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Mast cell population in the frog brain: distribution and influence of thyroid status

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SUMMARY

In the developing frog brain, the majority of mast cells (MC) are distributed in the pia mater, and some immature MC are located adjacent to the blood capillaries in and around the neuropil. In the adult brain, MC are more numerous than in pre- and pro-metamorphic tadpoles; they are mainly located within the pia mater and are particularly numerous in the choroid plexuses. Many MC are found within the brain ventricles juxtaposed to the ependymal lining. MC are rarely observed in the brain parenchyma. In the adult brain, MC number is much higher than in the brain of post-metamorphic froglets. In the latter, MC number is nearly 2-fold over that found in the pre-metamorphic brain. Treatment of pre- and pro-metamorphic tadpoles with 3,5,3′-triiodothyronine (T_3) and thyroxine (T_4) stimulates overall larval development but does not induce a significant change in MC population within the brain. By contrast, treatment with 6-n-propyl-2-thiouracil (PTU) delays larval development and leads to a significant numerical increase of brain MC. In the adult, PTU treatment also has a similar effect whereas hypophysectomy causes a drastic decrease of MC population. The negative effects of hypophysectomy are successfully counteracted by a two-week replacement therapy with homologous pars distalis homogenate. In the adult frog, MC population seems to be refractory to thyroid hormone treatment. The present study on frog brain suggests that pituitary–thyroid axis may be involved in the regulation of MC frequency.

Key words: mast cells, amphibia, brain, thyroid hormones, pituitary.

INTRODUCTION

Mast cells (MC) have been described intracranially, in the dura mater, leptomeninges and choroid plexuses in mammals (Dropp, 1972; Dropp, 1976; Theoharides, 1990; Michaloudi and Papadopoulos, 1999) and birds (Silverman et al., 1994; Silver et al., 1996; Zhuang et al., 1996; Silverman et al., 2002). They are also reported in the parenchyma of rat brain, particularly in the thalamus with a predominantly perivascular location (Goldschmidt et al., 1985; Dimitriadou et al., 1990; Manning et al., 1994). In the medial habenula of ring doves, MC are known to occur with a particularly high frequency and here they lie in close contact with neuronal and glial elements (Zhuang et al., 1993; Wilhelm et al., 2005). The secretory products of MC are released in the central nervous system (CNS) and can alter the function of both neural and vascular elements (Esposito et al., 2001; Wihelm et al., 2005; Khalil et al., 2007).

Some studies have established that brain MC population is not static in the sense that it is influenced by the developmental, physiological or behavioral status of the animal (Dropp, 1972; Lambracht-Hall et al., 1990; Gill and Rissman, 1998; Zhuang et al., 1999; Wilhelm et al., 2000). However, the mechanisms by which the size of MC population in the CNS is regulated are far from settled. In the musk shrew, a primitive eutherian mammal, MC are numerous in the brain during postnatal development but only rarely are MC observed in the adult brain (Gill and Rissman, 1998). In the rat brain, during postnatal development, MC migrate from the pia mater into their adult positions in the thalamus (Persinger, 1981; Lambracht-Hall et al., 1990). In the hedgehog brain, MC number increases during hibernation (Flood and Krüger, 1970) whereas in the rat brain, MC number declines in response to physiological stress (Theoharides et al., 1995) or handling (Persinger, 1983). Gonadotropin-releasing hormone immunoreactivity has been observed in MC situated in the habenula of the ring dove brain (Silverman et al., 1994; Silverman et al., 2002; Khalil et al., 2003); these cells increase in number rapidly during courtship but are reduced drastically in number following gonadectomy (Zhuang et al., 1993). The magnitude of MC increase can reach a 10-fold level following exposure to either testosterone or dihydrotestosterone in the male and to 17β-estradiol in the female (Wilhelm et al., 2000). It is interesting to note that MC isolated from human bladder contain estrogen receptors (Pang et al., 1995), and that MC secretory activity in rats is sensitive to estrogen and progesterone (Vliagoftis et al., 1990; Vliagoftis et al., 1992). Nevertheless, the vast 'popularity' of MC in the mammalian body is attributable to their important role in the immune response.

Some old papers indicate that thyroid hormones (TH) could affect MC of various tissues (Constantinides and Rutherdale, 1954; Wegelius and Asboe-Hansen, 1956; Santini, 1962). In the neonatal rat brain, administration of an anti-thyroid agent, propylthiouracil, increases MC number whereas treatments with TH or thyrotropic hormone (TSH) up to five days of age lead to a decrease in brain MC number (Sabria et al., 1987). That thyroid status can have a role in MC population dynamics is further strengthened by results such as a higher MC frequency in the bone marrow of hypothyroid rats as compared with that in euthyroid, thyrotoxic or hypothyroid-thyroxine-replaced animals (Siebler et al., 2002); this is supported by the expression of 3,5,3′-triiodothyronine (T_3) receptor α1 (TRα1) in the nucleus and cytoplasm and of TRα2 and TRβ1 in

the cytoplasm of rat skeletal MC. A recent study has demonstrated that rat and mouse MC, together with other cells of the immune system, contain T_3, and that MC T_3 concentration seems to be regulated by TSH (Csaba and Pállinger, 2009). These facts point to TH as possible candidates playing a regulatory/modulatory role in MC dynamics.

The founding stone for this line of investigation in non-mammalian vertebrates was laid by our recent preliminary data, which indicates that thyroid status does influence MC population in the frog brain (Chieffi Baccari et al., 2009). The aim of the present study is to provide a detailed neuroanatomical map of MC distribution in the brain of the adult frog *Rana esculenta* and to investigate further the influence of thyroid status on brain MC population in the adult as well as during development. The amphibian larval brain is a major target for TH with a wide variety of changes taking place during metamorphosis in both its anatomical and functional characteristics (for a review, see Tata, 2006). Circulating levels of TH are relatively low during pre-metamorphosis; they increase gradually during pro-metamorphosis and metamorphic climax and then decrease in adult frogs (Regard et al., 1978; Weber et al., 1994). In the present study, we have examined the effects of an anti-thyroid agent, 6-n-propyl-2-thiouracil (PTU), thyroxine (T_4) and T_3 on the brain population of MC in *R. esculenta* tadpoles (in pre- and pro-metamorphosis) and adults. Furthermore, to understand the involvement of pituitary gland, adult frogs were hypophysectomized and a pool of them received pars distalis replacement therapy.

