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Tyrosine hydroxylase activity and dopamine turnover of rainbow trout (*Oncorhynchus mykiss*) brain: the special status of the hypothalamus

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Abstract

The dynamics of catecholamine (CA)-synthesis enzymes have been poorly studied in fish. Tyrosine hydroxylase (TH), the rate-limiting enzyme of CA synthesis has been only studied in *in vitro* conditions. In the present report the *in vivo* CA synthesis and the CA metabolism were studied in different regions of the forebrain of the rainbow trout. Levels of norepinephrine (NE), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and the rate of accumulation of 3,4-dihydroxyphenylalanine (DOPA) were determined by HPLC following a treatment with hydroxybenzylhydrazine (NSD), a potential inhibitor of DOPA decarboxylase. Kinetics of the accumulation of DOPA and of the decline of DOPAC were in agreement with those found in rat, evidencing that the accumulation of DOPA following NSD can be used in trout to quantify the *in vivo* enzymatic activity of tyrosine hydroxylase. Experiments using treatment with NSD or with methyl-p-tyrosine reached a same conclusion: the DA neuronal activity in trout is much higher than NE neuronal activity. However, the hypothalamus had high DA levels *vs.* low *in vitro* and *in vivo* TH activities and exhibited a low CA turnover.

Introduction

Many studies have reported distributions of catecholamines (CA) in the fish brain by immunocytochemistry or quantitative neurochemistry. Antibodies against tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DβH) or CA are classically used and allow a qualitative analysis of the neuronal population. The determination, by HPLC, of the levels of dopamine (DA), norepinephrine (NE) and major related catabolites allow a quantification of CA metabolism in the fish brain (Nilsson 1989; Dulka *et al.* 1992; Saligaut *et al.* 1992a, b). However, quantitative studies on the dynamics of CA-synthetizing enzymes are less numerous; DβH has been studied in the catfish (Senthilkumaran *et al.* 1995). The rate-limiting enzyme of CA synthesis – TH – (Levitt *et al.* 1965) has been only studied in *in vitro* conditions in the hypothalamus and in the telencephalon of the rainbow trout (Saligaut *et al.* 1993). The hypothalamus had a low *in vitro* TH activity, in contrast with a high CA content. In those *in vitro* studies, brain homogenates were incubated in optimal conditions (30°C; saturating concentrations of both the substrate – tyrosine – and the pterin cofactor) (Saligaut *et al.* 1993). The question arises as to whether these artificial conditions correctly reflect endogenous conditions. *In situ* levels of the natural pterin cofactor are low and may be rate limiting (Lloyd and Weisz 1978; Zigmond *et al.* 1989). Moreover, the enzymatic activity of TH depends upon phosphorylation, which could be altered in *in vitro* conditions (Joh *et al.* 1978; Zigmond *et al.* 1989) and the *in vitro* optimal temperature is 30°C whereas trout are a cold water poikilothermic species.

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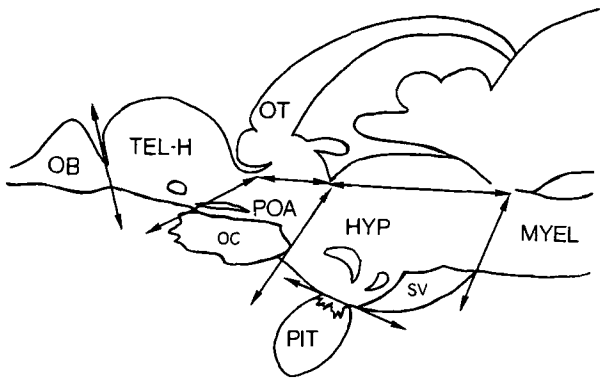

Fig. 1. Schematic diagram of a sagittal section of the rainbow trout brain. Brain was dissected as represented by arrows. OB: olfactory bulbs; TEL.H: telencephalic hemispheres; POA: preoptic area; HYP: caudal hypothalamus; MYEL: myelencephalon; OT: optic tectum; OC: optic chiasma; SV: saccus vasculosus.

