

Neuroanatomical Distribution of Cannabinoid Receptor Gene Expression in the Brain of the Rough-Skinned Newt, *Taricha granulosa*

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Key Words

CB1 · *Taricha granulosa* · Rough-skinned newt · Amphibian · In situ hybridization

Abstract

Type I cannabinoid receptor (CB1) is a G-protein coupled receptor with a widespread distribution in the central nervous system in mammals. In a urodele amphibian, the rough-skinned newt (*Taricha granulosa*), recent evidence indicates that endogenous cannabinoids (endocannabinoids) mediate behavioral responses to acute stress and electrophysiological responses to corticosterone. To identify possible sites of action for endocannabinoids, in situ hybridization using a gene and species specific cRNA probe was used to label CB1 mRNA in brains of male *T. granulosa*. Labeling of CB1 mRNA in the telencephalon was observed in the olfactory bulb and all areas of the pallium, as well as the bed nucleus of the stria terminalis and nucleus amygdalae dorsolateralis. The labeling of CB1 mRNA was also found in regions of the preoptic area, thalamus, midbrain tegmentum and tectum, cerebellum, and the stratum griseum of the hindbrain. A notable difference in CB1 labeling between this amphibian and mammals is the abundance of labeling in areas associated with olfaction (anterior olfactory nuclei, nucleus amygdalae dorsolateralis, and lateral pallium), which hints that endocannabinoids might modulate responses to odors as well as pheromones. This widespread distri-

bution of CB1 labeling, particularly in sensory and motor control centers, fits with prior results showing that endocannabinoids modulate sensorimotor processing and behavioral output in this species. The distribution of CB1 in the brain of *T. granulosa* was in many of the same sites previously observed in the brain of the anuran amphibian, *Xenopus laevis*, as well as those of different species of mammals, suggesting that endocannabinoid signaling pathways are conserved.

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Introduction

In mammals, the cannabinoid receptor (CB1) is the chief molecular target of the active component of marijuana, Δ^9 -tetrahydrocannabinol (THC) [Razdan, 1986]. The CB1 receptor is a G-protein coupled receptor that is localized in presynaptic membranes [Herkenham et al., 1990], and is activated by postsynaptic retrograde messengers (endocannabinoids), which suppress neurotransmitter release [Maejima et al., 2001; Alger, 2002; Howlett et al., 2004]. The neuroanatomical distribution of CB1 in mammals is well documented in the rat, primate, and canine brains [Herkenham et al., 1990; Pettit et al., 1998; Tsou et al., 1998; Ong and Mackie, 1999]. However, the distribution of CB1 in brains of non-mammals has not been studied except for one anuran amphibian, *Xenopus laevis* [Cesa et al., 2001; Cottone et al., 2003], and more

recently, in the African cichlid fish, *Pelvicachromis pulcher* [Cottone et al., 2005].

Although there is conservation of cells expressing CB1 mRNA (or containing the protein) in homologous brain areas in mammals and anamniotes, of which most occur in regions of the forebrain [Herkenham et al., 1990; Ong and Mackie, 1999; Cottone et al., 2003, 2005], there are also significant differences among species. Species differences in CB1 brain distribution are seen within the mammalian central nervous system [Herkenham et al., 1990; Ong and Mackie, 1999]. Whether such discrepancies in CB1 distribution in the amphibian brain exist among species is unknown and unpredictable given that anurans and urodeles are now suggested to have polyphyletic origins from different ancestral fish [Feller and Hedges, 1998].

In the rough-skinned newt (*Taricha granulosa*), the CB1 receptor has been cloned and characterized, revealing that CB1 receptors in this caudate amphibian are structurally and pharmacologically highly conserved compared to mammalian CB1 receptors [Soderstrom et al., 2000]. Behavioral studies also found that levonantradol and CP 55,940, CB1 agonists, suppress courtship clasping and locomotor activity in males of this species [Soderstrom et al., 2000]. Because CB1 receptors can affect behaviors in male *T. granulosa*, and because of the paucity of information regarding the evolution of the cannabinoid signaling system, the present study focused on the localization of cells expressing the CB1 receptor gene using in situ hybridization (ISH) procedures and brains from adult males of this species.

Materials and Methods

Mature adult males of *T. granulosa* ($n = 10$) were collected from local ponds (Lincoln County, OR) and transported to the laboratory. Animals were kept in large, cylindrical tanks (91 cm diameter, 78 cm depth; stock density ≤ 50 animals/tank) containing dechlorinated water (to a depth of 40 cm) at a controlled temperature and photoperiod (7°C; 12 h light:12 h dark) and fed earthworms (1 worm/5 newts every other day). All procedures were performed under the guidelines of the US Public Health Service's 'Guide to the Care and Use of Laboratory Animals' and approved by the Oregon State University Animal Care and Use Committee.

A cannabinoid receptor cDNA fragment was amplified using cDNA from *T. granulosa* brain tissue and gene specific primers (forward; 5'-CAG CCT CAT TCA CAG CTT CA-3', reverse; 5'-CAT GCC TGT GCT GAC AGT CT-3'). The cDNA fragment was transformed into a plasmid (pCR TOPO 4) and amplified in *E. coli* using the TOPO TA Cloning[®] Kit for Sequencing (Invitrogen[™], San Diego, Calif., USA). The cRNA probe contained 714 bases and complimented nucleotides 1,240–1,954 of CB1 in *T. granulosa*

(Accession # AF181894) [Soderstrom et al., 2000]. Following the methods of Maniatis et al. [1982] and Birnboim and Doly [1979], large scale plasmid-preps were performed by alkaline lysis. The plasmid was then linearized with either *SpeI* or *NotI* restriction enzymes. The linearized plasmid was used as a template in an in vitro transcription reaction to produce the cRNA probe using the RNA polymerases T7 or T3 (depending on fragment orientation) to yield sense and anti-sense probes, respectively. In vitro transcription was performed in the presence of 500 μM each of ATP, CTP, and GTP, and 6 μM UTP, and 6 μM [³⁵S]-UTP (ICN, Aurora, Ohio, USA). Finally, probes were extracted with phenol/chloroform (pH = 5.2) and two ethanol precipitations in the presence of 0.4 M sodium chloride, and re-suspended in 50 μl 0.1% sodium dodecyl sulfate (SDS). The probes were stored at -80°C until use, which was within 24 h.

The in situ hybridization technique was based, with modification, on the methods of Zoeller et al. [1997]. Animals were killed by rapid decapitation and brains were removed, embedded in Histoprep Frozen Tissue Embedding Medium (Fisher Scientific, Pittsburgh, Pa., USA), and stored at -80°C until sectioned. Whole brains were sectioned at a thickness of 20 μm at -20°C using a cryostat. The sections were then thaw-mounted on Superfrost Plus[®] positive charged microscope slides (Shandon, Inc., Pittsburgh, Pa., USA), and stored at -80°C until use. The mounted tissue slices were prepared for fixation and prehybridization washes by allowing them to thaw at room temperature. The tissue slices were fixed for 30 min (4% paraformaldehyde) in 1 \times phosphate buffered saline (PBS) then rinsed twice in 1 \times PBS for 2 min. Acetylation followed by immersing the tissue in 0.45% sodium chloride containing 0.1 M triethanolamine-hydrochloride (pH = 8.0), and 0.25% acetic anhydride (added just before use) for 10 min. After a 2 min rinse in 1 \times standard saline citrate (SSC), the tissue was dehydrated through a series of increased concentrations of ethanol (70% for 1 min, 80% for 1 min, 95% for 2 min, and 100% for 1 min) then delipidated in chloroform (5 min). Finally, the tissue was partially rehydrated by immersion in 100% ethanol (2 min) and 95% ethanol (2 min). The tissue was then allowed to dry for 30 min. Tissue sections were then covered in hybridization solution (50% deionized formamide, 0.1% sodium pyrophosphate, 10% dextran sulfate, 2 \times SSC, 25 $\mu\text{g/ml}$ tRNA, 1 \times Denhardt's solution, and 200 mM DTT (dithiothreitol)) containing the appropriate volume of [³⁵S]-UTP-labeled cRNA probe (2,000,000 cpm/slide). Finally, parafilm coverslips were placed over the hybridization solution, and the slides were placed on a rack in a Tupperware container with 50 ml of water in the base to provide a humid environment. The container was then placed in an incubator for 20 h at 52°C.