MATERIALS AND METHODS

Larval stages and experimental groups

Tadpoles of *Rana esculenta* L. were collected over two breeding seasons (2006 and 2007) near Naples, Italy. They were maintained in plastic tanks at a constant temperature (16–18°C) and lighting (12 h:12 h light:dark cycle) and fed *ad libitum*. Two pools of tadpoles at pre-metamorphic (26–27, hindlimbs developing) and pro-metamorphic (29–30, forelimbs emerged) stages (Witschi, 1956) were used. Each pool of tadpoles was then divided in four groups of 25 tadpoles each: group 1, intraperitoneal (i.p.) injections of PTU (40 μg of PTU per 50 μl of Ringer solution per injection); group 2, i.p. injections of T_3 (0.2 μg of T_3 per 50 μl of Ringer solution per injection); group 3, i.p. injections of T_4 (0.2 μg of T_4 per 50 μl per Ringer solution per injection); group 4, control group received only the vehicle. Injections were given on alternate days for a week. To avoid a high rate of mortality, the doses were chosen on the basis of our preliminary experiments. The mortality rate under our experimental conditions was less than 10%. In addition, it was shown earlier that this dose range of PTU and T_3 is effective in the regulation of frog metabolic activity (Di Meo et al., 1995).

Five to eight tadpoles from each experimental group were anaesthetized by immersion in 1% solution of MS-222 (Sigma Chemical Co., St Louis, MO, USA) and killed. Heads were fixed for light microscopy as described below.

Adult animals and experimental groups

No sexual dimorphism was recorded either in the neuroanatomical distribution or in total number of MC in the adult brain. Consequently, we simply chose to use only adult males of *R. esculenta* (*ca*. 20 g body mass). These were collected from the same area as the tadpoles and were kept under similar conditions in the laboratory and fed *ad libitum* with mealworms. The treatments were as given below.

Hormonal and anti-hormonal treatment

Each experimental group was composed of 10 adult frogs. Treatment groups were as follows: (1) 40 μg of PTU per 100 μl of Ringer solution per dose; (2) 400 μg of PTU per 100 μl of Ringer solution per dose; (3) 0.2 μg of T_3 per 100 μl of Ringer solution per dose; (4) 2 μg of T_3 per 100 μl of Ringer solution per dose; (5) 0.2 μg of T_4 per 100 μl Ringer solution per dose; and (6) 2 μg of T_4 per 100 μl of Ringer solution per dose. The substances, dissolved in Ringer solution, were injected into the dorsal sac on alternate days for two weeks. Control frogs (N=10) were injected with the same volume of vehicle.

Hypophysectomy and replacement therapy

A batch of 40 adult males was hypophysectomized, and 10 frogs each were killed after one and two weeks. One day after hypophysectomy, the remaining 20 hypophysectomized males were injected with homologous pars distalis homogenate (an equivalent of 1 pars distalis per dose per frog: pars distalis was removed using a sterilized surgical kit and homogenized at 4°C in amphibian Ringer solution, pH 6.4) into the dorsal sac on alternate days, and animals were killed after one and two weeks. Ten sham-operated animals were used as control.

Frogs from each experimental group were killed 24 h after the last injection. They were anaesthetized by immersion in 1% solution of MS-222 (Sigma Chemical Co.) and then killed. The brains were rapidly dissected and fixed in Bouin's fluid.

All experiments were performed in accordance with local and national guidelines governing animal experiments.

Histology and histochemistry

Serial paraffin sections (transverse, 6 μm-thick) were stained with 0.2% Toluidine blue in Walpole buffer at pH 4.2 (Gabe, 1968). Acidic Toluidine blue reveals the metachromatic property of MC by binding to sulfated glycosaminoglycans in the granules (Enerbäck et al., 1986; Markey et al., 1989). Some sections were stained with Alcian blue/safranin (AB/safranin); brain sections were first stained in 1% AB in 3% acetic acid (pH 2.2) and followed by 0.5% safranin in HCl (pH 1.3), according to a previously described technique (Chieffi Baccari et al., 1998). The combination of these two dyes can distinguish granules containing low-sulfated glycosaminoglycans (AB-positive) from granules containing high-sulfated glycosaminoglycans (safranin-positive) (Enerbäck et al., 1986; Markey et al., 1989).

MC count and anatomical distribution

To assess MC number and distribution alternate sections of the entire brain from each experimental subject (a minimum of five tadpoles and four adult frogs per experimental group) were stained for Toluidine blue metachromasia. The total MC number per brain or brain area was obtained by simply multiplying the total number of brain sections by the mean number of MC per section.

Morphometric analysis consisted of digitization of transverse sections viewed under a Nikon Eclipse E600 light microscope, Melville, NY, USA) with an attached JVC TK-C1381 photocamera (Tokyo, Japan) connected to a Pentium II computer (Intel Corp., Assago, Italy) running Lucia ScMeas on Mutech software (Fairfield, NJ, USA). Cell counting was done twice, on the same set of slides, by two different investigators, and the mean of the two was used for statistical analyses. Keeping in mind the limited nuclear diameter range (5–7 μm) and a highly variable cell size (~20 μm spherical MC; ~50 μm elongated MC), our method of cell counting, which cannot rule out that the same MC may appear in two adjacent

sections, may tend to overestimate the number of MC per brain. Values of MC number per brain or brain area were compared by analysis of variance (ANOVA) for multi-group comparisons followed by Student's *t*-test for between-group comparisons. All data were expressed as means ± standard deviation. The level of significance was taken at $P<0.005$, $P<0.05$, $P<0.01$.