The present study reports for the first time in fish an evaluation of the *in vivo* TH activity in different brain regions. HPLC was used to measure the accumulation of L-3,4-dihydroxyphenylalanine (L-DOPA; the product of the hydroxylation of tyrosine) and the decline of 3,4-dihydroxyphenylacetic acid (DOPAC; a catabolite of DA) following the administration of hydroxybenzylhydrazine (NSD), an inhibitor of DOPA decarboxylase (Demarest and Moore 1980; Gonzalez *et al.* 1988). The *in vivo* and *in vitro* TH activities were compared in the hypothalamus and in the telencephalon of trout; some control experiments were also made in the rat brain. The *in vivo* TH activity and the decline of NE and DA levels following a short-term treatment with alpha-methyl-p-tyrosine (MPT) were simultaneously determined in different regions of the forebrain of the rainbow trout. This decline of NE and DA was used as an additional criterion of CA turnover (Trudeau *et al.* 1993; Senthilkumaran *et al.* 1995).

Materials and methods

a) Experimental animals

Preliminary experiments (kinetics, comparison of *in vivo* and *in vitro* activities of TH) needed a lot of animals to obtain optimal conditions and were performed on immature female rainbow trout (200 ± 20 g). The comparison of CA metabolism of different brain regions was made on larger female rainbow trout (1000 ± 50 g). NE, DA, DOPAC and *in vivo* TH activity had similar values in the two populations when expressed as quantity by mg protein (present data and personal data). All the fish were kept in aerated and recirculated fresh water at 13 ± 1°C under a natural photoperiod (Laboratoire de Physiologie des Poissons, INRA, Rennes). They were killed by decapitation at the same time of day (between 14.00 and 15.00h) to avoid possible nycthemeral changes of CA (Popek 1983; Le Bras 1984). The brain was dissected as shown in Figure 1 into the olfactive bulbs (OB), the telencephalic hemispheres (TEL.H), or the telencephalon (TEL; OB included), the preoptic area (POA) and the hypothalamus (HYP) (Saligaut *et al.* 1990). Female Wistar rats (Le Genest, France), weighing about 200 ± 20 g, were used in some control experiments. They were kept under a natural photoperiod and had free access to food and water.

b) In vivo TH activity

The method that we used to quantify the *in vivo* TH activity has been currently used in mammals. DOPA decarboxylase was inhibited by hydroxybenzylhydrazine (NSD) and the subsequent accumulation of DOPA was measured (Demarest and Moore 1980; Gonzalez and Porter 1988). Fish or rats received a buffered salt solution (controls) or a solution of NSD dihydrochloride (Sigma, USA) at a dose of 100 mg kg^{-1} (650 µl kg^{-1} body weight). This dose gave the maximal enzymatic blockade of DOPA decarboxylase in rat (Demarest and Moore 1980) and in trout (our results; data not shown). The buffered salt solution contained 116 mM NaCl, 5.5 mM KCl, 1.8 mM $CaCl_2$, 5 mM glucose, 0.8 mM $MgSO_4$, 10 µM NaH_2PO_4 and 250 mM Hepes buffer. Animals were killed at different times following the treatment with NSD. The brain structures were quickly removed and homogenized (Elvejheim Potter) in cold water (300 µl for the brain structures of the rainbow trout, 500 µl for the hypothalamus of rat, 750 µl for the striatum). One aliquot of the medium was used for protein determination (Bradford 1976). Two hundred and thirty µl of the homogenate were quickly diluted with the same volume of an antioxidant medium (0.1%

EDTA, 0.1% $Na_2S_2O_5$, 0.2% cysteine) containing 0.4 M perchloric acid. After centrifugation (10,000 g, 3°C, 20 min) alpha-methyldopa (α-MD; Sigma, USA) was added as an internal standard. Catecholaminergic compounds were extracted by aluminium oxide (BAS; West Lafayette, USA) fixation at pH 7.75, elution with 100 µl 0.1 N HCl, and assayed by HPLC-ECD (Saligaut *et al.* 1992b). A mobile phase (0.1 M phosphate buffer, pH 3.2), containing 6% methanol and 5 mM heptan sulfonic acid (Waters, USA) permitted a good separation of α-MD, DOPA and other aminergic compounds in a C18 reverse phase column (250 × 4 mm, Merck, Germany). A standard solution of DOPA, NE, α-MD, DOPAC, DA, prepared in 0.1 N HCl, gave respective retention times of about 6.1, 7.3, 10.2, 13 and 18 min. Recovery after aluminium oxide extraction reached 50% (DOPAC), 60% (DOPA, α-MD) and 90% (NE, DA).