After incubation, the parafilm coverslips were removed by dipping the slides in 1 \times SSC. The tissue was then washed four times for 15 min in 1 \times SSC, following which slides were placed in two 20 min washes of 2 \times SSC/50% deionized formamide at 52°C then rinsed twice for 10 min in 2 \times SSC at room temperature. Tissue was incubated in RNase wash buffer (0.5 M sodium chloride, 0.01 M Tris, 1 mM EDTA; pH = 8.0) at 37°C for 10 min, followed by 30 min incubation in RNase A (Sigma, St. Louis, MO; 100 $\mu\text{g/ml}$ in RNase wash buffer) at 37°C. Tissue was then rinsed twice in 2 \times SSC for 10 min and placed in two additional 20 min washes of 2 \times SSC/50% deionized formamide at 52°C and two rinses for 10 min in 1 \times SSC at room temperature. Finally, the slides were placed in 70% ethanol twice for 5 min, after which they were allowed to dry for at least 30 min. After drying, slides were individually dipped in

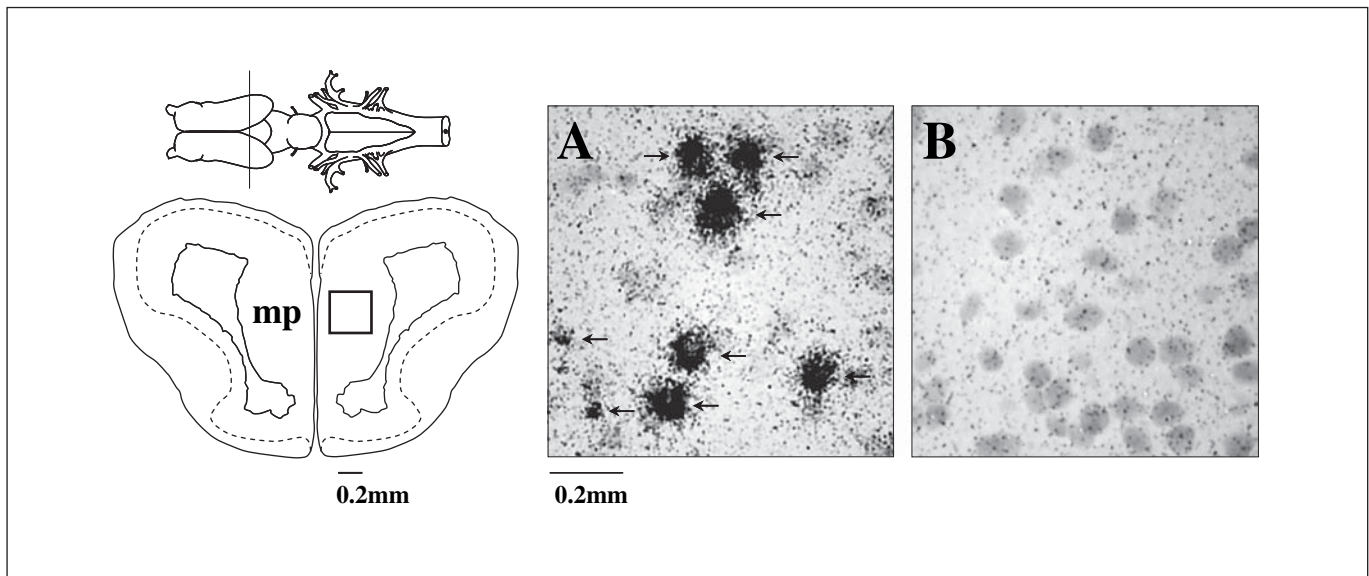


Fig. 1. CB1 ISH labeling in the brain of male *T. granulosa*. **A** High magnification (200 \times) photomicrograph of labeled cells in the medial pallium from a brain tissue section incubated with the CB1 anti-sense ^{35}S -labeled riboprobe. Arrows indicate labeled cells. **B** Photomicrograph (200 \times) of negative control showing no signal in cells of the medial pallium from an adjacent brain tissue section incubated with the CB1 sense ^{35}S -labeled riboprobe. All tissue

counterstained with methyl green. Brain schematic at left top indicates approximate level of photomicrographs. Below the schematic, a mirror image line drawing of the brain level in frontal section (ventral at bottom) from low magnification (40 \times). Box in right side of line drawing identifies location of photomicrographs in the medial pallium (mp). Scale bars, for both low and high magnification are shown at bottom.

Kodak NTB-2 emulsion film (Rochester, NY) at 42°C, dried for 3 h at room temperature, and exposed for 28 days at 4°C. Following exposure, slides were developed in Dektol developer (Kodak, or VWR Rochester N.Y., USA) for 2 min, placed in a stop bath (deionized, distilled H₂O) for 30 s, and fixed in full strength fixer (Kodak, Rochester, N.Y., USA) for 5 min (all chemicals were between 12 and 14°C). The slides were then washed in running tap water for 5 min. Afterward the tissue was counterstained with 0.1% methyl green for 30 s, followed by a 2–3 min wash in running tap water, and finally dehydrated in 50% ethanol for 30 s. The slides were dried for at least 15 min, cover-slipped using Permount Histological Mounting Medium (Fisher Scientific, Santa Clara, Calif., USA), and analyzed using light microscopy.

Nomenclature used to refer to neuroanatomical regions was based on the work of Herrick [1927], Northcutt and Kicliter [1980], Roth [1987], Schmidt and Roth [1990], Marin et al. [1997a, b], Sanchez-Camacho et al. [2001], and Stuesse et al. [2001].

Results

Based on ISH procedures and species-specific riboprobe, CB1 receptors appear to be widespread in the central nervous system of *T. granulosa*. Distinct populations of CB1 ISH labeled cells were found, but some popula-

tions were not confined by neuroanatomical boundaries both rostro-caudally and dorso-ventrally. Labeling densities varied from cells that were extremely intense in appearance (often solid black) to cells that labeled just above background (fig. 1A). In some cases, within densely populated areas of intensely labeled CB1 cells, individual cell distinction was difficult to discern due to close proximity with other labeled cells. The sense strand of the CB1-R gene was devoid of any labeling above background (fig. 1B).