RESULTS

Effects of PTU, T_3 and T_4 treatment on brain MC population during development

In the brain of control tadpoles at stages 26–27 (pre-metamorphic), MC were observed, antero-posteriorly, in the pia mater around the olfactory–vomeronasal nerves complex, in adherence to the basement of the olfactory bulbs, in the medial septum as well as inside lateral and third ventricles juxtaposed to the ependymal cell lining. They were also present dorsally in the anterior choroid plexus in the vicinity of habenulae, in the rhombencephalon (in the fourth ventricle) and in the posterior choroid plexus (Pinelli et al., 2010). Only sporadically were some immature MC observed adhering to the blood capillaries within the brain parenchyma where they were never observed in association with neurons. Some of the MC were metachromatic with Toluidine blue and safranin-positive with AB/safranin, which indicated that the MC were mature cells as earlier described for the MC in other frog tissues (see Chieffi Baccari et al., 1998; Chieffi Baccari et al., 2003; Esposito et al., 2002).

Intraperitoneal administration of T_3 and T_4 accelerates larval development; just one week after T_3- and T_4-injections the pre-metamorphic tadpoles had reached the advanced pro-metamorphic stage overlapping with the beginning of climax (stages 30–31). Therefore, the MC population of these experimental groups was compared with a batch of control tadpoles at stages 30–31. At the same time, however, PTU administration delayed larval development because it inhibits the synthesis of TH by blocking the thyroid peroxidase activity (Oppenheimer et al., 1972; Leonard and Visser, 1986). Infact, PTU-injected tadpoles in the 26–27 stage group did not advance in their development but they all showed a significantly higher MC number (807±165 MC per brain section) as compared with the controls (309±75 MC per brain section) at the same stage of development (Fig. 1A). A numerical increase of MC occurred mainly in the diencephalon (including third ventricle and anterior choroid plexus) and mesencephalon (optic ventricles). In the PTU-treated tadpoles, brain MC population was composed of mature and immature cells. They were, in fact, either round or elongated in shape and showed different intensities of Toluidine blue metachromasia. When stained with AB/safranin, some MC contained only AB-positive granules, while some others contained a mixture of AB/safranin granules and still others contained only safranin-positive granules (Fig. 2A,B).

In control tadpoles at pro-metamorphosis (stage group 29–30), mature MC were very abundant in the forebrain. Here, they were distributed along the meningeal lining of the medial septum, inside the lateral ventricles, and many of them were associated externally with blood vessels, showing a clear bilateral symmetry in their distribution. MC were observed in the diencephalon, both externally in the pia mater and internally inside the third ventricle. Many were located within the anterior choroid plexus and some others were observed in the infundibular recess. Only a few MC were observed inside the mesencephalon near the cerebellum. In the rombencephalon, MC were present in the fourth ventricle and in the pia mater; numerous MC were observed in the posterior choroid plexus (Pinelli et al., 2010).

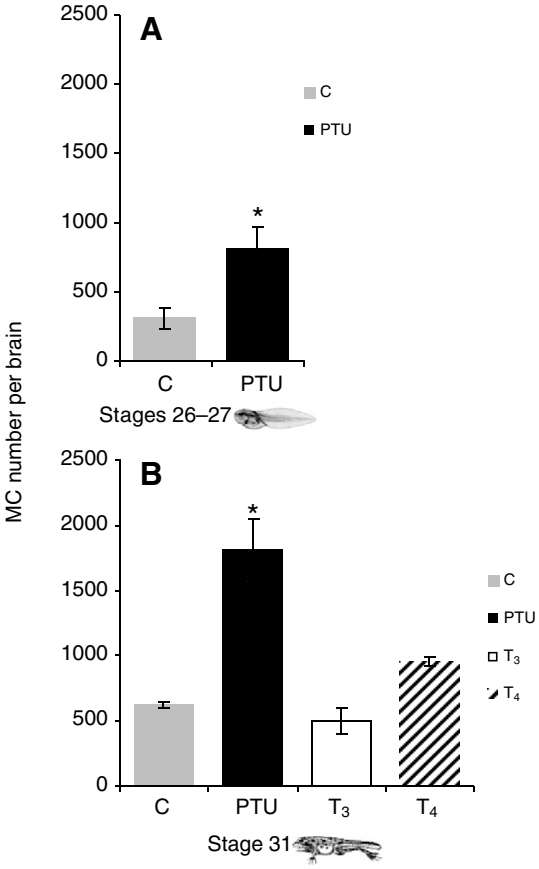

Fig. 1. (A) Mast cell (MC) number per brain in pre-metamorphic (stages 26–27) control tadpoles (C) and 6-n-propyl-2-thiouracil (PTU)-injected tadpoles (PTU); (B) MC number per brain at stage 31 (early metamorphic climax) in control tadpoles (C), PTU-injected tadpoles (PTU), 3,5,3′-triiodothyronine (T_3)-injected tadpoles (T_3) and thyroxine (T_4)-injected tadopes (T_4). Each bar represents the mean ± standard deviation. MC number in PTU-injected tadpoles at both stages 26–27 and stage 31 (early metamorphic climax) was significantly (*$P<0.01$) higher than the control.

Pro-metamorphic tadpoles (stage group 29–30) treated with T_3, T_4 and PTU for one week had all reached the early metamorphic climax (stage 31). The frequency of MC in PTU-treated tadpoles increased in all brain areas. Indeed, PTU-injected tadpoles had a significantly higher MC number (1812±240 MC per brain) in the brain as compared with that observed either in the control (628±27 MC per brain) or in the T_3-treated (507±98 MC per brain) and T_4-treated (952±36 MC per brain) tadpoles (Fig. 1B). No significant difference in brain MC number was observed between the control group and T_3-treated and T_4-treated tadpoles. At stage 31, the MC in the brains of control and treated tadpoles (PTU, T_3, T_4) were large and elongated. In all groups the MC were filled with strong metachromatic granules staining red with AB/safranin (Fig. 2C,D).

