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Dopaminergic systems in the European eel: characterization, brain distribution, and potential role in migration and reproduction

Marie-Emilie Sébert · Finn-Arne Weltzien · Christine Moisan · Catherine Pasqualini · Sylvie Dufour

Abstract In fish like in mammals, dopamine (DA) is a major catecholaminergic neurotransmitter that contributes to many functions of the nervous system like sensory perception, tuning of sensori-motor cues, and hypothalamic and pituitary functions. In the eel, DA inhibits gonadal development, and juvenile silver eels remain blocked at a prepubertal stage if their reproductive migration does not occur. From data in other teleosts and vertebrates, it is suggested that DA would be involved also in the last steps of eel reproduction (oocyte maturation, ovulation, and spermiation) as well as in eel reproductive migration (locomotion and olfaction). Investigating dopaminergic systems in the eel may help in understanding the mechanisms of its complex life cycle and provide new data for its conservation and reproduction. In this article we review the biosynthesis and catabolism of catecholamines and discuss available methods to investigate brain dopaminergic systems in vertebrates and their application to the eel. Immunocytochemistry, in situ hybridization, and different tracing methods are used to map dopaminergic neurons and projections in the brain and pituitary and infer their potential functions. Moreover, variations in dopaminergic activity may be approached by means of quantitative methods like quantitative real-time RT-PCR and HPLC. These tools are currently used to study dopaminergic systems in the eel brain, their anatomy, regulation, and potential roles with special emphasis on the regulation of reproduction and reproductive migration.

Keywords Teleosts · Catecholamine · Locomotion · Olfaction · Puberty

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Introduction

First described for their role in the cardiovascular function (Holtz, 1939; McGeer et al., 1978),

catecholamines (CAs) were then discovered in the central nervous system (CNS) (Vogt, 1954). Through the various CAs, DA is widely present in the CNS where the differential location of DA neurons between brain areas illustrates an implication in various central functions (for review, Smeets & Gonzalez, 2000). For instance, DA modulates the visual and olfactory sensory perception, tuning of sensory-motor cues, and hypothalamic (food intake, thermoregulation) and pituitary (e.g. prolactin secretion) functions. In fish, various studies report that DA is also involved in such different functions than locomotion (Mok & Munro, 1998b), reproduction (for review, Dufour et al., 2005), and aggressive and dominant behaviors (for review, Winberg & Nilsson, 1993).

In this article we will review methodological approaches and results concerning DA systems in teleosts with special focus on the European eel in which DA plays a critical role in sexual maturation. Indeed DA has been shown to inhibit synthesis and release of gonadotropins, the pituitary hormones which stimulate gonadal development (for review, Dufour et al., 2003). Following this, when silver eels leave continental waters to undertake a 6,000-km long migration to reach their reproductive area they remain blocked at a prepubertal stage. The silver stage is the last stage observed in natural conditions, and migratory and reproductive conditions continue to be a mystery. Moreover DA may also be involved during migration and in the last steps of gonadal development and spawning. Keeping in mind that eel stocks are menaced worldwide (Wirth & Bernatchez, 2003), it is important to elucidate the roles and regulation of DA to develop sustainable conservation plans and commercial aquaculture for this species. In addition, eels belong to the phylogenetically ancient group of Elopomorphs and therefore represent a relevant model to study evolution of dopaminergic control of central functions.

Dopamine metabolism

Biosynthesis

Catecholamines are synthesized from the amino acid tyrosine and therefore have a common structure including a hydroxylated benzene ring and an amine tail. Their synthetic pathway is well conserved among vertebrates as reported in phylogenetical studies (Reiner, 1994). Moreover, the synthetic pathway is similar in various tissues like neurons and non-neural chromaffin cells (Blaschko, 1939; Holtz, 1939). Catecholamine metabolism in fish has been reviewed by Nilsson (1983) and more recently by Randall and Perry (1992).

The synthetic cascade of CAs, also named the "Blaschko pathway" (Blaschko, 1939), begins with the hydroxylation of tyrosine into L-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH), a cytoplasmic enzyme (Fig. 1). This first step is considered as the rate-limiting one (Levitt et al., 1965) in CA synthesis, whatever the end product is. Then, L-DOPA is decarboxylated to DA by the cytoplasmic enzyme aromatic L-amino acid decarboxylase (AADC). Because the available enzymatic equipment determines what end product a particular neuron will produce, dopaminergic neurons would

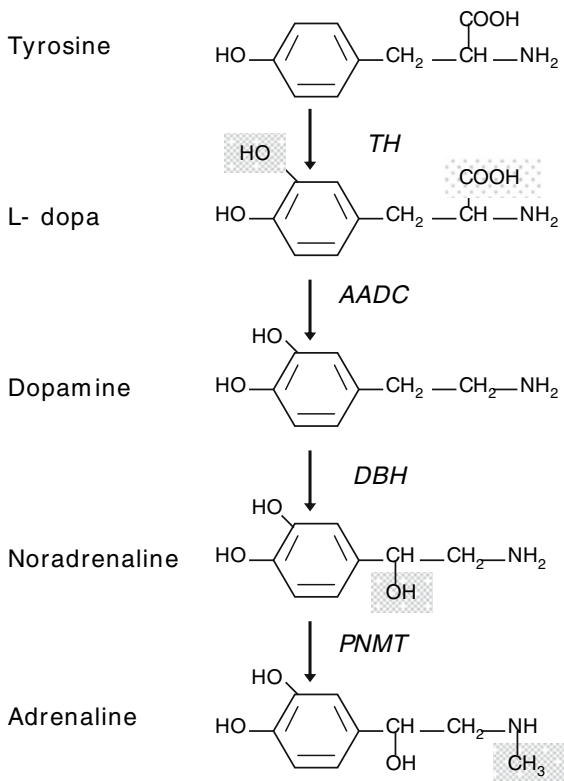

Fig. 1 Biosynthesis of catecholamines. Enzyme abbreviations: TH, tyrosine hydroxylase; AADC: amino acid decarboxylase; DBH, dopamine-β-hydroxylase; PNMT: phenylethanolamine-*N*-methyl transferase

enclose only TH and AADC. In contrast, the neurons using NA or A as neurotransmitters contain additional enzymes and produce DA as an intermediary product. Like TH and AADC, DA is found in the cytosolic space where it is taken up in storage vesicles until its release by dopaminergic neurons. In noradrenergic and adrenergic neurons, the dopamine-β-hydroxylase (DBH) converts DA to NA inside the storage vesicles. The final step in the Blascho pathway consists of the methylation of NA by the cytoplasmic enzyme phenylethanolamine *N*-methyltransferase (PNMT) to produce A. In teleosts, DA and NA are the most frequent CAs in the CNS where they act as neurotransmitters, whereas A is mainly produced in the periphery by the chromaffin tissue.