In the telencephalon of *T. granulosa*, CB1 labeled cells were observed at the level of the rostral tip of the lateral ventricle in the nucleus olfactorius anterior pars medialis (internal granular layer; medial to the lateral ventricle) (fig. 2A) including a few CB1 labeled cells that were more rostral of this level. Slightly caudal of this level, there were greater numbers of CB1 cells with a marked increase in labeling (fig. 2B). The most intense CB1 ISH labeling in the olfactory bulb was observed at the most caudal level of the internal granular layer, medial to the lateral ventricle, and extending throughout the region dorso-ventrally just medial to the lateral ventricle (fig. 2C). CB1 labeled cells were also observed in the nucleus olfactorius

anterior dorsalis (internal granular layer; dorsal to the lateral ventricle) at the level of rostral tip of the lateral ventricle through the medial part of the nucleus olfactorius anterior (fig. 2A–C). At the level of the medial part of the nucleus olfactorius anterior and just rostral to the extreme end of the primordial hippocampi, populations of CB1 labeled cells of this region appeared contiguous with the population of labeled cells that extended dorsally in the medial olfactory nucleus (fig. 2B, C). CB1 labeled cells were also observed in the nucleus olfactorius pars ventralis (internal granular layer; ventromedial to the lateral ventricle), just rostral to the extreme end of the primordial hippocampi, and extended to the extreme end of the primordial hippocampi (fig. 2C, D). Most labeled cells of the internal granular layer (ventromedial to the lateral ventricle) were slightly more medial to the lateral ventricle (fig. 2C, D). The internal granular layer was the only cell layer of the olfactory bulb that had CB1 ISH labeling, however in a few individuals a labeled cell was observed lateral to the lateral ventricle in the ventral region of the mitral layer (fig. 2C).

At the level of the extreme rostral end of the primordium hippocampi, CB1 ISH labeled cells were observed in both the primordium pallii dorsalis (dorsal pallium) and primordium pallii hippocampi (medial pallium) (fig. 2D). From the extreme rostral end of the primordium hippocampi to the level of the extreme posterior edge of the postoptic and commissural habenularum, intensely labeled cells were found to extend rostro-caudally in both the dorsal and medial pallium (fig. 2D–N). At their most rostral, populations of CB1 ISH labeled cells in both the dorsal and medial pallium appeared to merge rostro-caudally with the populations of labeled cells seen in the dorsal and medial olfactory nuclei, respectively (fig. 2C, D). Also, much like CB1 ISH labeled cells in the dorsal and medial olfactory nuclei, labeled cells of the dorsal and medial pallium often appeared contiguous showing no distinction across neuroanatomical boundaries.

Caudal to the accessory olfactory bulb, approximately at the level of the middle of the septum, CB1 ISH labeled cells were observed at their most rostral in the nucleus olfactorius dorsolateralis (lateral pallium) (fig. 2F), typically limited to one or two cells, each with less intense labeling than the cells in the dorsal and medial pallium. Cells with this level of labeling were found throughout the lateral pallium, extending rostro-caudally to the caudal poles of the telencephalon (fig. 2F–N).

Also at the level of the middle of the septum, CB1 ISH labeling was observed in the nucleus accumbens septi (nu-

cleus accumbens) (fig. 2F). This was usually limited to one or two labeled cells and did not occur in all individuals.

At the level just rostral to the septum ependymale, CB1 ISH labeling was observed in the nucleus medialis septi (medial septum) and nucleus lateralis septi (lateral septum) (fig. 2G). These labeled cells were less common and only found in two individuals.

Abbreviations of major neuroanatomical landmarks used in figure 2

Aac	area acusticolateralis
Acc	nucleus accumbens
Ad	anterodorsal tegmentum
Adl	amygdala dorsolateralis
Aob	accessory olfactory bulb
apoa	anterior preoptic area
Av	anteroventral tegmentum
Bn	bed nucleus of the decussation of the fasciculus telencephali
bnst	bed nucleus of the stria terminalis
Cb	cerebellum
Dp	dorsal pallium
Dth	dorsal thalamus
Epl	extragranular plexiform layer
Gl	glomerular layer
H	habenula
Igl	internal granular layer
Ip	interpeduncular nucleus
Lc	locus coeruleus
Lp	lateral pallium
Ls	lateral septum
mc	mitral cell layer
Mp	medial pallium
mpoa	magnocellular preoptic area
Ms	medial septum
Ml	mitral layer
Na	nucleus of the amygdala
Nc	nucleus cerebelli
Nri	nucleus reticularis isthmi
ppoa	posterior preoptic area
Ra	raphe nuclei
Rm	nucleus reticularis medius
Rp	reticularis parvocellularis
Rs	nucleus reticularis superior
Se	septum ependymale
Sol	solitary tract
St	striatum
T	tectum mesencephali (optic tectum)
Te	thalamic eminence
Vh	ventral hypothalamus
Von	ventral olfactory nucleus
vpoa	ventral preoptic area
Vth	ventral thalamus