Neuroanatomical distribution of MC in the adult brain

No sex differences were recorded in our preliminary examination, and this description is applicable to either sex. The distribution of MC in the brain of *R. esculenta* is schematically reported in representative transverse rostro-caudal sections (Fig. 3). The majority of MC were elongated in shape, filled with secretory granules that were safranin-positive and metachromatic with Toluidine blue

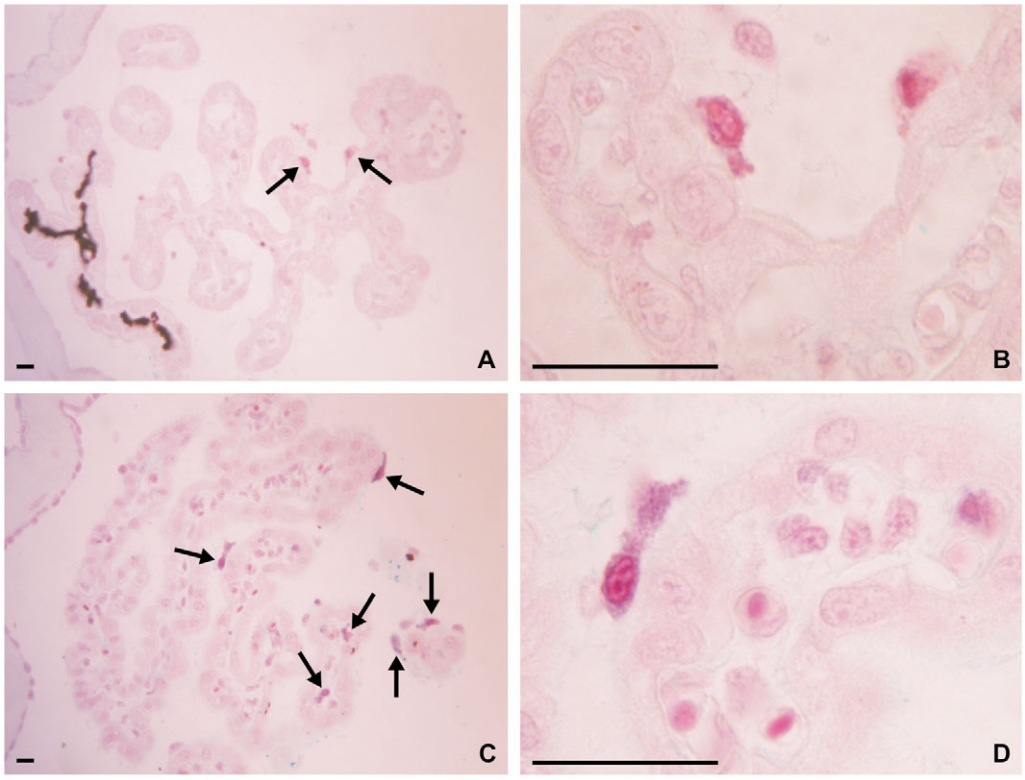

Fig. 2. Paraffin-embedded sections of the choroid plexus of 6-n-propyl-2-thiouracil (PTU)-injected tadpoles at stages 26–27 (A,B) and at stage 31 (C,D). The mast cells (MC) (arrows) have their cytoplasm filled with safranin-positive granules. Alcian blue/safranin reaction. Scale bars, A,C=100 μm; B,D=50 μm.

(Fig. 4). The MC were located within the pia mater and many of them were closely associated with blood vessels and often with melanocytes (Fig. 4A,I,J). Only a few roundish MC were observed within the brain parenchyma in areas such as the striatum, medial septum and midbrain tegmentum (Fig. 3).

Telencephalon

Mature MC of variable shapes were particularly numerous in the proximity of blood vessels surrounding the olfactory–vomeronasal nerves complex (Fig. 4A). MC were frequently observed inside the nerve tissue.

At the level of olfactory bulbs, numerous MC were observed in close association of the dorso-laterally and ventrally running blood vessels (Fig. 3A). Furthermore, ventrally, MC were also present in the glomerular layer of the main olfactory bulbs in close proximity of olfactory nerves branches that here constitute the olfactory glomerules (Fig. 3A). Many MC were also observed within the accessory olfactory bulbs, very likely associated with the vomeronasal nerve branches (Fig. 3A).

In the telencephalon, MC were observed throughout the rostro-caudal extent; they were located in the pia meter all around the pallial subdivisions (dorsal, lateral and ventral) as well as internally along the medial septum (Fig. 3B–D; Fig. 4B,C). As mentioned earlier, the majority of MC were located within the pia mater but several such cells were observed within the neuropil in a perivascular location in the striatum and medial septum (Fig. 3B–D). The latter were prevalently round in shape and located in close association of the blood vessels penetrating the brain.

Few mast cells were observed, in a perivascular position, in the ventral telencephalon at the level of the preoptic recess (Fig. 3C,D); these MC were particularly conspicuous and taut with secretory granules (Fig. 4F). A few, roundish and mature MC were observed inside the lateral ventricles (Fig. 3B,C; Fig. 4D,E).

Diencephalon

Also diencephalon is a brain region richly laid with MC. Located in the pia mater, MC were particularly abundant around the epithalamus and hypothalamus; they were always associated with blood vessels and the meningeal lining. Very rarely were MC observed inside the brain parenchyma, not in association with blood vessels. Dorsally, numerous MC were observed in the anterior choroid plexus, in which they showed a preferential perivascular distribution in habenular region (Fig. 3F; Fig. 4G) and laterally in the dorsal thalamic area (Fig. 3F; Fig. 4H). In this latter area, MC were strikingly elongated in shape as if they were migrating (Fig. 4H). Some mature MC were also observed in the epiphysis and inside the dorsal part of the third ventricle (Fig. 3G). Ventrally, many MC were observed in association with the optic nerve (Fig. 3F,G). At the level of the hypothalamus, MC were numerous in the pia mater around the dorsal and lateral region of the infundibulum and at the boundary with mesencephalic tegmentum, where conspicuously big blood vessels are present (Fig. 3I,J; Fig. 4I,J). MC were rarely seen inside the infundibular recess. However, MC were present in the median eminence and the pituitary gland.

Mesencephalon

In the mesencephalon, MC were less frequent than in the forebrain. They were observed in the pia mater, dorsally and laterally, and a few MC were located within the optic ventricles (Fig. 3I,J).