c) In vitro TH activity

The *in vitro* TH activity was determined by assaying DOPA formed from L-tyrosine (Nagatsu *et al.* 1979) in brain homogenates. The optimal duration and temperature for incubation were used (13 min; 30°C) (Saligaut *et al.* 1993). The standard incubation medium (150 µl final volume; final concentrations in brackets) contained: 1 M acetate buffer pH 6 (66 mM), 10 mM L-tyrosine in 0.01 N HCl (1 mM), 10 mM 6-methyl-5,6,7,8 tetrahydropterin (6 MPH_4, 660 µM; Sigma) in 1.5 M mercaptoethanol, 50 µl of brain homogenate in 0.25 M sucrose (HYP or TEL), catalase (2,600 U; Boehringer Mannheim, Germany), 12 mM ferrous ammonium sulfate (830 µM), 0.15 M ascorbic acid (10 mM) and water. The reaction was stopped with 600 µl of cold 0.5 M perchloric acid containing α-methyl DOPA (Sigma) as an internal standard in an ice bath. Catechol compounds were extracted by aluminium oxide (BAS, West Lafayette, USA)-fixation at pH 8.0, elution with 0.1 N HCl-, and assayed by HPLC-ECD. A mobile phase (0.1 M phosphate buffer, pH 3.85), containing 6% methanol and 5 mM heptan sulfonic acid, permitted a good separation of DOPA and α-MD (retention time of about 5.1 and 10.2 min, respectively). TH activity was proportional to the amount of tissue (100–900 µg protein) and remained linear for periods up to 15 min of incubation (Saligaut *et al.* 1993).

d) Effects of MPT

The decline of DA and NE levels was measured following the inhibition of TH by MPT (250 mg kg^{-1}, ip; alpha-methyl-*p*-tyrosine methyl ester, Sigma, USA) (Trudeau *et al.* 1993; Senthilkumaran *et al.* 1995). Fish were sacrificed at 0 and 240 min following treatment with MPT. NE and DA were assayed by HPLC in OB, TEL.H, POA and HYP as previously reported in the section of *in vivo* TH activity.

e) Statistical analysis

All the results are expressed as mean ± SEM. Data of the kinetic studies and changes of CA metabolism following MPT were analysed by one-way analysis of variance (ANOVA) followed by a Fisher test. Data on the comparison of *in vivo* and *in vitro* TH activities in TEL and HYP and their interactions were analysed by two-way analysis of variance (two-way AOV).

Results

a) Enzymatic activity of TH

In the first experiment, fish were killed 0, 30, 60, 90 and 120 min following the treatment with NSD. Kinetics of the accumulation of DOPA and of the decline of DOPAC, levels of NE, DA were determined in the TEL (olfactory bulbs included) and HYP (Fig. 2). DOPA was not detectable in control fish, but increased significantly in the TEL and HYP ($p < 0.01$ and $p < 0.05$, respectively) and reached a plateau 90 min following the treatment with NSD. DOPAC levels decreased in the TEL (about 50%; $p < 0.05$) up to 60 min following the treatment with NSD. DOPAC was no longer detectable by our HPLC method in the HYP by 30 min following the treatment with NSD. By contrast, the NE and DA levels did not change throughout the experiment. In a similar manner, DOPA accumulated ($p < 0.01$), DOPAC decreased (about 75%; $p < 0.01$), but NE and DA levels did not change in the striatum and in the HYP of rat treated with NSD (Table 1).