Many studies have focused on the regulation of TH, the rate-limiting enzyme. Available data report different ways of TH control. First, works in mammals report that synthesized end products like NA, A (Spector et al., 1965, 1967), or L-DOPA (Demarest & Moore, 1980; Reymond & Porter, 1982) may ensure a rapid negative feedback on TH synthesis, thereby regulating CA production. It seems that similar mechanisms exist also in fish as suggested in the trout (*Oncorhynchus mykiss*): Linard and coworkers reported that treatment with an inhibitor of AADC resulted in L-DOPA accumulation in the telencephalon and the hypothalamus of female trout. The L-DOPA content increased in a linear fashion before reaching a plateau, suggesting a negative feedback from L-DOPA on its own production (Linard et al., 1996). Second, the phosphorylation of TH is also a short-term positive regulating way for CA synthesis because it increases TH affinity for its cofactor, oxygen (Joh et al., 1978; Zigmond et al., 1989). Finally, modulation of TH expression, transcription, and translation constitutes a long-term regulation mechanism for CA neurons. Such long-term TH regulation has recently been discussed in the eel in response to steroid feedback (Weltzien et al., 2006). Besides the regulation of the rate-limiting enzyme, the last steps of the "Blaschko pathway" may be submitted to hormonal control in some vertebrate groups. For instance, adrenocortical steroids have been shown to stimulate DBH and PNMT (Nilsson, 1983); in rainbow trout, in chromaffin tissue, cortisol has no effect on the PNMT but significantly increases DBH activity (Jonsson et al., 1983) and thereby NA synthesis.

Degradation

As with their synthesis, CA degradation is a well-conserved process among vertebrates. DA catabolism involves two major enzymes: monoamine oxidase (MAO) and catechol-*O*-methyl transferase (COMT) (Fig. 2). MAO is located in the external membrane of mitochondria. In fish only one form of MAO has been reported (Setini et al., 2005) in contrast to tetrapods in which two isoforms exist (MAO A, MAO B) (McGeer et al., 1978; Cooper et al., 1986; Siegel et al., 1989). COMT is located in the cytoplasm and in the synaptic cleft, but it is also largely involved in the degradation of circulating CAs. During nerve activity DA is released in the synaptic cleft where it may act as a neurotransmitter, binding to postsynaptic G-protein coupled DA receptors (DA receptors 1–5) (for review, Callier et al., 2003). However, about 70% of the released DA will be taken up rapidly by a membrane presynaptic transporter coupled to the co-transport of Na^+ and Cl^- ions (McGeer et al., 1978; Cooper et al., 1986; Siegel et al., 1989; Chen et al., 2004). Once in the presynaptic terminal, free DA may be deaminated and transformed to its corresponding aldehyde by MAO and further converted by aldehyde dehydrogenase into dihydroxyphenylacetic acid (DOPAC, Fig. 2). Alternatively, the remaining fraction of DA released in the synaptic cleft may be inactivated by COMT into O-methylated catabolites. Following this, DOPAC may be O-methylated to the homovanillic acid (HVA) by COMT prior to be released directly or via the cerebrospinal fluid in the circulation.

In the European eel, it seems that MAO is the principal degradative pathway because DOPAC was easily detected, while HVA remained undetectable except in olfactory bulbs (see Section "Estimation via the study of synthesizing enzyme"). The predominance of the MAO or the COMT catabolic pathway follows species-specific variations. DOPAC is found as the primary metabolite of DA catabolism in goldfish (*Carassius auratus*; Dulka et al., 1992) and Atlantic cod (*Gadus morhua*; Ehrenström & Johansson, 1987). In contrast, HVA is preferentially found in the crucian carp (*Carassius carassius*; Nilsson, 1989, 1990), while in a selacian, the dogfish, both O-methylation and deamination appear important (Mazeaud & Mazeaud, 1973). Although these variations are linked to species-specific differences, it

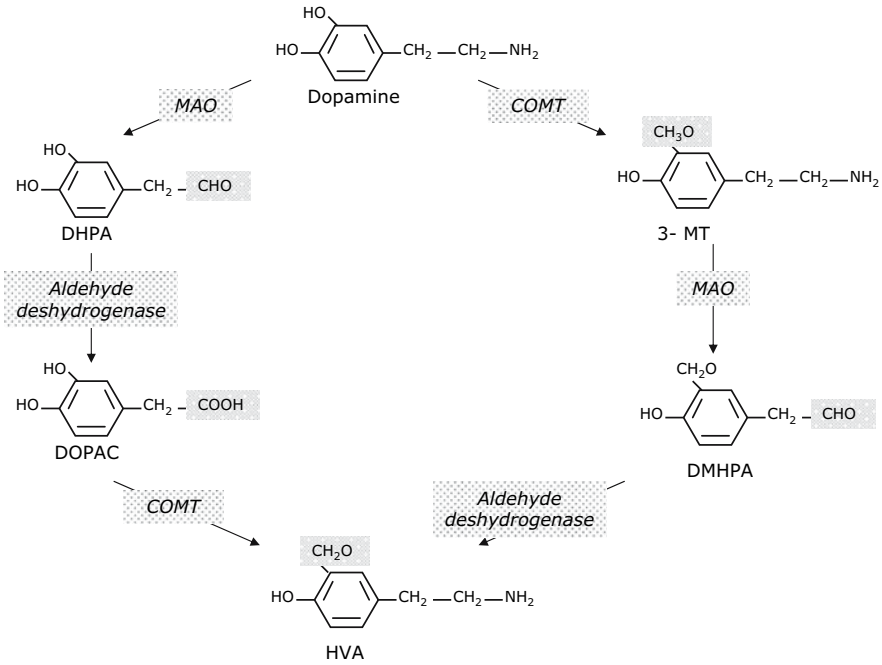

Fig. 2 Catabolism of dopamine. Catabolite abbreviations: DHPA, 3,4-dihydroxyphenyl-acetaldehyde; DMHPA, 3-methoxy-4-hydroxyphenyl-acetaldehyde; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 3-MT, 3-methoxytyramine. *Enzyme abbreviations*: MAO, monoamine oxidase; COMT, catechol-*O*-methyl transferase

seems that it also depends on the analytical technique used.

Methodological approaches of dopaminergic system investigations

Cartography of dopaminergic systems

Formaldehyde-induced fluorescence method (FIF)

The FIF method was developed following the discovery that sections of formaldehyde-fixed adrenal medulla that produces CAs were fluorescent (Eränkö, 1955). The procedure described by Falck and coworkers consists in the exposure of freeze-dried tissue to formaldehyde vapor (Falck et al., 1962; Erankö, 1967); during this exposure CAs and indole-amines become intensively fluorescent. This technique allowed labeling of catecholaminergic cells and fibers in various tissues including the rat brain (Dahlström & Fuxe, 1964). Later, the treatment of tissue with glyoxylic acid was preferentially used because it was more sensitive compared to the FIF method. However, neither of these methods allow for discrimination between individual CAs or between CAs and indoleamines. With these major limitations, the FIF method is not suitable for studying CA systems in their complexity.

Immunocytochemistry

Immunocytochemistry is the cytological method classically used to map brain cellular bodies and their projections. Because DA is stored in vesicles in the cytosolic space, using antibodies against DA enables the localization of DA neurons and fibers. One may also indirectly label DA neurons and their projections using antibodies against DA-synthesizing enzymes (TH, AADC). However, this indirect labeling necessitates a further distinction between DA, NA, and A neurons because DA is a transitory product in NA and A synthesis.

In the European eel, brain localization of DA and TH immunoreactive (-ir) cells and fibers has been performed (see procedure details in Roberts et al., 1989; Kapsimali et al., 2000; Vidal et al., 2004; Weltzien et al., 2006). In addition to being a useful tool in cartographical studies, immunocytochemistry may be used as a semi-quantitative measure of protein expression. This may in some aspects be biologically more relevant compared to in situ hybridization in which gene expression is visualized.