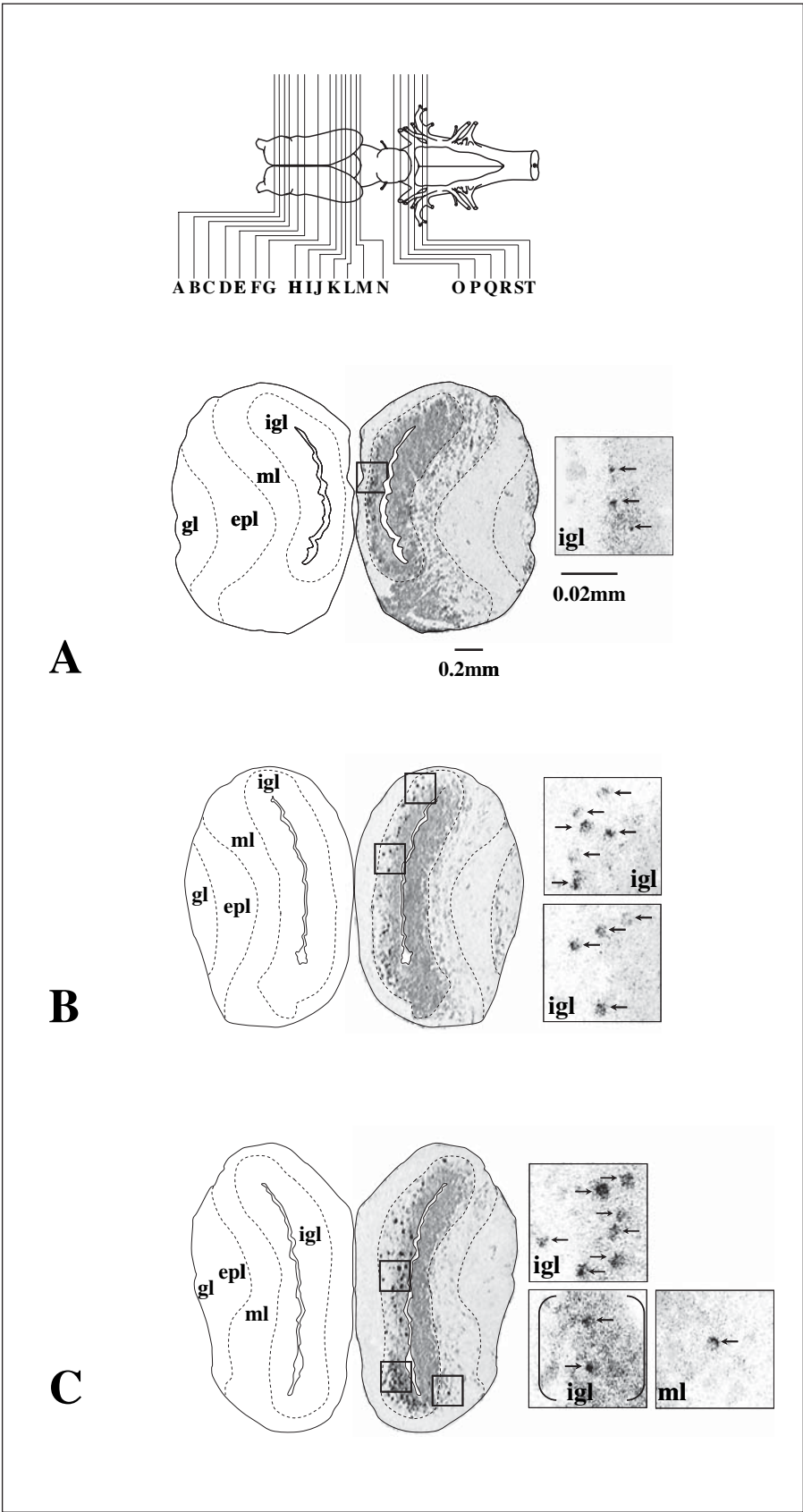
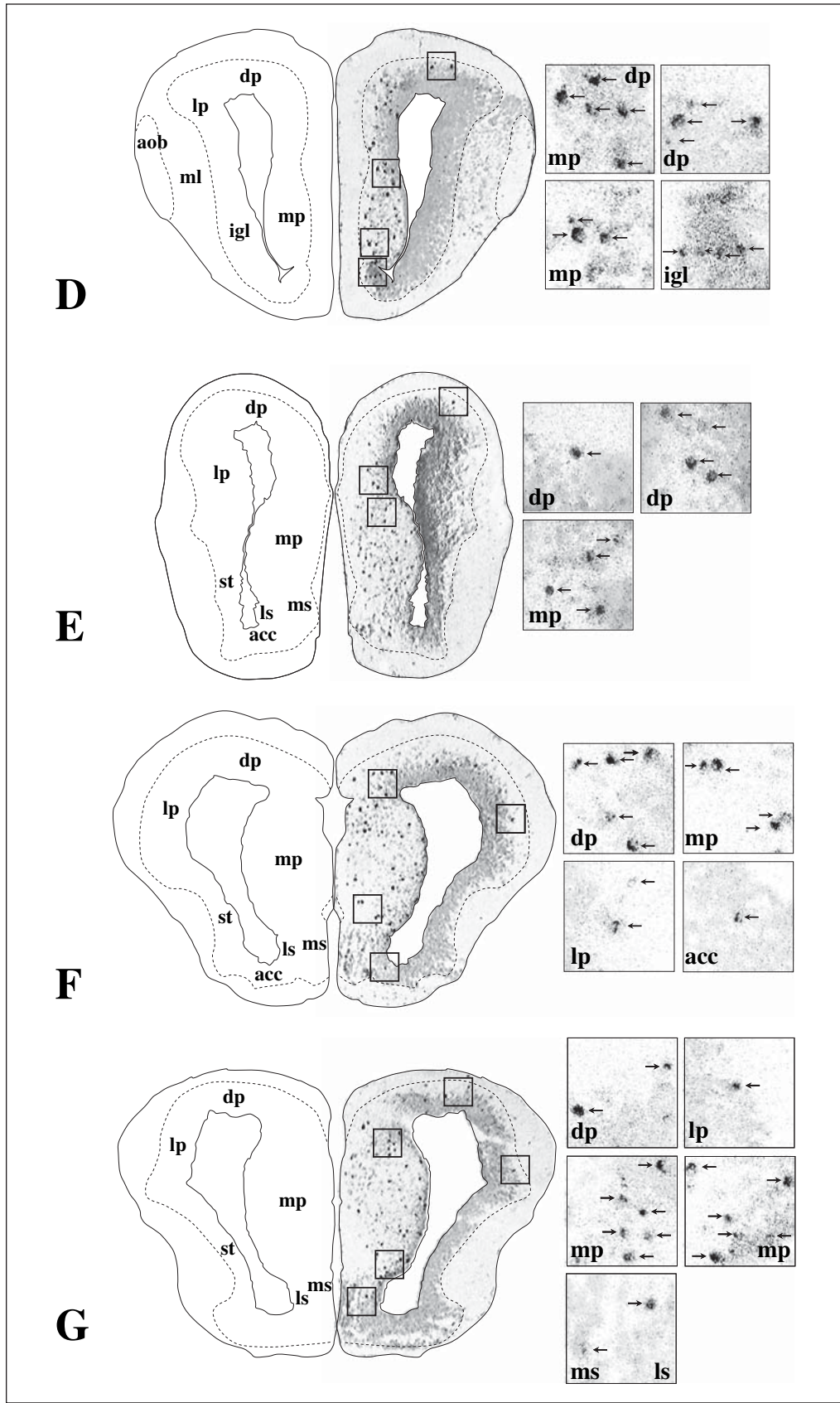
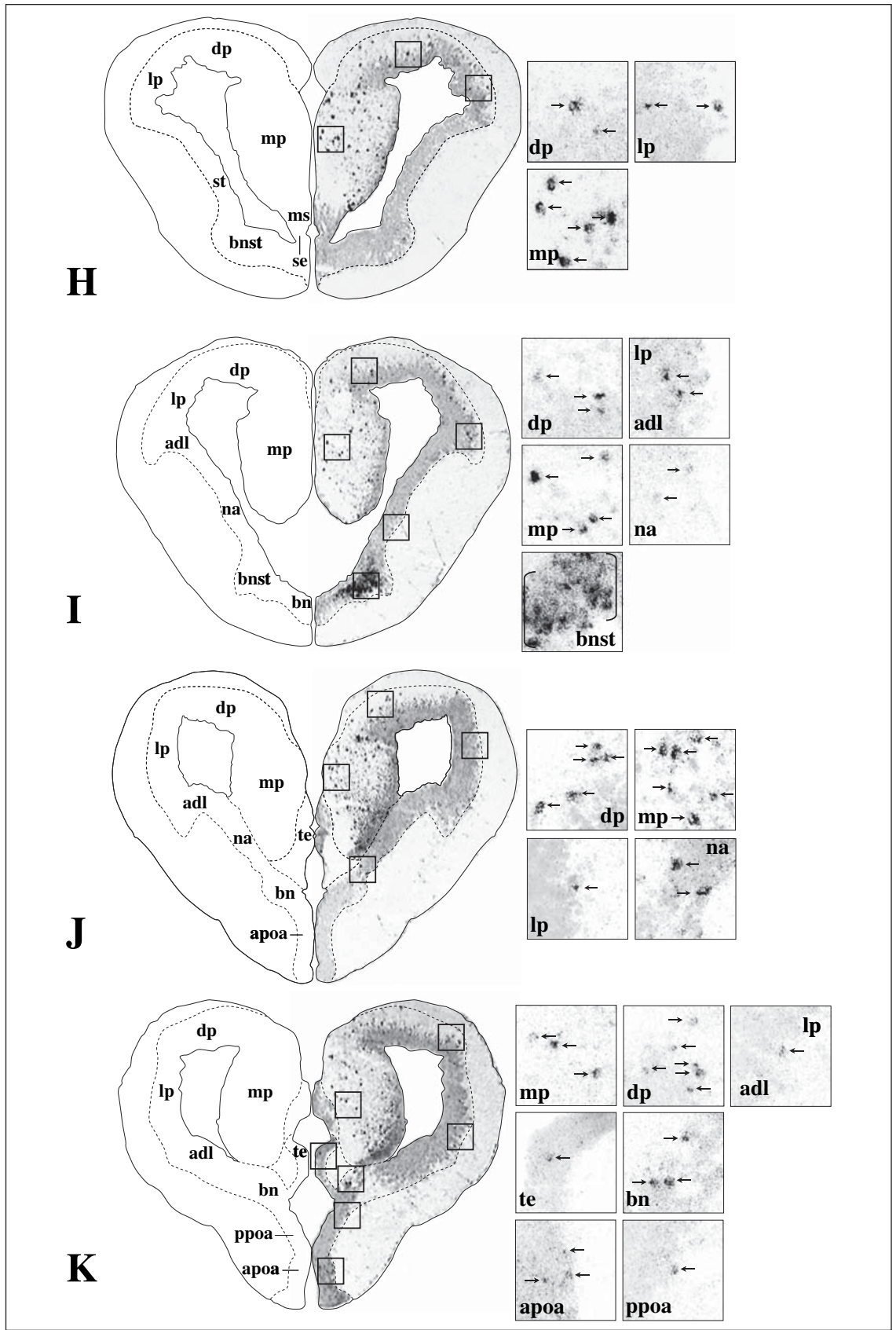
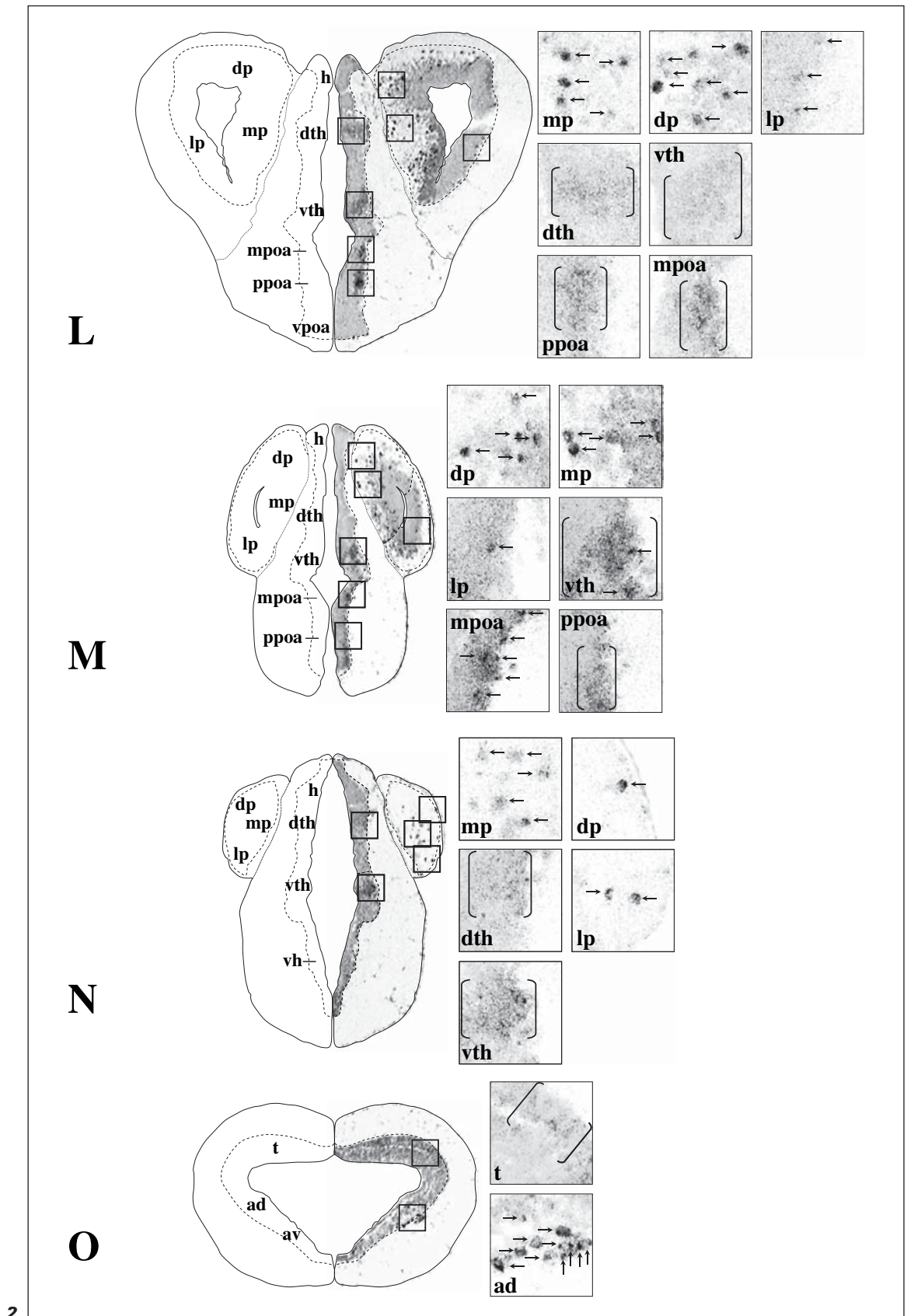