In a second experiment, *in vitro* and *in vivo* ac-

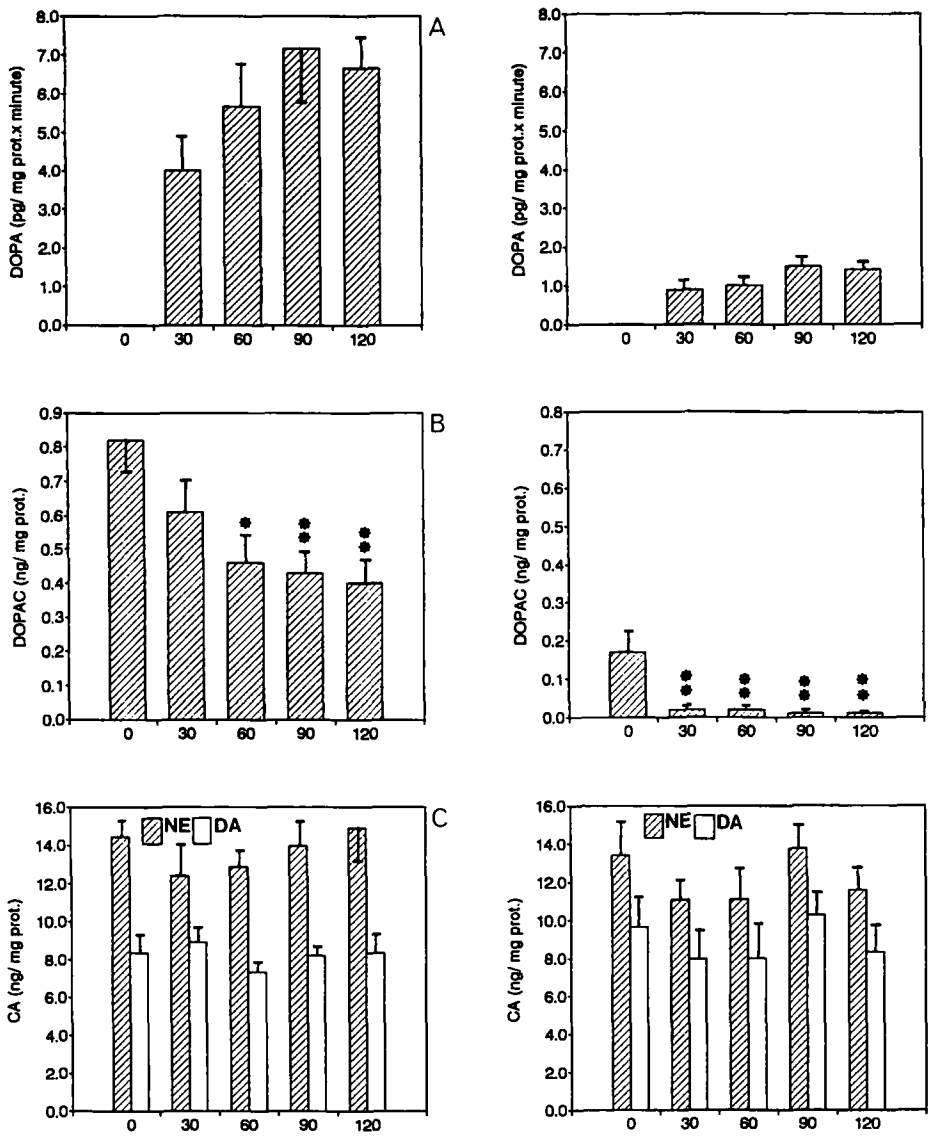

Fig. 2. Effects of NSD, a blocker of Dopa decarboxylase, on the catecholamine metabolism of the telencephalon (left part) and of the hypothalamus (right part) of the rainbow trout. (A) DOPA accumulation (*in vivo* TH activity), (B) DOPAC decline and (C) catecholamine contents (NE, DA). n = 7 per group. Trout were killed 0, 30, 60, 90, 120 min following the treatment with NSD (single injection; 100 mg kg^{-1} ip). Data are expressed as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$: when compared with control group (t=0) (Fisher test).

tivities were compared in both the TEL and HYP of the rainbow trout (Table 2). *In vivo* TH activity was about 1000 fold lower than in *in vitro* conditions ($p < 0.01$). The TH activity of the TEL was about 4 fold higher than that of the HYP in both *in vivo* and *in vitro* experiments ($p < 0.01$), two-way ANOVA showed no significant statistical interactions.

b) CA metabolism of different brain areas

The metabolism of CA was studied in different regions of the forebrain of the rainbow trout (Fig. 3). The OB contained largely DA (about 95%) and exhibited the highest *in vivo* TH activity and DOPAC levels ($p < 0.01$ in comparison with the other brain regions). The TEL.H and POA had low *in vivo* TH activity and were characterized by the presence of

Table 1. Effects of NSD on NE, DA, DOPAC levels (in 10^{-9} g mg protein^{-1}) and accumulation of DOPA (*in vivo* TH activity; in 10^{-12} g mg protein^{-1} min^{-1}) in the striatum (STR.) and in the hypothalamus (HYP.) of rats treated or not with NSD (100 mg kg^{-1} ip; decapitation 30 min following NSD).