Like this, it constitutes a nice support for brain CA quantitative assays by HPLC.

In situ hybridization (ISH)

Compared to immunocytochemistry, ISH labels cellular bodies that express the gene of interest, whereas cellular projections are not labeled. Because successful hybridization depends on the exact match between the gene of interest and the synthesized riboprobe, ISH specificity is higher compared to immunocytochemistry especially when using polyclonal antisera. As a result, ISH generally gives higher sensitivity. In addition, ISH can be a precious support for gene expression analyses by quantitative real-time (qrt) RT-PCR. The cloning of a cDNA for European eel TH has opened qualitative and quantitative studies on TH expression in this species (Boularand et al., 1998). Recently, steroid effects on dopaminergic cells in specific brain areas of the European eel have been clearly observed by ISH (see Weltzien et al., 2006).

Retrograde tracing

As a support to the various methods of immunolabeling, the complexity of dopaminergic system organization may be approached through neuronal tract tracing. One of the available tract-tracing methods consists of incorporation of tracers in cell bodies (anterograde tracing) or near-peripheral endings (retrograde tracing). Among available tracers, lipophilic carbocyanine dyes like 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindicarbocyanine perchlorate (DiI) are widely used. In fact, they are not cytotoxic and may be used for labeling live neurons in vivo as well as in vitro (Godement et al., 1987). Such dyes provide intense and long-lasting staining, and their good resolution allows labeling of fine processes. Moreover, it is useful for multi-labeling studies because they are available in different colors. In aldehyde-fixed tissues, the crystals of DiI, inserted into nerve terminals, diffuse along the plasma membrane of cells in a retrograde direction. In the European eel, this method was applied according to previous works in goldfish (Anglade et al., 1993) to trace the origin of hypophysiotropic fibers (Weltzien et al., 2006).

Evaluation of dopaminergic activity

Assay of catecholamine content

Radioenzymatic labeling of catecholamines This technique involved radioactive labeling of amines by the transfer of a tritiated methyl group from *S*-adenosyl methionine by using the enzyme COMT. Labeled CAs were then separated by chromatography, and the radioactivity of the amine derivatives was counted. This method was used in previous studies by Le Bras (1984) to study circadian variations of CAs in various tissues in eel. However, the method was long and constraining, and therefore it is not used anymore.

Quantification by high-performance liquid chromatography (HPLC) DA content in the brain and other tissues can be assayed by high-pressure liquid chromatography (HPLC). This gives a sensitive measurement of CA content. The simultaneous measurement of CA metabolites allows an estimation of the turnover rate. This was recently performed in different brain areas and pituitary of the eel according to the method previously described by Caroff et al. (1986) and is briefly summarized below.

(a) *Sample preparation*. Brains from female silver eel were dissected into five parts (olfactory bulbs, telencephalon including the rostral preoptic area, diencephalic and mesencephalic areas, cerebellum, and medulla oblongata) (Fig. 3) prior to be frozen in liquid nitrogen and stored at −80°C until further processing.

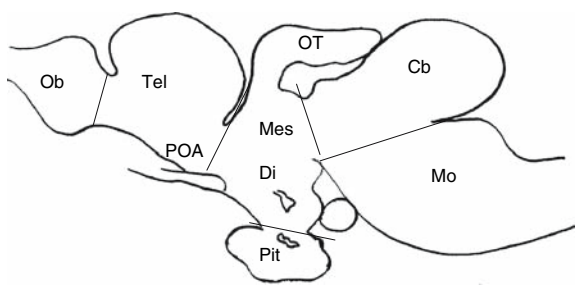

Fig. 3 Dissection of eel brain for sampling. Abbreviations: Ob, olfactory bulbs; Tel, telencephalon; POA, preoptic area; TeO, optic tectum; Di, diencephalon; Mes, mesencephalon; Cb, cerebellum; Mo, medulla oblongata; Pit, pituitary

(b) *Analysis*. Brain parts were weighed, sonicated (4°C) in 225 µl $HClO_4$ 0.1 M (with 25 µl of NAC-5-HT as internal standard), and centrifuged at 12,000 *g* for 10 min (4°C), and the supernatant was filtered (0.45 µm). The analysis was performed with an HPLC system with electrochemical detection. Composition of the mobile phase: citric acid 35 mM, Na_2HPO_4 12.5 mM, sodium octyl sulfate 0.25 mM, EDTA 0.05 mM, and 8% of acetonitrile, pH = 3.35. The injection of 20-µl filtered supernatant in the reversed-phase chromatographic column (Ultrasphere ODS 5 µm) with ion pairing gave a direct measure of the CA content in the brain area concerned by extrapolation to an external standard.

This method is a suitable tool to approach neuronal activity through the measurement of the CA metabolite/CA ratio. Thus, the activity of dopaminergic neurons in the European eel may be estimated by the calculation of DOPAC/DA ratio because DOPAC is the major DA catabolite in this species (see Section "Assay of catecholamine content").

Estimation via the study of synthesizing enzyme

Quantification of enzyme activity Because TH is the rate-limiting enzyme in the biosynthesis of CAs, calculation of TH activity may be used as a measure of CA activity. Chaube and Joy have measured TH enzymatic activity in catfish, *Heteropneustes fossilis*, in different brain parts using the method of Shiman et al. (1971) (Chaube & Joy, 2002a, b, 2003a, b). Briefly, brains were dissected, homogenized, centrifuged, and passed through a Sephadex column to obtain an eluate containing the enzyme. TH activity was measured adding L-tyrosine as substrate and also compounds required for the enzymatic reaction. After 30 min, the reaction was stopped and the amount of L-DOPA formed was assayed by spectrophotometry and tissue protein content determined by the method of Lowry et al. (1951). TH activity is expressed in nmol L-DOPA formed per mg protein per hour. This method allows a direct measure of enzyme activity and has been used to estimate the effect of specific treatments on dopaminergic activity. For instance, Chaube and Joy have studied steroids–CA interactions in female catfish, *H. fossilis* (Chaube & Joy, 2002a, b). They demonstrated that estradiol may modulate TH activity which is of particular interest because both DA and NA may inhibit and stimulate, respectively, GtHs' secretion (see Section "Characterization of dopaminergic systems in the European eel").

Enzyme inhibitors may also be used to indirectly assay the activity of an enzyme. Once the metabolic pathway is blocked, the accumulated product can be assayed. For instance, studies on TH activity may be performed using hydroxybenzyhydrazine, which blocks AADC prior to further assay of the amount of accumulated L-DOPA by HPLC, as reported for rainbow trout (Linard et al., 1996).

Quantification of enzyme gene expression Recently, a qrtRT-PCR assay to measure the gene expression of TH has been developed in the eel. In this qrtRT-PCR assay, sensitive quantification of TH mRNA is given relative to a reference gene, ARP (acidic ribosomal phosphoprotein P0). Because the procedure details have been recently published (Weltzien et al., 2005), we will not discuss this here. As regulation of TH expression is a means to answer an enhanced and sustained neuronal activity, this method is a precious tool to approach DA modulation after special treatments.

Characterization of dopaminergic systems in the European eel

Cartography

Eel is a suitable model to study dopaminergic system organization principally for two reasons. First, eel is a support for phylogenetical studies because, as a member of the Elopomorpha, an ancient teleostean group (Lauder & Liem, 1983), it may provide knowledge on ancestral brain organization in vertebrates. Second, its brain presents well-separated areas which are easier to dissect relative to that of many other fishes.