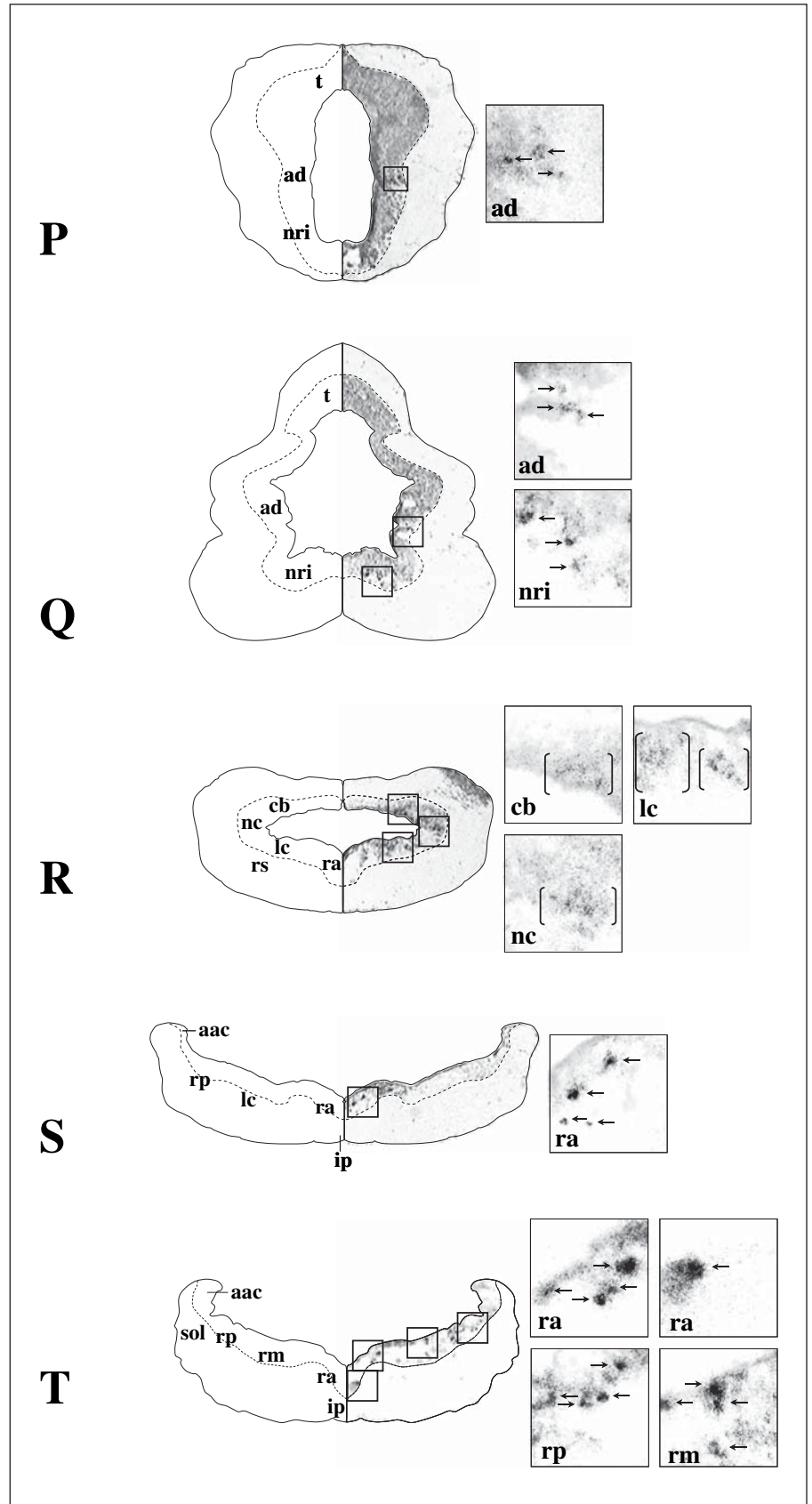
Fig. 2. Photomicrographs depicting the distribution of CB1 ISH labeling in the brain of the adult male rough-skinned newt. On the left, low magnification (40×) photomicrographs of entire right-side frontal sections (ventral at bottom) of a representative brain level with observed CB1 ISH labeling are mirrored by line drawings indicating major neuroanatomical areas. On the right, high magnification (400×) photomicrographs of CB1 ISH labeled cells. Small boxes on the low magnification right-side photomicrograph indicate actual location of high magnification photomicrograph. Brain schematic at top of figure indicates approximate level of each representative section with its corresponding letter indicated. Arrows indicate distinct labeled cells, whereas brackets identify areas of CB1 ISH labeling that lack individual cell distinction. Scale bars, for both low and high magnification, are located in **A**.