		DA	NE	DOPAC	DOPA
STR	controls	73.4 ± 4.2	0.6 ± 0.1	9.9 ± 1.7	0
	NSD	82.0 ± 2.6	0.6 ± 0.1	2.5 ± 0.3**	480 ± 30**
HYP	controls	2.9 ± 0.4	12.6 ± 1.9	0.6 ± 0.2	0
	NSD	2.5 ± 0.2	11.6 ± 0.7	ND	110 ± 5**

Results are expressed as mean ± SEM; n=4 to 8 per group; **p < 0.01 (statistical analysis by Fisher test) ND: not determined.

Table 2. In vitro and *in vivo* TH activities (DOPA, in 10^{-12} g mg protein^{-1} min^{-1}), levels of DA and NE (in 10^{-9} g mg protein^{-1}) in the telencephalon (TEL) and in the hypothalamus (HYP) of immature rainbow trout.

	In vitro TH activity	*In vivo* TH activity	DA	NE
TEL	6360 ± 28	7.7 ± 1.1++	8.3 ± 1.2	14.4 ± 1.2
HYP	1950 ± 70**	1.1 ± 0.4**++	9.7 ± 1.9	13.2 ± 2.3

Results are expressed as mean ± SEM (n=5 to 7 per group). **, ++: p < 0.01 (two-way ANOVA; analysis between *in vivo* and *in vitro* conditions (+) and between brain regions (*)).

NE. Equal levels of NE and DA were found in the HYP, suggesting that the HYP contains both DA and NE neurones. However, high levels of DA (about 10 ng mg^{-1} protein) were associated with a low *in vivo* TH activity in this brain region.

The blockade of CA synthesis by MPT decreased significantly the DA levels in the OB and POA (respectively about 66% – $p < 0.01$ and 32% – $p < 0.05$) (Fig. 3) but did not change DA levels in the TEL.H and HYP. MPT did not significantly modify DOPAC and NE levels in the brain regions studied.

Discussion

The *in vivo* TH activity was determined in trout by assaying the accumulation of DOPA in NE and DA neurons following a DOPA decarboxylase inhibition by NSD. DOPA could not be detected in zero time treatment groups, suggesting that DOPA decarboxylase is not a rate-limiting enzyme of CA synthesis, as it appears to be in mammals. The accumulation of DOPA was approximately linear during the first 60 min following NSD treatment and reached a plateau in both the HYP and the TEL. Previous studies reported a linearity of accumulation up to 30 min in both the striatum and median eminence in rats (Demarest and Moore 1980). A plateau was also observed in rat (Reymond and Porter 1982) which was suggested to be due to a feedback inhibition of TH by the build-up of DOPA. Such an hypothesis can be made in trout because catechol compounds also compete with pterin at the cofactor site *in vitro*, as they do in mammals (Saligaut *et al.* 1993). However, the efficacy of the blocker cannot be quantified by the accumulation of DOPA. The decline of levels of DOPAC following NSD is more interesting in this way. DOPAC is a major DA catabolite and has been used as an index of DA release in fish (Dulka *et al.* 1992). The decline of DOPAC following NSD is then consistent with the view that DA released in the synaptic cleft is derived from newly synthesized DA. However, the decarboxylase inhibition could not be complete in trout (about 50%) because NSD leads to an incomplete decrease of DOPAC levels (about 50% in TEL) after 60 min. The hypothesis of a residual release of DA, maintained from a storage pool, cannot be neglected. A partial blockade of decarboxylase has already been reported in rat: inhibition of about 55, 60 and 70%, respectively in the median eminence, the HYP and the striatum (Reymond and Porter 1982). In our study, the decrease of DOPAC was about 80% in the striatum. Our data demonstrate that NSD inhibits CA synthesis in fish as it does in mammals. The accumulation of DOPA up to the plateau is a good index of CA synthesis in trout. Some differences between trout and rat could result from: i) a lower