Several early studies have been performed on the European eel using the FIF method (Lefranc et al., 1969, 1970; L'Hermitte & Lefranc, 1972; Fremberg & van Veen, 1977). Later, these histofluorescence

data were elaborated through the distribution of DA- (Roberts et al., 1989) and TH-immunoreactive nuclei (Kapsimali et al., 2000). Moreover, following the cloning of TH in this species, ISH has been performed to characterize the brain distribution of this enzyme (Weltzien et al., 2006; Dufour S. et al., unpublished data). Here, we summarize available literature data on the distribution of dopaminergic cells (Fig. 4) in this species (for details see: Roberts et al., 1989; Kapsimali et al., 2000). The nomenclature used follows the brain atlas of the Japanese eel (Mukuda & Ando, 2003).

Forebrain: eels have well-developed *olfactory bulbs* (Ob), and numerous dopaminergic cells with short processes are located in the external cellular layer, while a few cells with longer processes can be found in the internal glomerular layer. In the *telencephalon* (Tel), a high number of DA cells are found in the ventral area (Va), while fewer cells are found along the anteroposterior axis. These nuclei form a distinctive mediolateral band across each hemisphere from the rostral to the caudal telencephalon until the anterior commissure. This organization is a common feature among vertebrates.

Diencephalon: in the *preoptic area* (POA), DA cells were found in the posterior parvocellular preoptic nucleus (PPp) and in the suprachiasmatic nucleus (SC). Moreover, like in goldfish (Kah et al., 1987), neurons have been observed in the antero-ventral preoptic nucleus (NPOav) and projecting to the proximal pars distalis of the pituitary (Vidal et al., 2004; Weltzien et al., 2006). NPOav is involved in the control of reproduction (see Paragraph 4). In the *thalamus* few TH-ir cells are present in the ventromedial nucleus (Kapsimali et al., 2000) although Roberts et al. (1989) did not report DA-ir cells in this region. In the *pretectum* neither DA- nor TH-ir nuclei have been found. Nevertheless, dopaminergic cells have been reported in the pretectum of other teleosts (Meek, 1994) as well as in reptiles and birds. In the *periventricular posterior tuberculum* (TPp), strong DA- and TH-immunoreactivity was found. In the *hypothalamus*, DA cells formed two groups: one located in the dorsal periventricular hypothalamic nucleus and another associated with the paraventricular organ (PVO). These last neurons line the ventricular walls and extend short, club-like processes to the ventricle. Such cells are DA-ir but not TH labeled, and they are thought to accumulate CAs (presumably DA) from the CSF of the third ventricle. The PVO is considered as a primitive feature (for review: Smeets and Gonzalez, 1990) common to teleost fishes (goldfish: Hornby et al., 1987; zebrafish: Rink & Wulliman, 2001; various species: Bradford & Northcutt, 1983) and other anamniotes (*Rana nigromaculata*, *R. catesbeiana*: Nakai et al., 1977).

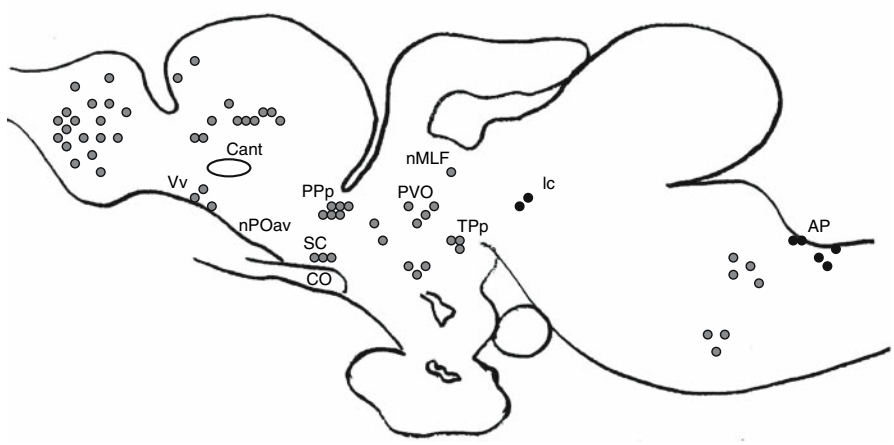

Fig. 4 Schematic organization of dopaminergic systems in the European eel brain. *Abbreviations*: AP, area postrema; Cant, anterior commissure; CO, optic chiasm; lc, locus coeruleus; nMLF, nucleus of medial longitudinal fascicle; Ob, olfactory bulbs; PPp, posterior parvocellular preoptic nucleus; PVO, paraventricular organ; Vv, ventral nucleus of the ventral telencephalic area; SC, suprachiasmatic nucleus; TPp: periventricular posterior tuberculum. Gray points: dopaminergic neurons (DA-ir/TH-ir); dark points: hypothetic noradrenergic neurons

Mesencephalon: in the eel both TH- and DA-ir cells were found in the dorsal tegmentum. This may be associated with the nucleus of the medial longitudinal fascicle (nMLF; Roberts et al., 1989; Kapsimali et al., 2000). In contrast, a dopaminergic group is usually described in the ventral mesencephalon of mammals, but its presence is submitted to great variation among vertebrates and even within the same vertebrate class. In this way, studies have not revealed TH- and DA-ir cells in the ventral mesencephalon either in the eel (Roberts et al., 1989; Kapsimali et al., 2000) or in other teleosts (Smeets & Gonzalez, 2000; Rink & Wullimann, 2001). In contrast, this ventral mesencephalic group was evident in the lungfish *Protopterus* (Sarcopterygian) (Reiner & Northcutt, 1987).

Rhombencephalon: DA-ir cells were found rostrally in the *locus coeruleus* (lc), whereas four other groups were defined caudally. Numerous cells are found in the *area postrema* (AP) at the border between the hindbrain and the spinal cord. Although few immunocytochemical studies have been made for NA in fish (e.g. three-spined stickleback, *Gasterosteus aculeatus*: Ekström et al., 1986), it is generally accepted that the neurons in the lc contain mostly NA, whereas the more caudal groups may also contain A, DA, or L-DOPA as end product. This view is supported by immunocytochemical localization of enzymes involved in NA synthesis (zebrafish, *Danio rerio*: Kaslin & Panula, 2001; *Apteronotus leptorhynchus*: Sas et al., 1990). No NA-producing cell bodies have been reported rostral to the lc in fish. In conclusion, studies in the eel show an organization of the dopaminergic systems basically similar to that of other teleostean fishes like rainbow trout and goldfish (Meek & Nieuwenhuys, 1998.), zebrafish (Ma, 1994, 1997, 2003; Wulliman et al., 1996), and medaka (*Oryzias latipes*; Kapsimali, Vernier et al., unpublished data). The CA distribution in brains of various bony fishes is reviewed by Meek (1994). The organization of CAs has also been studied in chondrostean fishes (Adrio et al., 2002) and cartilaginous fish (Meredith & Smeets, 1987). The CA organization as well as their metabolic and catabolic pathways seems highly conserved in vertebrates. An overview on the catecholaminergic system, including its organization and functional properties among vertebrates, has been recently published (Smeets & Gonzales, 2000).