(For fig. 2D–2T see next pages.)









2

Just caudal to the septum endymale, but rostral to the eminentia thalami (thalamic eminence), CB1 ISH labeling occurred in the rostral portion of the nucleus amygdalae dorso-lateralis (amygdala dorso-lateralis; the dorso-lateral region of the amphibian amygdala pars lateralis). This labeling was observed along the border of the ventral region of the lateral pallium at the prominentia lateralis (lateral cellular prominence) (fig. 2I). This labeling was also seen caudally at the level of the hippocampal commissure (fig. 2K). Similarly to labeling in the amygdala dorso-lateralis, CB1 ISH labeling occurred in the nucleus amygdalae (nucleus of the amygdala; the ventro-medial region of the amphibian amygdala pars lateralis) caudal to the septum endymale, but rostral to the thalamic eminence, extending caudally to the level of the hippocampal commissure (fig. 2I, J). CB1 labeled cells of the nucleus of the amygdala often bordered the ventro-medial boundary of the bed nucleus of the decussation of the fasciculus lateralis telencephali ((lateral) bed nucleus; lateral region of the amphibian amygdala pars medialis) (fig. 2J).

Caudal to the septum endymale, but rostral to the level of the thalamic eminence, the most distinct and intense CB1 ISH labeling observed in the entire *T. granulosa* brain occurred in the prominentia ventralis (bed nucleus of the stria terminalis) (fig. 2I). In the bed nucleus of the stria terminalis, CB1 ISH labeling was so intense throughout that individually labeled cells were not distinguishable. The CB1 labeling in the bed nucleus of the stria terminalis also extended dorso-laterally into the nucleus of the amygdala and medially into the bed nucleus of the decussation of the fasciculus medialis telencephali ((medial) bed nucleus; medial region of the amphibian amygdala pars medialis).

CB1 ISH labeling in the thalamic eminence was observed in most individuals at the level of the hippocampal commissure and was usually limited to one or two labeled cells (fig. 2K). Also at this level, CB1 ISH labeling was seen at its most rostral in areas of the preoptic area. In the nucleus preopticus pars anterior (anterior preoptic area) (fig. 2K) and nucleus preopticus pars posterior (posterior preoptic area), cells with low intensity labeling were observed. Caudally at the level of the ventral habenular nucleus and extending to the level of the rostral dorsal habenular nucleus, more intense CB1 ISH labeling of the posterior preoptic area was evident, (mixed among these cells were a few lightly labeled cells) (fig. 2L, M). This pattern was also seen dorsal to the posterior preoptic area in the pars magnocellularis of the preoptic nucleus (magnocellular preoptic) area as well (fig. 2L, M).

Much like regions of the preoptic area, both the pars dorsalis thalami (dorsal thalamus) and pars ventralis thalami (ventral thalamus) showed CB1 ISH labeling, but also often lacked individual cell distinction. Cells in the dorsal thalamus typically had light labeling (fig. 2L–N), and in some individuals no labeled cells were seen. The CB1 ISH labeling in the ventral thalamus often appeared more intense than that of the dorsal thalamus (fig. 2M, N), occasionally indicating individual cell distinction (fig. 2M).

CB1 ISH labeling was lacking in much of the midbrain of *T. granulosa* caudal to the extreme posterior edge of the postoptic commissure and the commissural habenularum. However, in the rostral midbrain, at the level of the III nerve roots, intense CB1 ISH labeling of cells was observed particularly in lateral aspects of the tegmentum dorsali mesencephali (anterodorsal tegmentum) (fig. 2O). At this level, CB1 ISH labeled cells were distinct. CB1 labeled cells in the dorsal tegmentum extended caudally to the level of the isthmus [Lowry et al., 1997] (fig. 2P, Q), where low intensity labeling was seen in the tegmentum isthmi (nucleus reticularis isthmi) (fig. 2Q).

Just caudal to the level of the nucleus posterior tecti (inferior colliculus), CB1 ISH labeling occurred in the cerebellum dorso-laterally in the corpus cerebelli, and in the nucleus cerebelli (fig. 2R). There was also labeling at the approximate area of the locus coeruleus. As with regions of the preoptic area and thalamus, CB1 ISH labeling in regions at this level of the brain typically lacked individual cell distinction due to relatively low density labeling.

CB1 labeling occurred in the hindbrain just caudal to the cerebellum at the level of the rostral medulla (fig. 2S, T). Specifically, in the rostral area of the hindbrain, CB1 ISH labeling occurred in cells of the stratum griseum [Herrick, 1914]. However, this labeling was only in the very medial region of the raphe nucleus [Sanchez-Camacho et al., 2001], more specifically, at the location of the raphe magnus, just dorso-medial to the griseum centrale rhombencephali [Stuesse et al., 2001], and medial to the locus coeruleus [Marin et al., 1997a, b]. At a slightly more caudal level of the rostral medulla, CB1 ISH labeling extended through cells of the stratum griseum, with the exception of the area acousticolateralis. More specifically, CB1 ISH labeling occurred in the raphe nucleus [Sanchez-Camacho et al., 2001], in the raphe magnus, again just dorsal-medial to the griseum centrale rhombencephali [Stuesse et al., 2001]. CB1 ISH labeling also occurred in the nucleus reticularis medius, in the motor nucleus of the tegmentum. Finally, CB1 ISH labeling occurred in

cells located in the lateral aspects of the stratum griseum, just dorsal to the parvocellular reticular nucleus. No CB1 ISH labeling was observed caudally to these regions of the hindbrain.