Further caudal, at the mesencephalon–rhombencephalon border, a conspicuous batch of MC was observed in the dorsal tegmentum just near nuclei isthmi (Fig. 3K).

Rhombencephalon

At the rhombencephalon level, numerous MC were located around blood vessels that surround the nervous tissue, either laterally or

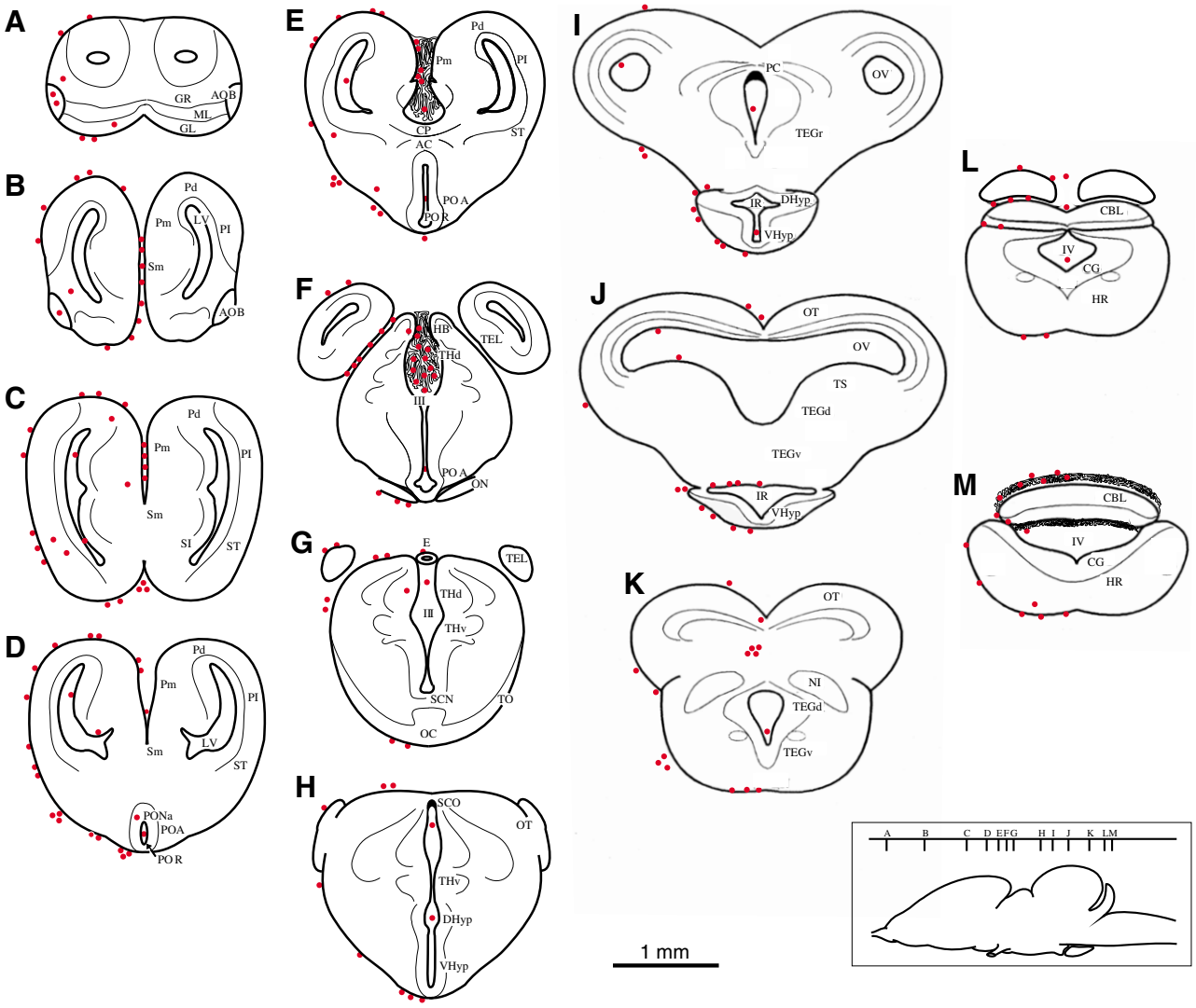

Fig. 3. Camera lucida drawings of rostro-caudal progressive transverse sections of the adult brain of *Rana esculenta*. Uppercase alphabet (A–M) in the schematic lateral profile of brain (bottom right) indicate the level of transverse sections. The mast cells (MC) are indicated as red dots. The density of red dots in a given neuroanatomical area is indicative of low, moderate or high frequency of MC. AC, anterior commissure; AOB, accessory olfactory bulb; CBL, cerebellum; CG, central gray; CP, pallial commissure; DHyp, dorsal hypothalamus; E, epiphysis; GL, glomerular layer; GR, granular layer; HB, habenula; HR, hindbrain reticular formation; IR, infundibular recess; LV, lateral ventricle; ML, mitral cell layer; NI, nucleus isthmi; OC, optic chiasma; ON, optic nerve; OT, optic tectum; OV, optic ventricle; PC, posterior commissure; Pd, dorsal pallium; Pl, lateral pallium; Pm, medial pallium; POA, preoptic area; PONa, anterior preoptic nucleus; POR, preoptic recess; SCN, suprachiasmatic nucleus; SCO, subcommissural organ; Sl, lateral septum; Sm, medial septum; ST, striatum; TEGd, dorsal tegmentum; TEGr, rostral tegmentum; TEGv, ventral tegmentum; TEL, telencephalon; THd, dorsal thalamus; THv, ventral thalamus; TO, optic tract; TS, torus semicircularis; VHyp, ventral hypothalamus; III, third ventricle; IV, fourth ventricle.

ventrally (Fig. 3M). Numerous MC were also observed dorsally, associated with blood capillaries running between rombencephalon and cerebellum as well with those of the posterior choroid plexus (Fig. 3M; Fig. 4K). In the posterior choroid plexus, Toluidine blue-stained MC were often masked by large-sized melanocytes (Fig. 4K). Along the lateral and ventro-lateral sides of the rhombencephalon, several MC could be observed at points where the rhombencephalic nerves were sprouting from.