Dopaminergic activity

The CA contents in the European eel have been investigated by various methods. Seasonal and circadian variations in the CA amount have been first studied using the FIF method (Popek, 1983) or radioimmunoenzymatic labeling (Le Bras, 1984). These authors reported that the CA content was generally higher during the light phase compared to the dark phase (Popek, 1983; Le Bras, 1984). In addition, modifications of acclimatized temperature (and season) induced some variations in CA content, as increases of DA and A and a decrease of NA have been shown when the water temperature increases (15–25°C) (Sébert et al., 1984).

Catecholamine content in eel brain (HPLC assay)

HPLC measurements of DA and DOPAC in the eel indicated variations at different stages of the biological cycle: DA and DOPAC contents in the olfactory bulbs were significantly higher in silver eels (migratory stage) compared to yellow eels (growth stage). However, the DOPAC/DA ratio, reflecting DA turnover, did not change (Giorgi et al., 1994).

We have recently assayed by HPLC various CAs (DA, NA, A) and DA metabolites (DOPAC, HVA) (for details, see Section "Methodological approaches of dopaminergic system investigations") in different brain regions as well as in the pituitary (Fig. 3) of female silver eels. The results (Sebert M. E. et al., unpublished data) are shortly reported below.

Dopamine Highest levels of DA (Fig. 5a) were found in the diencephalon (200 ± 10 ng/g wet tissue; average ± SEM) and olfactory bulbs (155 ± 33 ng/g tissue), while lowest levels were found in the cerebellum (16.1 ± 3.6 ng/g tissue). Intermediate DA levels were found in the rest of brain: the part including the telencephalon and preoptic area, the medulla oblongata, and the pituitary. These results are in agreement with those previously found in this species (Giorgi et al., 1994). In addition, a similar differential distribution of DA content through the brain is observed in other teleost species. Thus, generally highest DA contents are found in the telencephalon (including olfactory bulbs) and the diencephalon (crucian carp: Nilsson, 1989; goldfish: Dulka et al., 1992).

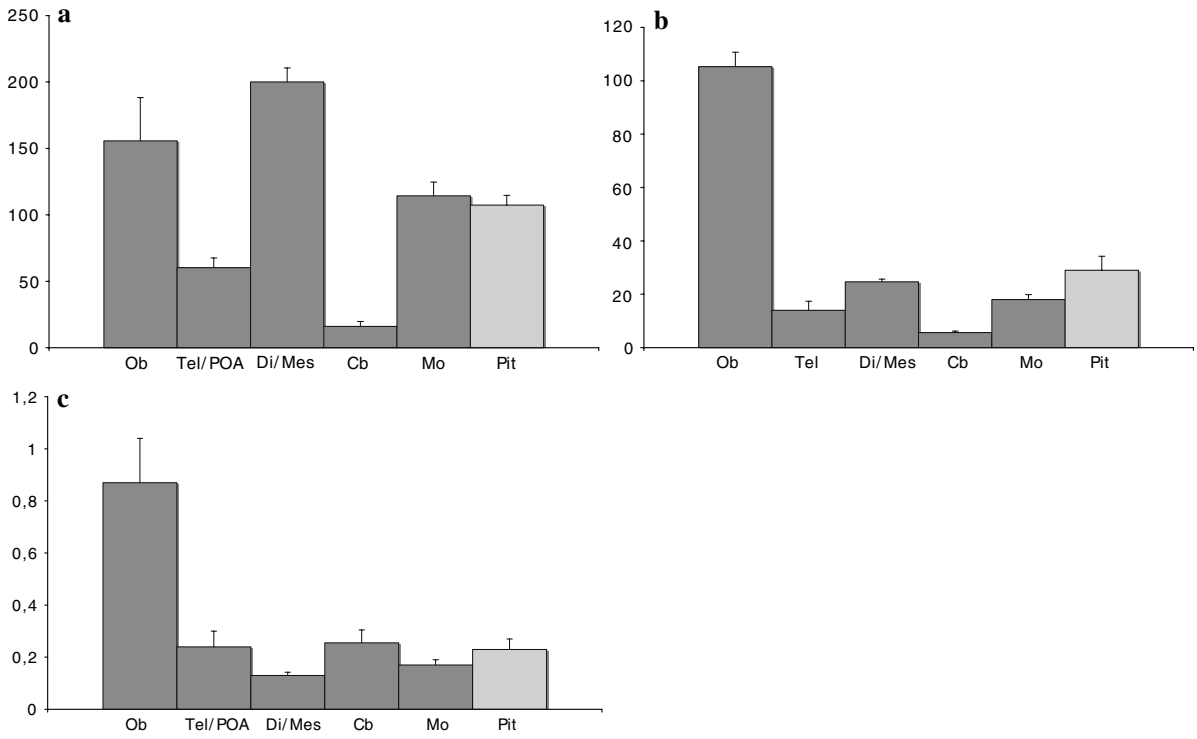

Fig. 5 Dopamine and its metabolite DOPAC contents in the brain and pituitary of the European eel assayed by HPLC. (**a**) Dopamine content; (**b**) DOPAC content; (**c**) DOPAC/dopamine ratio as indicator for dopamine turnover. Brain parts of female silver eels were dissected as shown in Fig. 3. Dopamine and DOPAC concentrations are expressed as ng/g brain tissue. Means are given ± S.E.M. ($n = 8$ eels). *Abbreviations*: Ob, olfactory bulbs; Tel/POA, telencephalon/preoptic area; Di/Mes, diencephalon/mesencephalon; Cb, cerebellum; Mo, medulla oblongata; Pit, pituitary

The high DA content in the olfactory bulbs may be illustrated by immunocytochemical data as shown using antiserum against DA (Roberts et al., 1989). The olfactory bulbs contain numerous cells that are highly TH- and DA-immunoreactive. A majority of these cells are interneurons with DA being synthesized and used locally which is reflected by the high DA contents.

In the diencephalon, elevated DA contents are partly explained by the richness of TH- and DA-ir projections that cross the diencephalon coming from the forebrain and the hindbrain. Moreover, the CSF-contacting cells located in the PVO in the hypothalamus are DA-ir but not TH-ir (Roberts et al., 1989; Kapsimali et al., 2000) indicating that the diencephalon contains cells that take up and concentrate DA from the CSF.

Dopamine metabolites In this study DOPAC and HVA, both DA metabolites, have been assayed, whereas previous works only reported DOPAC content. Our study demonstrates for the first time that DOPAC is the major metabolite of DA in all brain parts of the eel. Large concentrations of DOPAC (Fig. 5b) were found in the olfactory bulbs (105.3 ± 5.4 ng/g tissue). In the other brain areas, DOPAC contents were low with almost undetectable levels in the cerebellum (<10 ng/g tissue). In contrast, HVA was only detectable in the olfactory bulbs (70.5 ± 28.5 ng/g tissue, data not shown).

