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Localization of Corticotropin-Releasing Factor, Urotensin I, and CRF-Binding Protein Gene Expression in the Brain of the Zebrafish, *Danio rerio*

SARAH L. ALDERMAN AND NICHOLAS J. BERNIER*
Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

ABSTRACT

Our current understanding of the corticotropin-releasing factor (CRF) system distribution in the teleost brain is restricted by limited immunohistochemical studies and a lack of complete transcriptional distribution maps. The present study used in situ hybridization to localize and compare CRF, urotensin I (UI), and CRF-binding protein (CRF-BP) expression in the brain of adult zebrafish (*Danio rerio*). All three peptides were localized in the preoptic area, periventricular hypothalamic and tectal regions, and dorsal part of the trigeminal motor nucleus. CRF and UI were both expressed in several nuclei of the dorsal telencephalon, whereas CRF and CRF-BP were both expressed in the ventral nucleus of the ventral telencephalon. Sole expression of CRF and CRF-BP was apparent in the olfactory bulbs and superior raphe nucleus, respectively, whereas only UI was observed in the corpus mamillare, nucleus of the medial longitudinal fascicle, dorsal tegmental nucleus, nucleus lateralis valvulae, and nucleus interpeduncularis. A major finding of this study was the general regional overlapping of CRF-BP with its ligands and a tendency to be expressed in tandem with CRF rather than UI. Overall, the mRNA expression patterns outlined in this study support the stress-related neuroendocrine, autonomic, and behavioral functions generally ascribed to the vertebrate CRF system and suggest some unique functional roles for CRF and UI in the teleost brain. J. Comp. Neurol. 502:783–793, 2007.

Indexing terms: corticotropin-releasing factor; urotensin I; CRF-BP; teleost; brain; gene expression

The vertebrate corticotropin-releasing factor (CRF) system consists of highly conserved neuropeptides, receptors, and a binding protein that aid in the maintenance of homeostasis during stress in vertebrates. In fish, the neuropeptides include CRF (present in all vertebrates), urotensin I (UI; structurally related to the tetrapod urocortin, UCN), and two peptides similar to mammalian UCN-2 and -3 (Chang and Hsu, 2004). These ligands signal through two main G-protein-coupled receptors (CRF-R1 and CRF-R2; Pohl et al., 2001), and a third subtype is known to exist in the catfish, *Ameiurus nebulosus* (Arai et al., 2001). The final component of the vertebrate CRF system is a binding protein (CRF-BP; Seasholtz et al., 2002), which is structurally distinct from the CRF receptors (Behan et al., 1993) and capable of binding all members of the CRF ligand family (Seasholtz et al., 2002).

The most notable function of the CRF system in fish, as in other vertebrates, is its role in regulating circulating glucocorticoid levels in response to stress by way of the hypothalamus-pituitary-interrenal (HPI) axis. CRF produced in the nucleus preopticus stimulates the release of corticotropin (ACTH) from the adenohypophysis, which in turn stimulates the production and release of cortisol (the main glucocorticoid in fish) from the interrenal cells of the head kidney (Wendelaar Bonga, 1997). UI is also a potent

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*Correspondence to: Nicholas J. Bernier, Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1.
E-mail: nbernier@uoguelph.ca

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in vitro ACTH secretagogue in fish (Tran et al., 1990) and may be involved in the in vivo regulation of the HPI axis; however, its production in the nucleus preopticus remains to be shown. Apart from its role in HPI-axis regulation, the CRF system in fish, as in other vertebrates, may be involved in a variety of autonomic actions, including cardiovascular modulation (Le Mével et al., 2006), locomotor responses (Carr, 2006; Lowry and Moore, 2006), and altered food intake (Bernier, 2006). However, in contrast to the hypophysiotropic role of the nucleus preopticus, the source of CRF-related peptides involved in the extrahypophysiotropic actions of the CRF system in teleosts is not known. CRF-BP is believed to play an inhibitory role in CRF-related peptide signaling, based on its ability to attenuate the ACTH-releasing activity of CRF in vitro (Potter et al., 1991; Cortright et al., 1995), and its broad distribution in the mammalian brain suggests that it is involved in modifying other functions of the CRF system as well (Potter et al., 1992). Although the CRF-related peptides signal through the same receptors, CRF and UI have different potencies for in vitro ACTH- and thyroid-stimulating hormone (TSH)-releasing activity (Tran et al., 1990; Larsen et al., 1998), and differential responses have been observed upon intracerebroventricular (icv) injections of CRF and UI with respect to food intake (Bernier and Peter, 2001) and cardiovascular activity (Le Mével et al., 2006). The latter is reminiscent of the disparity in cardiovascular response to icv injections of CRF and UCN in mammals (Parkes et al., 2001) and is evidence for unique functional roles for the CRF-related peptides in the central nervous system (CNS).