Effect of PTU, T_3 and T_4 treatment on MC population of the adult brain

PTU treatment (both doses) induced a significant increase of brain MC number, while a treatment with either T_3 or T_4 (both doses) did not induce any significant alteration (Fig. 5). Particularly, in PTU-treated frogs MC number increased significantly in the telencephalon (634±28 MC per brain), diencephalon (529±18 MC per brain) and mesencephalon areas (250±25 MC per brain) as compared with the same areas of control frogs (437±35 MC per brain, 336±4 MC per brain, 145±15 MC per brain, respectively). The cytoplasm of these MC in PTU-treated frogs was packed with metachromatic and safranin-positive secretory granules.

Effects of hypophysectomy and replacement therapy with pars distalis homogenate on adult brain MC population

As shown in Fig. 5, frogs hypophysectomized for either one or two weeks, showed a strong decrease of MC number (less than 1 MC per section) in all brain areas with respect to control frogs. Replacement therapy with homologous pars distalis homogenate

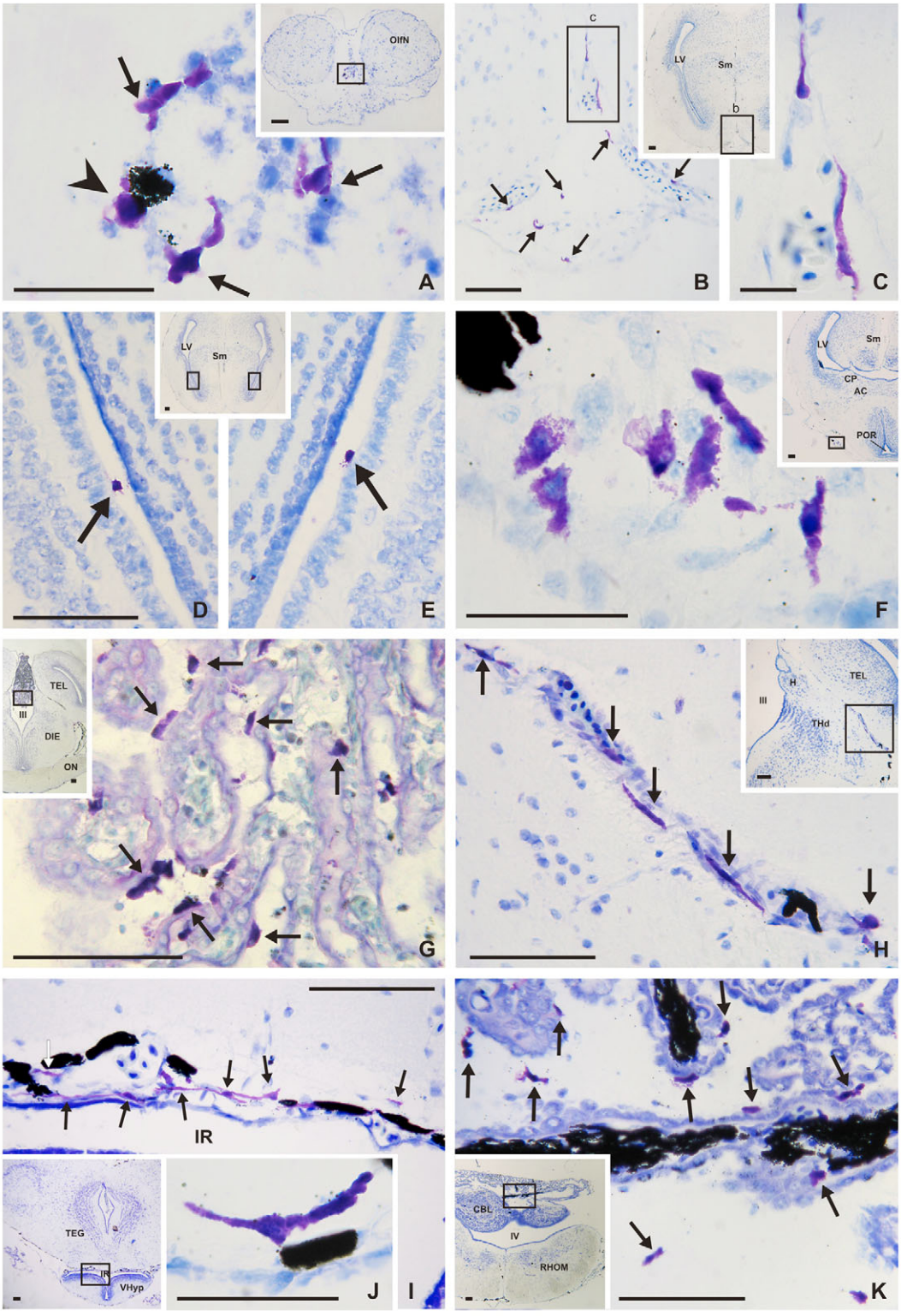

Fig. 4. Occurrence of mast cells (MC) in representative areas of the brain of control adult frog. Paraffin sections stained with acidic Toluidine blue. Insets show a general view of the brain section at low magnification. Each figure, unless otherwise specified, is an enlargement of the boxed area in the corresponding inset. (A) MC (arrows) in between the olfactory nerves. Arrowhead indicates a MC closely apposed to a melanocyte. (B) Enlargement of the perivascular MC (arrows) in the ventromedial telencephalon. (C) Enlargement of the boxed area in B, showing MC in the most ventral part of the medial septum. (D,E) Arrows indicate roundish MC located symmetrically on both sides of the telencephalic region, inside the lateral ventricles. (F) Numerous MC in the meningeal lining of the external ventrolateral telencephalic area. (G) Numerous MC (arrows) in the anterior choroid plexus, juxtaposed to the blood capillary endothelium. (H) Elongated MC (arrows) at the level of dorsal thalamus (see inset). (I) Elongated MC (arrows) at the level of the dorsal hypothalamus. (J) An elongated MC from the same region of another brain sample, at higher magnification. (K) Numerous MC (arrows) in the posterior choroid plexus, mostly in close proximity to blood capillaries. DIE, diencephalon; TEL, telencephalon; TEG, tegmentum; RHOM, rombencephalon; OlfN, olfactory nerve. For abbreviations see legend of Fig. 1. Scale bars, B,C,D,E,G,H,I,K=100 mm; A,F,J=50 mm.

induced an increase of MC population in all brain areas (Fig. 5). This increase was statistically significant already at one week in the telencephalon, and by two weeks the MC number increased significantly in all brain areas.