The DOPAC/DA ratio (Fig. 5c), often used as an estimate of dopaminergic activity (Cooper et al., 1986), differed between brain areas in accordance with previous studies (Giorgi et al., 1994). The DOPAC/DA ratio was four-fold higher in the olfactory bulbs (0.87) compared to other brain areas and the pituitary. This difference would be even higher if the olfactory bulb HVA content was taken into consideration to approximate the DA turnover (HVA + DOPAC/DA). As shown by immunocytochemical data, the olfactory

bulbs contain numerous DA cells with short processes, with HVA and DOPAC being produced in an abundant and local manner, in agreement with the high metabolites/DA ratio. In the diencephalon, high levels of DA but low levels of DOPAC were found. In fact, a large part of the diencephalic DA content is due to the richness of dopaminergic fibers in this brain region. However, a majority of these fibers only cross the diencephalon, while their terminals are in projecting areas outside the diencephalon. Moreover, in rainbow trout it has been suggested that the low DOPAC/DA ratio observed in the diencephalon may be related to a low activity of diencephalic neurons themselves. Indeed, treatment with a TH inhibitor did not significantly decrease DA levels in the trout diencephalon (Linard et al., 1996).

Noradrenaline and adrenaline Noradrenaline was found in different brain regions of the eel (Fig. 6), notably in the telencephalon and the diencephalon. In rainbow trout, the highest NA levels were also found in the telencephalon, the POA, and the hypothalamus (Linard et al., 1996). Few NA immunocytochemical studies have been done in fish (three-spined stickleback: Ekström et al., 1986; elephantnose, *Gnathonemus-petersii*: Meek et al., 1993). One can also "indirectly" localize NA-ir neurons by immunocytochemistry using antibodies against DBH. In this way cells that are DBH-ir cells may contain A or NA as end product. If CA assay has revealed low or undetectable A contents these DBH-ir cells may be considered as noradrenergic. This indirect approach has been used in several species (*Apteronotus leptorhynchus*: Sas et al., 1990; goldfish: Hornby et al., 1990; sturgeons, *Acipenser baeri*, *Huso huso*: Adrio et al., 2002).

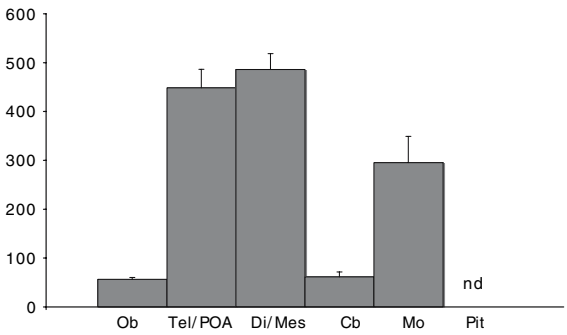

Fig. 6 Noradrenaline content in the brain and pituitary of the European eel brain assayed by HPLC. Brain parts of female silver eels were dissected as shown in Fig. 3. *Abbreviations*: See Fig. 5

In fish it seems that noradrenergic cell bodies are confined to the hindbrain with projections to the forebrain. The presence of noradrenergic fibers and terminals throughout the brain explains why we found a high content of NA in the telencephalon and the diencephalon. In spite of the absence of noradrenergic cell bodies, DA and NA as neurotransmitters would be present in both telencephalon and diencephalon.

The presence of A in the fish brain remains unclear. In our study, A levels were very low or undetectable (<10 ng/g tissue, data not shown). However, previous studies reported that A represented approximately 10% of total CA content in the European eel brain (Le Bras, 1984; Sébert et al., 1984, 1986). The fact that our assay was performed in smaller samples-brain dissected in five parts-may explain our difficulty to measure A contents. The presence of A is largely debated in goldfish (Baumgarten, 1972; Juorio, 1973), while A was not found in crucian carp (Nilsson, 1989, 1990). In addition to these divergent results, mapping of A-ir neurons has not been performed in fish because antibodies against A are not available yet.

Tyrosine hydroxylase expression assayed by quantitative real-time RT-PCR

Recently, the regional TH mRNA expression in the eel brain (Fig. 7) has been described using a newly developed quantitative real-time RT-PCR assay (Weltzien et al., 2005). TH mRNA expression was considerably higher (×4) in the olfactory bulbs relative to the other brain regions. This result is in good agreement with the cartography of TH-positive cell bodies by in situ hybridization and immunocytochemistry (see Section "Cartography of dopaminergic systems"). Specifically, numerous TH-labeled cells are found in the olfactory bulbs (Weltzien et al., 2006). This high expression of TH, the rate-limiting enzyme of DA synthesis, also concords with HPLC measurements of DA and its metabolites (Fig. 5). Indeed, high levels of DA as well as DOPAC in the olfactory bulbs suggest an intensive dopaminergic activity in this region. In the other brain areas, as discussed above, fewer DA cell bodies are present relative to those in the

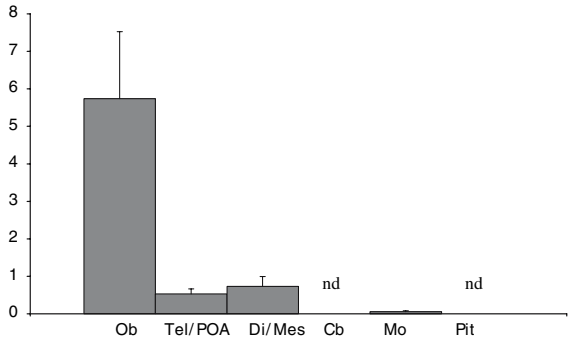

Fig. 7 Distribution of TH messenger RNA in the brain of European eel. TH messenger RNA levels were assayed by qrtRT-PCR and expressed relative to messenger RNA levels of a reference gene: ARP (acidic ribosomal phosphoprotein P0). Brain parts of female silver eels were dissected as shown in Fig. 3. Results are given ± S.E.M. ($n = 7$ eels). *Abbreviations*: See Fig. 5

olfactory bulbs. This explains the observed lower expression of TH in these areas.

Dopamine involvement in reproductive function

Neurohormonal control of reproduction in teleosts

In mammals, the reproductive function is under a complex neurohormonal control ensured by the brain-pituitary-gonad (BPG) axis. Gonadotropin-releasing hormone (GnRH), a hypothalamic decapeptide, stimulates the gonadotropic cells located in the proximal pars distalis (PPD) of the pituitary. The gonadotropes synthesize and release two gonadotropins (GtHs): luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Weiner et al., 1989). GtHs released in the general circulation act on the gonads and control the different steps of gonadal development (Fig. 8).

In fishes, GnRH plays a similar stimulatory role together with other factors like γ-amino butyric acid (Trudeau et al., 1993), neuropeptide Y (Peng et al., 1993), NA (Chang & Peter, 1983a, b), serotonin (Somoza & Peter, 1991), and glutamate (Trudeau et al., 2000). Unlike tetrapods, fishes are devoid of a hypophysial portal system. Instead, GnRH reaches the endocrine cells of the pituitary directly through hypothalamic nerve fibers (Ball, 1981) where GnRH binds to specific high-affinity receptors (for review: Kah et al., 2004). Moreover, in teleosts, GtHs may be submitted directly to a negative control by DA (Fig. 8; for review: Dufour et al., 2005) as first evidenced in goldfish (Peter et al., 1988). In maturing fishes, DA is involved in the control of spermiation, ovulation, and spawning (for review: Dufour et al., 2005). This role of DA does not seem linked with phylogenetical position within teleosts as it is observed in some species (salmonids, rainbow trout: Saligaut et al., 1999; cyprinids, carp: Lin et al., 1988; perciform, tilapia: Yaron et al., 2003), but not in others (seabream: Zohar et al., 1995). Moreover, when it is observed, the intensity of DA inhibition can be more or less pronounced according to species (for review: Dufour et al., 2005).