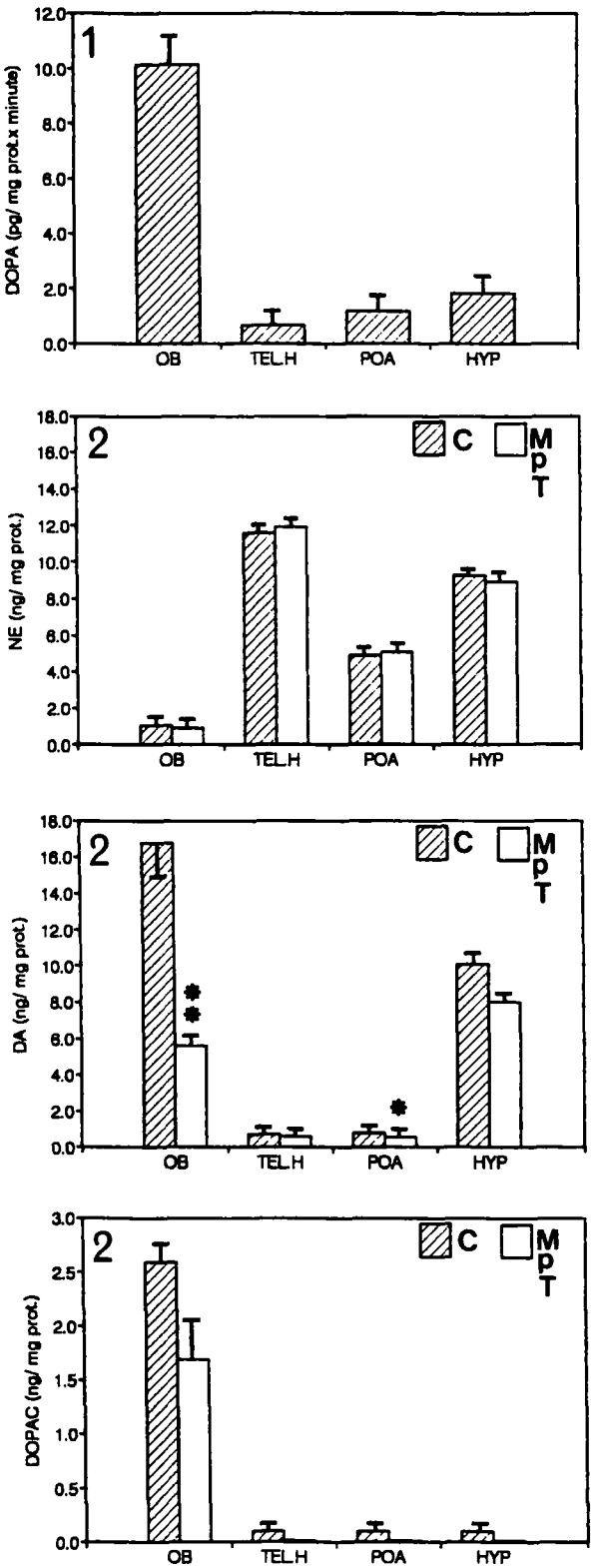

Fig. 3. In vivo TH activity (group 1) and levels of DA, NE, DOPAC (group 2) in different regions of the brain of the rainbow trout, treated or not with MPT (250 mg kg^{-1} ip). Group 1: determination of *in vivo* TH activity. Fish were killed 60 min following the treatment with NSD. Group 2: fish were killed at time 0 (controls–C; white/dark column) or 4 h following the treatment with MPT (250 mg kg^{-1} ip) (dark column); n=5 per group; results are expressed as mean ± SEM; $^{*}p < 0.05$; $^{**}p < 0.01$; statistical analysis by Fisher test.

efficacy of NSD to block CA synthesis and/or ii) a lower DA turnover in trout. NSD did not significantly alter CA levels in fish, like in rat. High intracellular stores could mask the decrease of newly synthetized CA.