DISCUSSION

To our knowledge this is the first detailed description of the neuroanatomical distribution of MC in the adult and larval brain of an amphibian. In the developing frog brain, MC are confined prevalently inside the pia mater, the vascularized meningeal layer surrounding the brain and the proximal parts of the cranial nerves sprouting from the brain, especially the vomeronasal and olfactory nerves; quite sporadically, MC are also observed along the blood capillaries penetrating the neuropil. In the adult brain, the MC become more numerous inside the pia mater surrounding the telencephalon and diencephalon and its invaginations in the choroid

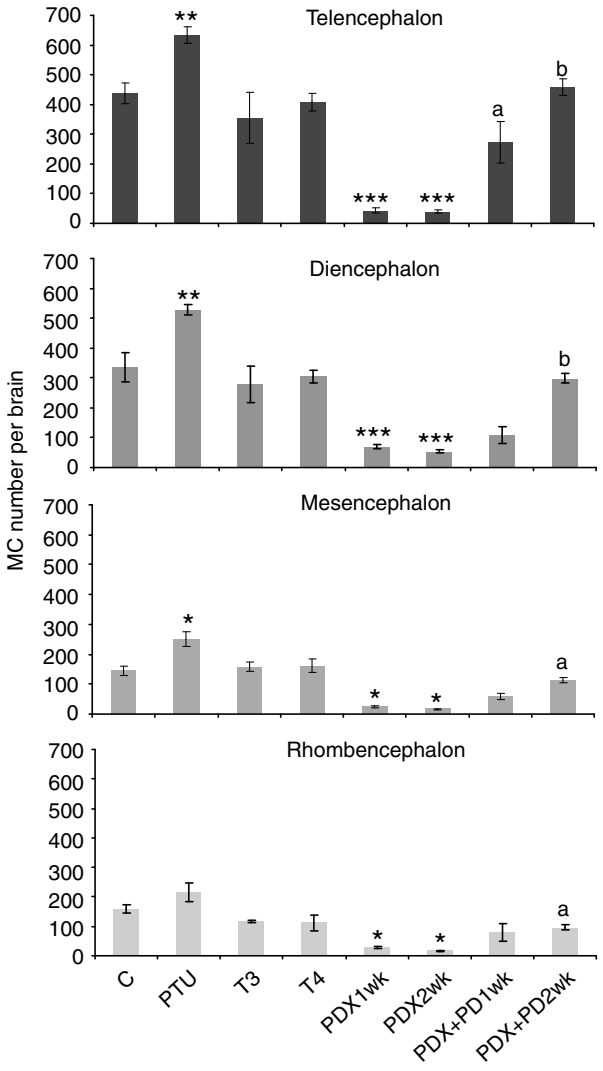

Fig. 5. Regional frequency of mast cells (MC) (MC number per brain). Control frogs (C), 6-n-propyl-2-thiouracil (PTU)-injected frogs, 3,5,3′-triiodothyronine (T_3)-injected frogs, thyroxine (T_4)-injected frogs, hypophysectomized frogs (PDX1week and PDX2weeks) and hypophysectomized pars distalis homogenate-injected frogs (PDX+PD1week and PDX+PD2weeks). Values represent the means ± standard deviations. Asterisks denote statistically significant alterations of experimental groups (PTU, T_3, T_4, PDX1week and PDX2 weeks) when compared with control (C). *P<0.05, **P<0.01, ***P<0.005; a, b denote statistically significant alterations of PDX+PD1week and PDX+PD2week *vs* PDX1week and PDX2weeks, respectively. a, P<0.05; b, P<0.01.

fissure. Within the brain parenchyma, most of the MC are observed in close adherence of blood capillaries penetrating the brain. Also in mammals in which there are several detailed accounts of MC distribution in the brain, all point to the fact that MC inside the brain (besides those that occupy the repeatedly described pial position) are located in a perivascular position (see Dropp, 1972; Dropp, 1976; Goldschmidt et al., 1985; Dimitriadou et al., 1990; Theoharides, 1990; Manning et al., 1994; Khalil et al., 2007). While we have not performed any ultrastructural analysis or specific coloration as in the rat (see Khalil et al., 2007), we would like to assume that most MC associated with blood capillaries inside the frog brain lie on the brain side of the blood vessel. Like in mammals and birds (Silver et al., 1996; Zhuang et al., 1999), a differential regional distribution of MC in the brain occurs in frogs. While in mammals and birds, MC have been described as concentrating much in the neuropil of the thalamus area (in birds, in the medial habenula) (Hough, 1988; Silver et al., 1996); in the adult frog, MC seem to occur in comparatively greater numbers in the areas adjacent to medial septum, striatum, epithalamus and midbrain tegmentum. Nevertheless, it is remarkable that they are frequently observed in the pia mater throughout the brain length, concentrating more at points related to the formation of choroid plexuses. It is interesting to note that MC are also observed along the ependymal lining of brain ventricles.