DA implicated in the control of pituitary function is secreted by neurons located in the NPOav in the preoptic area (Peter & McKeown, 1974; Vidal et al., 2004). These neurons project directly to the pituitary PPD (Kah et al., 1984, 1987; Anglade et al., 1993) where they release DA that acts on gonadotropes through D2-like receptors (Chang & Peter, 1983a, b; Chang et al., 1984; Omeljaniuk et al., 1987). The DA action on the gonadotropes may be direct or indirect. Indeed, DA inhibits basal and GnRH-induced LH release and acts on the GnRH intracellular signaling pathway (Peter et al., 1986, 1991; De Leeuw et al., 1986; Yu and Peter, 1992, Yaron et al., 2003). Moreover, DA may modulate pituitary sensitivity to GnRH by decreasing the number of GnRH-receptors in goldfish, catfish, and tilapia (De Leeuw et al., 1986, 1988; Omeljaniuk et al., 1989; Levavi-Sivan et al., 2004).

Dopamine involvement in the control of eel reproduction

In contrast to the DA inhibitory control of final maturation in teleosts, relatively few studies have addressed the possible role of DA in the inhibitory control of puberty. The few studies in juveniles showed no evidence for DA involvement in puberty. For example, administration of GnRH agonist and steroids was sufficient to induce precocious sexual maturation in rainbow trout (Crim & Evans, 1983). Treatment with a GnRH agonist alone or in combination with a DA antagonist led to the same

conclusion in two percomorphs (striped bass, *Morone saxatilis:* Holland et al., 1998; seabream, *Pagrus major*: Kumakura et al., 2003).

In contrast, preliminary data from Marcano and coworkers indicated that dopaminergic activity (DOPAC/DA ratio) decreases in hypothalamus at puberty in the Atlantic spadefish (*Chaetodipterus faber*; Marcano et al., 1995). Although this work apparently has never been followed up, the results suggest that decreased DA release was a cause of increased GtH release at the onset of puberty in female spadefish.

The involvement of DA in the control of puberty has been studied extensively in our laboratory using the eel as a model. In silver eels, only a combined treatment with GnRH agonist, DA antagonist (pimozide) and also pretreatment with sex steroids induces a significant increase of both LH synthesis and release as well as increased plasma vitellogenin levels (Dufour et al., 1988; Vidal et al., 2004). These results explain that the lack of pubertal development in the non-migratory silver eel has a dual origin: a deficit of GnRH stimulation and a strong DA inhibition (for review: Dufour et al., 2003). When and how the DA inhibition decreases to permit gonadal recrudescence remains enigmatic. In fact, maturing eels are not available in nature because they leave continents at the prepubertal silver stage and have never been caught after this stage. Improved knowledge of the regulation of dopaminergic activity, especially in relation to pubertal development, is of crucial importance, both as a fundamental biological question and also for development of eel aquaculture. This latter aspect has a dual objective: human food production and restocking or reducing the pressure on wild eel populations (Stone, 2003; Wirth & Bernatchez, 2003).

Regulation of dopamine inhibition of eel puberty and perspective works

Internal factors

Among internal factors that are likely to regulate dopaminergic activity, sexual steroids require special interest. Sexual steroids have been shown to exert both negative and positive feedbacks on the BPG axis, e.g. acting on GnRH and GtH synthesis and release (Montero & Dufour, 1996; Schmitz et al., 2005). Recently, their possible effects on the dopaminergic component of the BPG axis have been studied. Using ISH and qrtRT-PCR, an androgen-specific-positive effect on TH expression in the preoptic area in female silver eels was revealed (Weltzien et al., 2006). This suggests that sexual steroids could be involved in modulating the DA inhibitory tone during pubertal development, similar to what has been previously demonstrated during final maturation (female rainbow trout: Linard et al., 1995; Saligaut et al., 1999; Vacher et al., 2002; male goldfish: Dulka et al., 1992). This androgen specificity has not been described in other fish and may be related to species or reproductive stage (Weltzien et al., 2006).

Because other hormones like corticosteroids (Huang et al., 1999) and thyroid hormones (Volkoff, 1999; Pavlidis et al., 2000) are thought to interfere with the reproductive function, it would be interesting to investigate their effects on DA activity in the eel.

Environmental factors

As sexually mature eels have never been observed in natural conditions, it is supposed that silver eels undergo maturation during their reproductive migration and/or at their breeding area. Physiological (Kleckner, 1980; Sébert, 2003) and anatomical (Pankhrust, 1982; Braekevelt, 1988) predispositions as well as tracking studies (Tesch & Rohlf, 2003) indicate that, after leaving coastal waters, silver eels swim at great depth. Among environmental factors linked to a migration at depth, high hydrostatic pressure may be determinant for stimulation of sexual maturation and reproduction. Previous experiments with eels immersed in cages at specific depths showed a significant increase in pituitary LH content and gonadosomatic index (Fontaine et al., 1985). We are currently performing short- and long-term immersion of female and male silver eels, using the hyperbaric chamber with a water-recirculating system set up by Sébert and coworkers at UBO (Brest, France; Sébert et al., 1990). The effects of immersion on dopaminergic activity and other components of the BPG axis will be examined.

The dopamine involvement in the reproductive migration

Dopamine and locomotion

Silver eels are not sexually mature when they leave the continental shelf to reach their breeding area. The European eel swim up to 6,000 km before arriving in the Sargasso Sea, their supposed spawning area (Schmidt, 1923). Even though eels stop feeding at silvering, they demonstrate a striking swimming efficiency that has been recently reported to be 4–6 times higher than for other non-anguilliform fish (Van Ginneken, et al., 2005). The physiological mechanisms, including central regulations ensuring such swimming capacity, remain unknown and require attention.

In mammals, the locomotor function is well studied, and it is established that the neurotransmitters DA, NA, and serotonin are involved in this function. The richness of dopaminergic innervations in the mammalian midbrain was investigated several decades ago (McGeer et al., 1978). The importance of this region for coordination of locomotion was later demonstrated (Björklund & Lindvall, 1984) due to an involvement in various human pathological processes, including Parkinson's disease (Mason, 1984). Central in this respect is the mesostriatal sensorimotor system that consists of DA cells located in the substantia nigra (SN), projecting to the striatum (basal telencephalon). It has also been shown that DA exerts its role in locomotion through D1 and D2 receptors, both located in the striatum.

Recently, Rink and Wulliman have shown in zebrafish that dopaminergic neurons in the periventricular nucleus of the posterior tuberculum (TPp), homologous to the mammalian SN, ascend to the dorsal nucleus of area ventralis in the basal telencephalon (Rink & Wulliman, 2001). These authors identified the area ventralis as the striatum. These data on the similarity in location and distribution of the dopaminergic cells in fishes with those of mammals suggested a basic feature of brain organization for the control of locomotor function.

In eels, immunocytochemical studies have shown TH-ir cells in the TPp (Kapsimali et al., 2000). Furthermore, high expression of D1 receptors has been shown in the dorsal and the ventral nuclei of the area ventralis in the telencephalon (Kapsimali et al., 2000). However, whether neurons in the TPp project effectively to the telencephalon must be confirmed in the eel.