The *in vivo* activity of an enzyme depends upon its amount and its specific activity. The amount of TH, *in vitro* and/or *in situ* activities have already been simultaneously assayed in rats, respectively by immunoblot assay and determination of DOPA accumulation following a treatment with NSD (Gonzalez and Porter 1988; Labatut *et al.* 1988). In the present study we attempted to correlate the endogenous TH activity with the potent activity of the enzyme (*i.e.*, *in vivo* TH activity *vs. in vitro* TH activity assayed using optimal conditions). The *in vivo* TH activity of the TEL and the HYP was about 1000 fold lower than in *in vitro*. Such a difference is unusual: in the striatum of rat the *in vivo* TH activity was reported to be only 20 fold lower than *in vitro* (Labatut *et al.* 1988), suggesting that characteristics of enzyme or the environment might be different in rainbow trout and rat. *In vitro* experiments are classically performed at the optimal temperature, which is close to the *in vivo* temperature in rat, but not in trout; a temperature-mediated decrease of the specific activity of TH may be considered in the poikilotherm living in cold water. The *in situ* HYP has a high content of CA but also a low *in vivo* TH activity when compared with the TEL.H. The data presented here indicate that some specific endogenous factors of a TH activation, previously suggested in *in vitro* experiments (Saligaut *et al.* 1993), do not explain the rather high CA contents.

DOPA is a precursor of both DA and NE and will accumulate in both DA and NE neurones following the inhibition of DOPA decarboxylase. Moreover, the activity of TH depends also upon the nature of the catecholaminergic neurons in mammals (Demarest and Moore 1980). It was interesting to compare the activity of the two populations in trout. OB and TEL.H. contained

equal levels of CA (respectively DA and NE) but the *in vivo* TH activity was much higher in the OB. This suggests that the TH activity of DA neurons could be much higher than that of NE neurons which in turn suggests that the activity of DA neurons may be higher than that of NE neurons. The experiments in which only DA levels, but not NE levels, decreased 4h following the treatment with MPT support our hypothesis; in a steady state, the amount of CA released from or metabolized in axonal endings may equal the amount of CA synthetized in the tissue. According to this postulate, the amount of CA that disappears from the tissue of trout treated with MPT may be related with the amount of DOPA that accumulates in the tissue.

The HYP had high levels of NE and DA; however, DA neurones of the HYP may have a low activity because i) they exhibit a low DOPAC/DA ratio (index of DA release), ii) DA levels did not significantly decrease following the treatment with MPT.

The CA distribution that we find in the rainbow trout brain by HPLC is in agreement with the CA distribution observed by using the localization of TH, DA and DβH immunoreactive (iR) cells in teleosts. Most telencephalic and diencephalic TH iR neurons of teleosts are also DA iR, with a dominance of TH immunoreactivity in OB (*Apteronotus leptorhynchus*: Sas *et al.* 1990; *Gasterosteus aculeatus*: Ekström *et al.* 1990; *Anguilla anguilla*: Roberts *et al.* 1989; *Dicentrachus labrax*: Batten *et al.* 1993; *Carassius auratus*: Beltramo *et al.* 1994). Moreover, it was previously pointed out that the posterior periventricular nucleus (PVO – paraventricular organ) of the HYP showed differences in the distribution of TH and DA immunoreactivity. Some cerebro-spinal fluid (CSF) contacting neurons in the PVO of teleosts lack the enzyme TH but are CA immunoreactive (Meek *et al.* 1989; Sas *et al.* 1990; Meek and Joosten 1993). Such CA cells which pick up CA from the CSF and concentrate CA could explain why the HYP exhibits a high CA levels concomitant with a low TH activity.

In conclusion, the accumulation of DOPA can be used to quantify the endogenous CA synthesis. The experiments using NSD and MPT reached a same conclusion: DA turnover (*i.e.*, synthesis and release) is much higher than NE turnover in the trout brain. Our quantitative analysis agrees with qualitative immunocytochemical analysis in teleosts. The hypothalamus of trout has an apparently abnormal CA metabolism since it exhibits high levels of CA which cannot be related to a high CA synthesis. That was previously suggested in *in vitro* experiments, but is demonstrated for the first time in *in vivo* conditions in a quantitative way. A possible explanation could be the presence of CSF-contacting cells. Further experiments are however needed, focusing on the determination of such CSF-contacting cells in the hypothalamus of trout and their physiological function.

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