The brain MC population during development is not static; it changes dramatically like in the rat (Khalil et al., 2007). The magnitude of such change in the developing frog brain (a little over 2-fold), however, is a little less spectacular than in the rat (nearly 10-fold). In the frog, brain MC number is rather low in the pre-metamorphic stages, nearly doubles during the metamorphic climax and augments further during the adulthood. In the rat (Dropp, 1976; Lambracht-Hall et al., 1990; Michaloudi et al., 2007) and the dove (Zhuang et al., 1999), however, brain MC population increases during development and declines in a pre-adult stage of development. In rodents, it has been described that, during postnatal development, MC migrate from the pia mater into their adult positions in the thalamus (Persinger, 1981; Lambracht-Hall et al., 1990). In the dove, MC first appear in the pia mater appearing subsequently in the telencephalon and simultaneously in the medial habenula where they reach a maximum at peripubertal stage and decline thereafter (Zhuang et al., 1999). The decline in MC number is coincident with the peripubertal gonadal steroid surge (Zhuang et al., 1993). Although peritoneal MC have nuclear estrogen receptors (Parshad and Kathpalia, 1990; Vliagoftis et al., 1992), the mechanism by which gonadal steroids regulate MC number is unknown. Although we have no data on the presence of such receptors in the frog brain MC, we do know that circulating steroids in the developing tadpoles change dramatically and as such a role of steroids in the MC dynamics in frog brain cannot be excluded. Furthermore, while in the dove it is known that courtship may induce a dramatic increase in MC number in the medial habenula, presumably accounted for the high level of steroids, we have no idea whether in the adult frog brain seasonal changes in MC population related to the seasonally changing steroid profile of the animal occur. This needs to be investigated.

It is largely known that TH are central regulators of metamorphosis in amphibians (for a review, see Tata, 2006). In *Xenopus laevis*, *Rana catesbeiana*, *Rana pipiens* and *Bufo marinus* circulating levels of T_4 and T_3 are very low in pre-metamorphosis, progressively increasing during pro-metamorphosis and increasing further sharply at the onset of metamorphic climax (Regard et al., 1978; Mondou and Kaltenbach, 1979; Kikuyama et al., 1993; Weber et al., 1994; Krain and Denver, 2004).

Exposure of early stages of frog tadpoles to TH can precociously induce normal metamorphosis. Conversely, withholding the hormone by surgical or chemical ablation of the endocrine gland will prevent further larval development or metamorphosis.

The results of the present study indicate that T_3- and T_4-injections at pre- and pro-metamorphic stages do accelerate the larval development but they do not influence the number of brain MC. By contrast, PTU treatment at pre-metamorphic stage (stages 26–27) blocks larval development but it seems to bear no significant effect on pro-metamorphic tadpoles (stages 29–30). This last result is consistent with recent observations in the African clawed frog in which it was demonstrated that late pro-metamorphic tadpoles are

practically insensible to inhibitors of T_4 synthesis, such as thiourea or metilazolo (Degitz et al., 2005). However, either in pre- or in pro-metamorphosis, PTU injections lead to a significant increase (more than 2-fold) in the brain MC population. In pre-metamorphic tadpoles the numerical increase of MC occurs particularly in diencephalic and mesencephalic areas where histochemical procedures revealed a mixed population of mature and immature MC. In particular, some of them contained low-sulphated glucosaminoglycans while others had highly sulphated substances like heparin. Since it has been suggested that MC phenotype is determined by the surrounding tissue (Welle et al., 1997), it is likely that at this stage of development factors responsible for MC differentiation are still not sufficiently available.

It has been described that PTU treatment induces a drastic decrease in serum T_3 levels whereas T_3 treatment enhances serum levels of TH in *R. esculenta* (Di Meo et al., 1995). In adult frogs, we found that PTU administration induces a significant increase of MC number in telencephalon, diencephalon and mesencephalon. No significant differences were observed between T_3-injected and T_4-injected frogs and control. By contrast, hypophysectomy (one and two weeks) caused a decrease (up to 90% as compared with control) of MC number, with a recovery to normal following the replacement therapy for two weeks with homologous pars distalis homogenate. Our results only partially agree with the observations of Sabria et al. who reported that administration of PTU leads to an increase in MC number in rat brain whereas treatment with TH or TSH decreases the parameter (Sabria et al., 1987). Earlier, some authors (Clayton and Masouka, 1968; Ericson et al., 1972; Melander and Sundler, 1972; Wynford-Thomas and Stringer, 1982) described an increase in MC number in thyroid gland by administration of anti-thyroid drugs, and they hypothesized that these effects could be due to an increase in the levels of circulating TSH caused by the anti-thyroid agents. In the present study, PTU treatment induced a significant increase of MC number in both tadpoles and adult frogs, which could be accounted for a presumably higher TSH level. Accordingly, we found that hypophysectomy provokes a drastic decrease of brain MC population with a return to normal following replacement therapy with homologous pars distalis homogenate. This hypothesis is strengthened by the observation that at metamorphic climax MC number reaches the nearly highest level during development. During the advanced stages of frog development, plasma TH concentration is elevated and TSH is expressed; it is suggested that the negative feedback of TH on TSH is inoperative during the metamorphic climax (Manzon and Denver, 2004).

However, it must be taken into account that treatments with anti-thyroid agents cause an increase in the levels of TSH but they also decrease the circulating levels of TH. The fact that TH administration to either tadpoles or adult frogs was not able to alter brain MC number indicates that the effects of TH are not direct but are presumably mediated by some of the multiple processes in which TH are involved. Among these is the stimulation of secretion of adrenocorticotropic hormone (ACTH), growth hormone or somatostatin, which have a histamine-releasing action on MC *in vitro* (Tsai and Samuels, 1974; Hervas et al., 1975; Theoharides and Douglas, 1978; Irman-Florjanc and Erjavec, 1984; Macia et al., 1990). Consequently, it is reasonable to assume that the influence of TH on brain MC is mediated through the pituitary gland. In a previous work we had, in fact, observed that the pituitary gland influences MC population in frog Harderian gland and that among pituitary hormones, ACTH appears to be the most influential (Chieffi Baccari et al., 1991).

In conclusion, our findings indicate that pituitary–thyroid axis may be involved in the regulation of brain MC population. Without doubt, further studies are necessary to clarify as to which of the pituitary hormones mimics the effect of pars distalis homogenate.

LIST OF ABBREVIATIONS

AB	Alcian blue
ACTH	adrenocorticotropic hormone
CNS	central nervous system
MC	mast cells
PTU	6-n-propyl-2-thiouracil
T_3	3,5,3′-triiodothyronine
T_4	thyroxine
TH	thyroid hormones
TSH	thyrotropin, thyrotropic hormone

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