The CRF system is known to be broadly distributed in the mammalian CNS (de Souza and Grigoriadis, 1995); however, early immunohistochemical investigations on CRF-related peptide distribution in various fish species suggested a more restricted distribution (Yulis and Lederis, 1986; Olivereau and Olivereau, 1988; Zupanc et al., 1999), which may be partially attributed to the use of antibodies raised against mammalian peptides and low-sensitivity detection methods. Heterologous blocking of primary antisera to ensure specific localization of either CRF or UI has identified unique distribution patterns for the two peptides in *Catastomus commersoni* (Lederis et al., 1985), and the advent of higher sensitivity immunoperoxidase detection systems revealed a much broader distribution of CRF than previously reported for fish (Pepels et al., 2002). Thus our current understanding of CRF and UI peptide distribution in the CNS of teleosts is limited. Furthermore, there is a lack of studies, particularly in fish, that 1) identify central sites of production for CRF-related peptides and their binding protein, 2) compare the relative distribution of the CRF-related peptide expression, and 3) relate the distribution of CRF-BP expression to its ligands. Distribution of mRNA expression is of particular importance for neuropeptides, because it is often difficult to associate neurons of origin with immunoreactive fibers, making precise identification of production sites unascertainable. Additionally, although both CRF and UI signal through the same receptors, differences in ligand distribution, as well as the ability of CRF-BP to modulate the bioavailability of either ligand, may influence the in vivo functional roles of these peptides. Therefore, we have mapped the central mRNA distribution of the two fish CRF-related peptides, CRF and UI, and their shared binding protein, CRF-BP, in an important vertebrate model species, *Danio rerio*. Moreover, given the various genetic resources available for zebrafish research (i.e., sequenced genome, mutant and transgenic strains), the use of this species in the present study will provide the basis for future investigations into unique and conserved physiological roles of the CRF system.

MATERIALS AND METHODS

Animals

Adult zebrafish (*Danio rerio*) were maintained in 10-liter aquaria supplied with fresh oxygenated local well water at 28.5°C on a 12:12-hour simulated photoperiod and fed small amounts of flakes two or three times daily. The University of Guelph's Animal Care Committee approved care and use of the animals, as per the principles of the Canadian Council for Animal Care.

Abbreviations

CCe	corpus cerebelli
CM	corpus mamillare
CP	central posterior thalamic nucleus
D	dorsal telencephalic area
Dc	central zone of D
Dd	dorsal zone of D
DIL	diffuse nucleus of the inferior lobe
DiV	diencephalic ventricle
Dl	lateral zone of D
Dm	medial zone of D
Dp	posterior zone of D
DP	dorsal posterior thalamic nucleus
DTN	dorsal tegmental nucleus
ECL	external cellular layer
ENd	entopeduncular nucleus, dorsal part
ENv	entopeduncular nucleus, ventral part
GC	griseum centrale
Hc	caudal zone of periventricular hypothalamus
Hv	ventral zone of periventricular hypothalamus
LR	lateral recess of diencephalic ventricle
NIII	oculomotor nucleus
NIn	nucleus interpeduncularis
NLV	nucleus lateralis valvulae
NMLF	nucleus of the medial longitudinal fascicle
NVmd	trigeminal motor nucleus, dorsal part
PGZ	periventricular gray zone of the optic tectum
PM	magnocellular preoptic nucleus
PPa	parvocellular preoptic nucleus, anterior part
PPp	parvocellular preoptic nucleus, posterior part
PPv	periventricular pretectal nucleus, ventral part
PTN	posterior tuberal nucleus
RV	rhombencephalic ventricle
Sc	suprachiasmatic nucleus
SR	superior raphe
TeO	tectum opticum
TeV	tectal ventricle
TL	torus longitudinalis
TPp	periventricular nucleus of posterior tuberculum
TS	torus semicircularis
V	ventral telencephalic area
Val	lateral division of valvula cerebelli
Vd	dorsal nucleus of V
Vl	lateral nucleus of V
VM	ventromedial thalamic nucleus
Vs	supracommissural nucleus of V
Vv	ventral nucleus of V