Discussion

CB1 ISH labeling in the central nervous system of *T. granulosa* was widespread. The sense strand was devoid of any labeling above background, indicating that the (anti-sense) cRNA probe used in this study specifically labeled CB1 mRNA. Evidence for CB1 distribution as being highly conserved in the vertebrate brain has been shown with its localization in the brain of the anuran amphibian, *X. laevis*, particularly with its similarity to mammals in its distribution of CB1 in limbic regions of the forebrain including the medial pallium and hypothalamus [Cesa et al., 2001; Cottone et al., 2003]. This was the case with regard to CB1 ISH labeling in the brain of *T. granulosa* as well. Furthermore, the general pattern of greater CB1 expression in the forebrain and midbrain relative to the tectum, cerebellum, and brainstem of *T. granulosa* was consistent with other anamniotes studied thus far [Cottone et al., 2003, 2005]. Immunohistochemical and autoradiography studies in mammals indicate relatively high levels of CB1 in the cerebellum [Herkenham et al., 1990; Ong and Mackie, 1999].

As previously observed in mammals and *X. laevis*, the limbic system of *T. granulosa* was the site of numerous, intensely labeled CB1 ISH positive cells. In the limbic telencephalon, this included septal nuclei, regions of the amygdala, dorsal pallium, and the medial pallium which, in particular, was labeled throughout the entire region rostro-caudally. In the diencephalon, this included the hypothalamic preoptic area. In *X. laevis*, CB1 immunoreactivity is particularly abundant in the olfactory bulbs, lateral and medial pallium, amygdala, septum, and striatum [Cesa et al., 2001], whereas ISH labeling is most abundant in the olfactory bulbs, telencephalic pallium, and hypothalamus [Cottone et al., 2003]. Although in mammals the highest labeling abundance of CB1, depending on technique and species, has been in either the substantia nigra pars reticulata and globus pallidus, or substantia nigra pars compacta, all of which are followed by either the hippocampus or cerebellum [Herkenham et al., 1990; Rinaldi-Carmona et al., 1996; Ong and Mackie, 1999].

In spite of the conserved distribution of CB1, there were notable differences between CB1 ISH labeling seen

in *T. granulosa* in this study compared to that of the distribution of CB1 seen in the brain of *X. laevis* [Cesa et al., 2001; Cottone et al., 2003]. One of the most striking observations regarding differences in CB1 labeling between *T. granulosa* and *X. laevis* occurred within the olfactory bulb. In *X. laevis*, CB1 ISH labeling occurs in the granular, glomerular, and mitral cell layers [Cottone et al., 2003], whereas in *T. granulosa*, CB1 ISH labeling was present in the internal granular layer, but nearly absent in the mitral layer and completely absent from the rest of the olfactory bulb layers. Similarly, just caudal to the olfactory bulb, *X. laevis* has a population of CB1 labeled cells (and immunoreactive cells) in the striatum [Cesa et al., 2001]. In contrast, CB1 ISH labeling was lacking in the striatum of *T. granulosa*.

Another area that showed interspecific CB1 labeling differences was in the region of the hypothalamus. In *X. laevis*, CB1 ISH labeling, as well as immunoreactivity, in the hypothalamic anterior preoptic area indicates a large population of CB1 containing cells [Cesa et al., 2001; Cottone et al., 2003]. In contrast, CB1 ISH labeling in the anterior preoptic area of *T. granulosa* was nearly absent with the exception of a few lightly labeled cells. Another exceptional difference was the complete lack of CB1 ISH labeling in all other regions of the hypothalamus of *T. granulosa*, whereas in *X. laevis*, ISH labeling in the hypothalamus occurs in the suprachiasmatic and retrochiasmatic areas, as well as the infundibular walls of the tuberal hypothalamus [Cottone et al., 2003]. In *P. pulcher*, CB1-like expression is also found in the hypothalamus, particularly in the lateral infundibular lobes [Cottone et al., 2005]. Furthermore, as in most of the hypothalamus, no CB1 ISH labeling was observed in the pituitary of *T. granulosa*, as opposed to ISH and immunoreactive labeling of cells in the distal and neural lobe, respectively, of the pituitary gland of *X. laevis* as well as the immunoreactivity in the distal lobe of the pituitary of *P. pulcher* [Cesa et al., 2001; Cottone et al., 2003, 2005].

Similar to *X. laevis*, CB1 ISH labeling was observed in the thalamus of *T. granulosa*. However, unlike regions of the telencephalon, CB1 ISH labeling in the thalamus was relatively low, as seen in the mammalian thalamus [Moldrich and Wenger, 2000]. Because of the low level of CB1 labeling, there was a lack of individual, positive cell distinction. However, there were very distinct, localized areas within both the dorsal and ventral thalamus that had obvious CB1 labeling.

Within the midbrain, marked CB1 ISH labeling was observed in the tectum of *T. granulosa*. While neurons

positive for CB1-like immunoreactivity are found in *P. pulcher* as well [Cottone et al., 2005], neither ISH nor immunocytochemistry revealed the presence of either CB1 mRNA labeling or immunoreactive cells, respectively, in the tectum of *X. laevis* [Cottone et al., 2003]. Of note, the CB1 ISH labeling in the tectum of *T. granulosa* was extremely light. Also in the midbrain, CB1 ISH labeling in the tegmentum was highly localized with distinctly labeled cells, particularly in the anterodorsal tegmentum. Although this contrasts with the lack of CB1 ISH labeling in *X. laevis* in the anterodorsal tegmentum, CB1 immunoreactive cells are found in *X. laevis* in this region [Cesa et al., 2001; Cottone et al., 2003], while immunoreactive staining occurs in the mesencephalic tegmentum of *P. pulcher* [Cottone et al., 2005].

Finally, CB1 ISH labeling observed in the cerebellum and hindbrain was relatively low in *T. granulosa*, as seen in *X. laevis* as well as *P. pulcher* [Cesa et al., 2001; Cottone et al., 2003, 2005], and individual cells in the cerebellum were indistinct. Nevertheless, in different regions of the rostral hindbrain, CB1 ISH labeling in *T. granulosa* was intense in a few cells. Unlike most brain regions observed with CB1 ISH labeling, which was consistent across individuals, the labeling seen in the hindbrain varied substantially among individuals.

The disparity in regional CB1 ISH labeling between *T. granulosa* and *X. laevis* might reflect functional differences in those brain pathways integral to the different types of sensory input and behavioral output that are distinct in the two species and possibly even between anurans and urodeles in general. Although some differences seem likely due to species specificity, some differences may simply be due to differences in ISH technique sensitivity. We utilized a radiolabeled cRNA probe, as opposed to the DIG labeled probe used for *X. laevis* [Cottone et al., 2003]. The ³⁵S signal used was placed under conditions of extremely high stringency to reduce background noise, which might have reduced the resolution of putative CB1 containing cells in brain regions with low levels of the transcript.

The *T. granulosa* brain possessed an extensive distribution of CB1 ISH labeling with the most distinctive CB1 labeling occurring in regions of the forebrain. However, localization of the CB1 receptor protein itself in the brain of *T. granulosa* could be either more or less restricted than the localized sites of synthesis from this study indicate. The use of immunocytochemistry utilizing specific antibodies for the receptor protein itself would be of tremendous value in discerning the location of putative binding sites. Nevertheless, it remains plausible to suggest that

cannabinoid signaling might be playing an important role in sensory or associative processing in the various regions CB1 ISH labeling was observed.