Various experimental studies support the anatomical data to confirm the involvement of DA in locomotor function in fish as in mammals. For example, L-DOPA, a DA precursor (Fig. 1), enhances tail beat frequency and swimming speed in the European eel (Doyle & Roberts, 2004). L-DOPA also induced dominance in the Arctic charr (Salmonid), while high doses led to dyskinesia (Winberg and Nilsson, 1992). Administration of dopaminergic drugs like apomorphine, a DA agonist, stimulates swimming in *Oreochromis niloticus* and *O. Mossambicus* (Cichlids), and this effect is abolished by D1-antagonist (Mok & Munro, 1998). Apomorphine has been reported to modulate swimming and behavior in lampreys (Kemnitz et al., 1995).

Besides this direct implication of DA in locomotion, experiments show that DA may mediate the effect of various endocrine factors. In juvenile rainbow trout, the administration of GH increased swimming activity (Jönsson et al., 2003; Johansson et al., 2004) and dopaminergic activity (DOPAC/DA ratio) in both telencephalon and hypothalamus (Johansson et al., 2004). Moreover, the GH-stimulatory effects on swimming were abolished when fishes were treated with GH and D1 dopamine antagonist (Johansson et al., 2005), leading to the conclusion that DA is implicated in GH-induced swimming in trout.

Thyroid hormones are proposed to play a role in migratory behavior in salmonids (for review: Iwata, 1995, Specker et al., 2000) and cod (Comeau et al., 2000), and also in birds (Pathak & Chandola, 1984; Pant & Chandola-Saklani, 1993). Moreover, enhanced locomotor activity has been correlated with elevated thyroid hormone levels in American eels, *A. rostrata* (Castonguay et al., 1990), and most recently in *A. anguilla* glass eels (Edeline et al., 2005). Chaube and Joy have demonstrated in catfish *H. fossilis* that thyroid hormones modulate TH activity (Chaube & Joy, 2003b), which may suggest that DA could mediate thyroid hormone effects on migration as it may be the case for GH-induced swimming (see the above paragraph).

Further studies should aim at investigating the possible role of DA in the signaling pathways of endogenous factors (GH, thyroid hormones, and other

factors such as sexual steroids, cortisol, etc.), leading to the striking swimming capacity observed in silver eels. The role of dopaminergic activity in eel swimming could be investigated through swim trials performed using the swim tunnel system developed by Van den Thillart and coworkers (University of Leiden).

Dopamine and olfaction

Regulation of DA in the olfactory bulbs needs also attention because olfaction plays a major role in fishes for feeding (for review: Hara, 1994), reproduction, and migration (for review: Doving and Stabell, 2003). Like salmonids, eels return to their birth area for breeding, but the mechanisms of this homing behavior remain to be discovered. Various experiments suggest that adult salmons use olfaction in finding their way back to their original streams (for review: Doving and Stabell, 2003). In eels, olfaction has been shown to play a critical role in orientation during their spawning migration. Westin (1990) reported that silver eels, originating from glass eels imported from France for stocking in Sweden, showed a lower swimming speed as compared to indigenous silver eels, and the imported eels also failed to find their way out of the Baltic Sea. Barbin and coworkers (1998) tracked anosmic and control silver American eels during their estuarine migration. Compared to controls, the anosmic fishes showed longer migration times, and many of them did not leave the estuary. Because other relevant environmental cues were limited (no gradients of salinity and temperature, low electric-field, etc.), these eels seemed to use mainly the olfaction for orientation (Fig. 8).

In mammals, studies have shown that DA release in the olfactory bulbs is enhanced during learning, and DA antagonists inhibit olfactory memory formation (Coopersmith et al., 1991; Hsia et al., 1999; Davila et al., 2003; Pavlis et al., 2006). Available experimental data indicate that DA may also modulate the discrimination of olfactory input (Nowycky et al., 1983; Wilson et al., 1995; Hsia et al., 1999; Ennis et al., 2001).

As for locomotion, DA may take part in an intricate neurohormonal network that controls olfaction in fish. Thyroid hormones would be involved in homing in salmon (for review: Dittman & Quinn, 1996) and affect DA turnover in the olfactory bulbs (Morin et al., 1997). Besides the involvement of olfaction in homing this sensory function is required at the last step of reproduction. The detection of pheromones in water is crucial during the breeding phase to promote spawning success (goldfish: Demski & Dulka, 1984). Miranda and coworkers have recently demonstrated that male tilapia are able to discriminate between females of different reproductive stages (Miranda et al., 2005). Moreover, data from Bhatt and coworkers (2002) in a cyprinid, *Barilius bendelisis*, support this hypothesis that during the reproductive phase, pheromones as olfactive stimuli dominate over feeding stimuli to promote reproduction.

As silver eels are fasting, the olfactory dopaminergic system could be involved in two crucial final steps of the eel life cycle, namely, to reach the spawning area and during the actual spawning act. Our recent qrtRT-PCR studies showed a strong positive effect of androgens on TH expression in the silver eel olfactory bulbs, indicating that sex

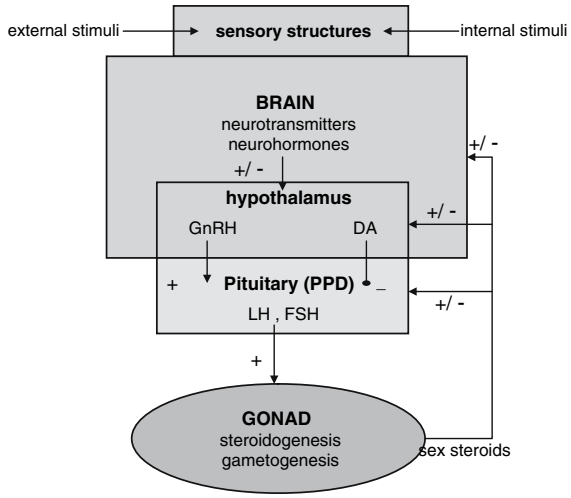

Fig. 8 Neurohormonal control of reproduction by the brain-pituitary-gonad (BPG) axis. Brain GnRH (gonadotropin-releasing hormone) stimulates the synthesis and release of pituitary gonadotropins (LH, luteinizing hormone; and FSH, follicle stimulating hormone), which activate gonadal activity (gametogenesis and steroidogenesis). An additional brain control is exerted in some teleosts by DA, which may inhibit the synthesis and release of gonadotropins. Sex steroids exert positive and negative feed at different levels of the BPG axis. Environmental and internal stimuli as sex steroids are integrated by the central nervous system and lead to modulation of the activity of GnRH and DA neurons

steroids may modulate DA mediation of olfactory processes in the eel (Weltzien et al., 2006). Further experimental studies, on artificially matured female and male eels, are necessary to investigate the role of DA in the mediation of pheromone effects, spawning behavior, and gamete release.

Conclusion

Dopamine has been shown to inhibit eel pubertal development and may also control the last steps of reproduction as in some other adult teleosts. In addition, DA is likely involved in locomotion and olfaction in fish as in mammals, functions implicated in reproductive migration and spawning. The recent development of various methodological tools allows investigation of dopaminergic systems in the eel. Anatomical data obtained by ISH and immunocytochemistry show that the organization of dopaminergic systems in the European eel is relatively similar to those of other fishes. Moreover, this organization is well conserved among vertebrates. Quantitative methods (HPLC, qrtRT-PCR) allow evaluation of variations in DA activity, and current studies investigate the effects of endogenous and environmental factors on these systems. A better understanding of the role and regulation of DA systems will contribute to gain basic and applied knowledge necessary to reproduce the eel life cycle in captivity and allow stock renewal.

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