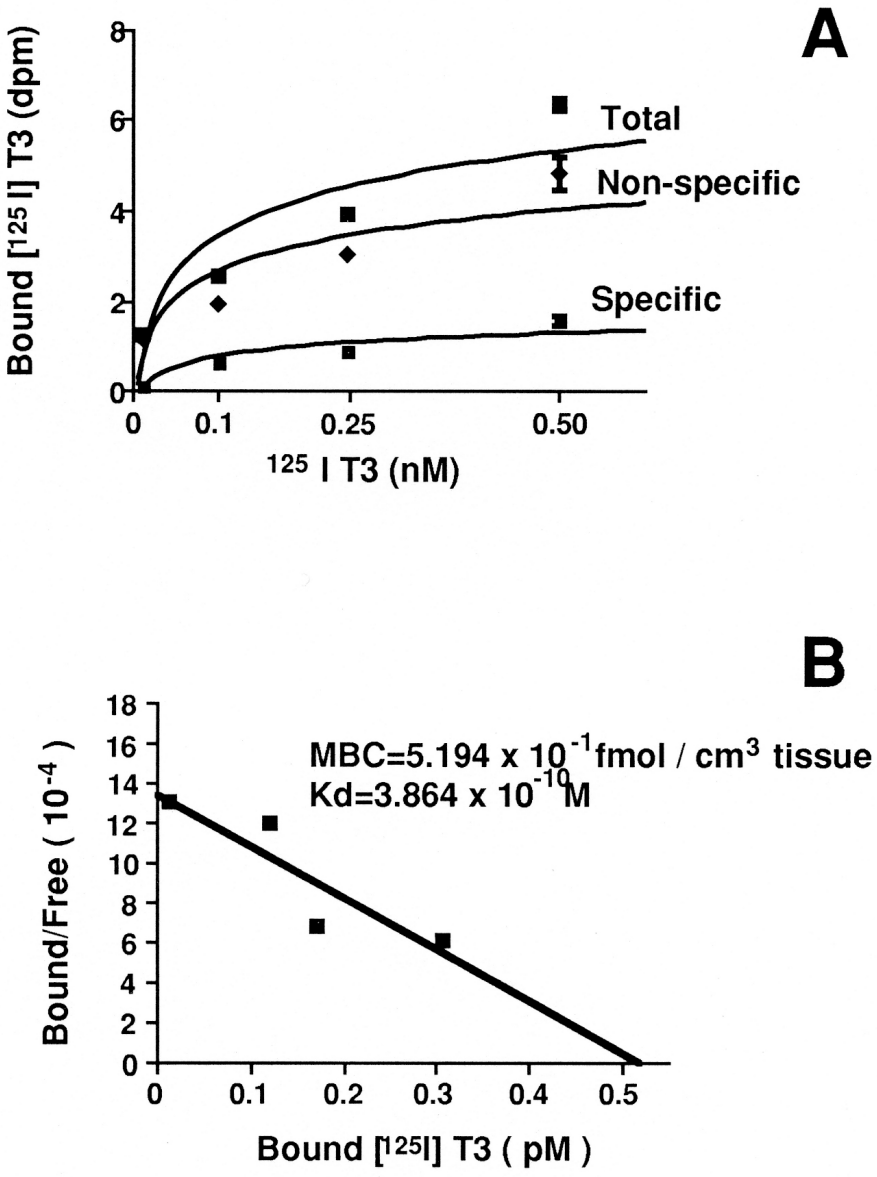

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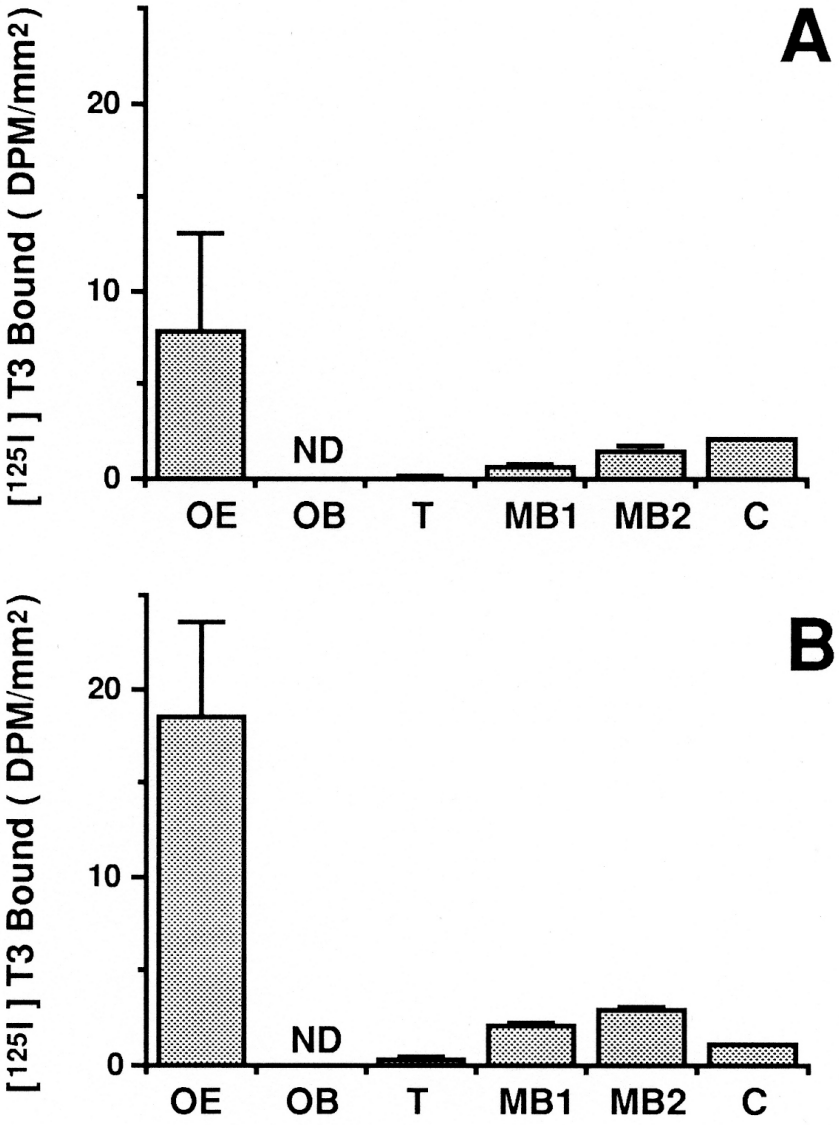