In the olfactory bulb, CB1 ISH labeled cells were found in the internal granular layer. In the amphibian brain, including that of a urodele, the granular cell layer includes large numbers of cells that synapse on mitral cells [Scalia et al., 1991], and contain the major inhibitory neurotransmitter, gamma amino-butyric acid (GABA) [Kratskin et al., 1989; Hamilton, 1992; Hollis and Boyd, 2005]. Granule cells use GABA through reciprocal synapses with second-order neurons, where the activity of the output neurons, the mitral cells, is under inhibitory control exerted by GABAergic interneurons, the granule cells [Mori and Shepherd, 1979; Jahr and Nicoll, 1980; Mori et al., 1981, 1984; Nowycky et al., 1981; Mori, 1987; Duchamp-Viret and Duchamp, 1993]. It is possible that in the *T. granulosa* brain, putative endocannabinoids synthesized and released from mitral cells could exert a retrograde attenuation of GABAergic inhibition from presynaptic granule cells. Endocannabinoids might further influence the processing of olfactory information in *T. granulosa* as CB1 ISH labeled cells were also observed in the lateral pallium. In the amphibian brain, mitral cell axons terminate in the lateral pallium, cells from which then project to different forebrain regions and the infundibular hypothalamus [Northcutt and Kicliter, 1980]. Given that intense cell labeling was observed in various brain regions pertaining to the processing of olfactory sensory information, it seems likely that endocannabinoids contribute to the processing of olfactory information. The involvement of endocannabinoids in this process is yet to be functionally examined.

The most intense labeling of CB1 ISH cells was observed in the bed nucleus of the stria terminalis of *T. granulosa* which suggests an important neurophysiological role of the CB1 system in this species. In mammals, the bed nucleus of the stria terminalis is considered part of the 'central extended amygdala' which, though spatially separate, includes the central amygdala [Alheid and Heimer, 1988; Alheid et al., 1995]. In the (anuran) amphibian brain, a central extended amygdala is thought to remain undivided, representing an ancestral condition [Roth et al., 2004]. Evidence suggests that the central extended amygdala of the urodele amphibian brain (*Plethodon shermani*), including the lateral portion of the bed nucleus of the stria terminalis, is functionally equivalent to that of mammals [Laberge and Roth, 2005]. In mammals, the bed nucleus of the stria terminalis receives projections from the basolateral amygdala and in

turn, projects to hypothalamic and brainstem target areas that mediate many autonomic and behavioral responses to adverse stimuli and participates in behavioral responses to anxiety and stress, possibly slower-onset, long-lasting responses that accompany sustained threats [Walker et al., 2003]. *Taricha granulosa* also exhibit a suite of endocrine and behavioral changes in response to acute stress including changes in hypothalamic-pituitary-adrenal endocrine axis [Lowry et al., 2001], corticosterone releasing factor (CRF)-induced changes in locomotion [Lowry et al., 1990, 1996], and glucocorticoid-induced suppression of clasping [Rose et al., 1998]. Furthermore, behavioral studies have shown that corticosterone (CORT) interacts with the peptide hormone, arginine vasotocin (AVT), homologous to mammalian arginine vasopressin (AVP), resulting in context-dependent behavioral responses to stress [Coddington and Moore, 2003]. Interestingly, a subset of cells in the bed nucleus of the stria terminalis of *T. granulosa* contain AVT [Lowry et al., 1997; Hollis et al., 2005]. We suggest that one of the sites that CORT and AVT interact to produce context-dependent behaviors might be in the bed nucleus of the stria terminalis, and that endocannabinoids might be playing a key role in this process. The basis for suggesting a role for endocannabinoids in mediating the interaction between CORT and AVT is that in mammals, AVP-expressing neurons of the paraventricular nucleus (PVN) are subject to rapid inhibitory glucocorticoid regulation via endocannabinoid release [Di et al., 2003]. Whether behavioral responses to stress in males of *T. granulosa* are modulated by an interaction between CORT and AVT via the CB1 system in the bed nucleus of the stria terminalis remains to be tested.

In contrast, there is electrophysiological evidence for endocannabinoids acting at the level of the hindbrain to influence sensorimotor processing associated with courtship clasping behavior of male *T. granulosa*. Endocannabinoid signaling mediates the CORT-induced suppression of rostromedial medullary neurons associated with courtship clasping behavior [Coddington and Moore, 2002]. Furthermore, cannabinoid agonists block AVT-induced enhancement of the same population of neurons [Coddington et al., 2003]. That intense CB1 labeling was found in the hindbrain, particularly in the rostral medulla, is consistent with the functional studies showing that endocannabinoids play an important role in regulating behavioral responses to stress and sensory input. An important observation from the present study is that the number of cells and the intensity of labeling in the hindbrain varied among individuals. This might indicate that

CB1 expression in the hindbrain is highly regulated in response to more subtle changes in the physiological state of the individual animal than other regions of the central nervous system of *T. granulosa*. Furthermore, the intense labeling seen in the hindbrain of *T. granulosa* indicates a strong influence of the endocannabinoids on the initial sensory input as well as motor output.

Given that labeling was observed in the cerebellum of *T. granulosa*, as well as other vertebrates studied thus far, another mechanism by which endocannabinoids might influence motor output is by action at the level of the cerebellum. The amphibian cerebellum has also been found to contain high levels of both the GABA_A receptor and GABA immunoreactivity, including the Purkinje cells [Franzoni and Morino, 1989; Tavolaro et al., 1993; Hollis and Boyd, 2005]. The interaction between cannabinoids and GABA in the cerebellum, including their influence on Purkinje cell and basket cell interaction, is evident in mammals [Galante and Diana, 2004; Szabo et al., 2004]. Interestingly, cannabinoids suppress GABA release from mammalian cerebellar granule cells, thus relieving post-synaptic inhibition [Howlett et al., 2004]. Thus, in *T. granulosa*, it seems likely that pathways leaving the cerebellum are also influenced by cannabinoids, of which attenuation of GABAergic inhibition would seem a likely possibility. This would also seem to be the case for regions of the hindbrain, given evidence for cannabinoid influence on GABAergic release in mammals [Vaughan et al., 1999]. Finally, although this study did not extend to the spinal cord of *T. granulosa*, there is evidence that CB1 may influence the motor output of amniotes due to its observed labeling distribution in the spinal cord of *X. laevis* and *P. pulcher* [Cottone et al., 2003, 2005]. Of interest, the localization of CB1 ISH labeling in the anuran spinal cord has also been related with nociception markers [Salio et al., 2002]. Given the little data available in nonmammals, the distribution of CB1 in the urodele spinal cord could provide valuable insight into the physiology the vertebrate central nervous system regarding both sensory input and behavioral output.

In summary, the CB1 receptor was found throughout much of the central nervous system of *T. granulosa*, with its distribution indicating highly conserved regionalization when compared to the neuroanatomical organization of CB1 in the brains of *X. laevis*, as well as *P. pulcher*, and those of mammals, which has been identified through a variety of techniques [Herkenham et al., 1990; Rinaldi-Carmona et al., 1996; Pettit et al., 1998; Tsou et al., 1998; Ong and Mackie, 1999; Cesa et al., 2001; Cottone et al., 2003, 2005]. Endocannabinoids, by signaling in a retro-

grade manner, are known to attenuate both excitatory (glutamate) and inhibitory (GABA and glycine) signals [Maejima et al., 2001; Howlett et al., 2004]. In mammals, the distribution of CB1 throughout much of the central nervous system suggests that this receptor influences many different brain pathways associated with sensory input, signal integration, and endocrine and behavioral output. Previously, the CB1 cannabinoid receptor of *T. granulosa* was characterized pharmacologically and molecularly, and was found to modify behaviors [Soderstrom et al., 2000]. The localization of CB1 throughout the brain of *T. granulosa* in this study indicates that the CB1 system likely exerts its influence on the behavior of this amphibian species through multiple neural pathways as well. These observations further support a conserved

CB1 distribution in the vertebrate brain where it imparts tremendous influence on the integrative pathways that direct appropriate responses to stimuli and neuroendocrine functions.

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