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Figure 5

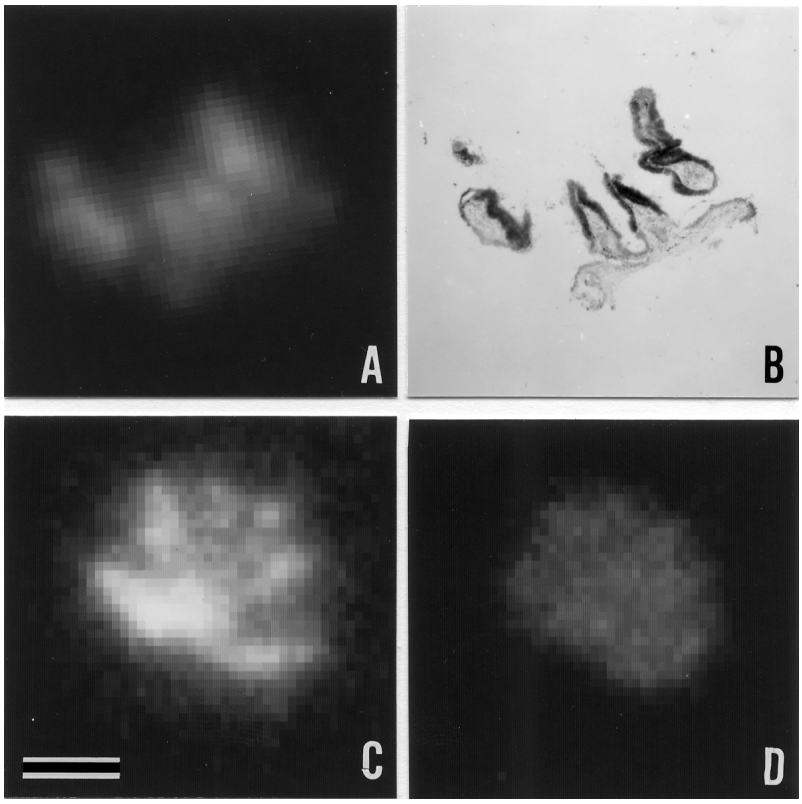

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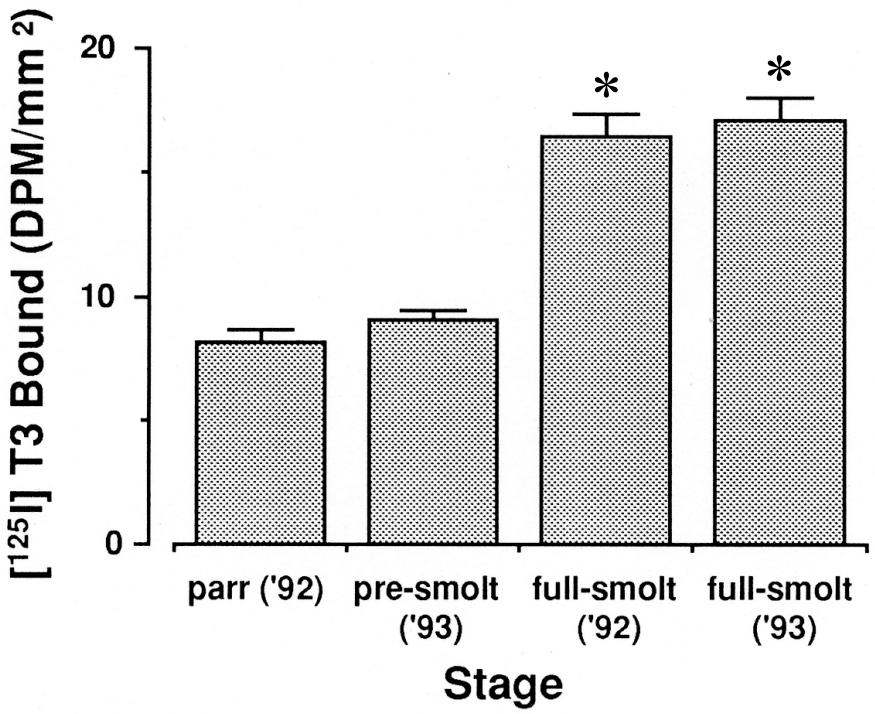
Figure 6



Kudo *et al.*
Figure 7