TABLE 1. Primer Pair Sequences Used To Generate Templates for the Synthesis of CRF, UI, and CRF-BP Riboprobes[1]

	Primer sequence (5′–3′)	Position	Product (bp)
CRF	Forward: CGA GAC ATC CCA GTA TCC AA	218	464
	Reverse: GAT GAC AGT GTT GCG CTT CT	682	
UI	Forward: TCC CAT TGG TCC TGC TCA TCA	10	390
	Reverse: CAG GTA TTT GCG GTT CAG T	400	
CRF-BP	Forward: GCT GTG CTT CCT CCT GTT G	23	482
	Reverse: CCT GAT TGG TGG AGC CTG A	505	

[1]All sequences are listed starting from the 5′ end, and their positions relative to the start codon and product sizes are indicated.

Riboprobe synthesis

Digoxygenin (DIG)-labelled riboprobes were generated to recognize zebrafish CRF (GenBank acc. No.: BC085458), UI (GenBank acc. No.: BX510372), and CRF-BP (GenBank acc. No.: XM678236) mRNA. Approximately 400–500 base pair cDNA fragments from the coding regions of each gene were generated by RT-PCR using the primer pairs listed in Table 1. The identities of the purified PCR fragments were verified by DNA sequencing and homology alignment, and the specificity of the CRF and UI probes was ensured by low sequence identity between the PCR fragments (17% homology). After ligation into the pGEM-T Easy Vector (Promega, Madison, WI), plasmid DNA was linearized using the restriction endonucleases Sal 1 or Nco 1 (Promega) and reverse transcribed in the presence of DIG-11-UTP using RNA polymerases T7 or SP6 (Promega) to create either antisense or sense riboprobes.

In situ hybridization

Adult male zebrafish (n = 6) were terminally anesthetized in 2 ml · liter^{-1} 2-phenoxyethanol, decapitated, and immersed in 4% paraformaldehyde (PFA) overnight at 4°C. The brains were dissected out intact, rinsed in phosphate-buffered saline (PBS; pH 7.4), and subjected to a further three overnight incubations in 30% sucrose, 1:1 30% sucrose:Cryomatrix (Thermo Shandon, Pittsburgh, PA), and Cryomatrix at 4°C before final embedding in Cryomatrix over dry ice. Transverse cryosections (12 μm) were thaw mounted onto Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA) and desiccated for 24–48 hours at 37°C. A set of six serial slides was created (three antisense genes, three sense genes) such that successive sections per slide were 72 μm apart. Sections were from the rostral tip of the olfactory bulbs through to the hindbrain just caudal to the cerebellum. Antisense and sense riboprobes for CRF, UI, and CRF-BP were hybridized to adult tissue sections as previously described (Craig et al., 2005). After verifying that no hybridization occurred with the sense probes, these sections were further processed using Nissl staining to confirm plane of sectioning and position of nuclei. Briefly, sections were rehydrated and stained for 30 minutes in 0.1% cresyl violet containing 1% acetic acid, differentiated in 95% ethanol, then dehydrated and mounted. Images were taken with a digital camera and OpenLab software. Photomicrographs were adjusted for brightness and contrast in Adobe Photoshop to increase the quality of the presentation; however, positive staining was not altered or enhanced. Line drawings were traced from original photomicrographs in Corel Draw and overlaid on serial sections to display the relative distribution of each gene. Nomenclature follows Wullimann et al. (1996).

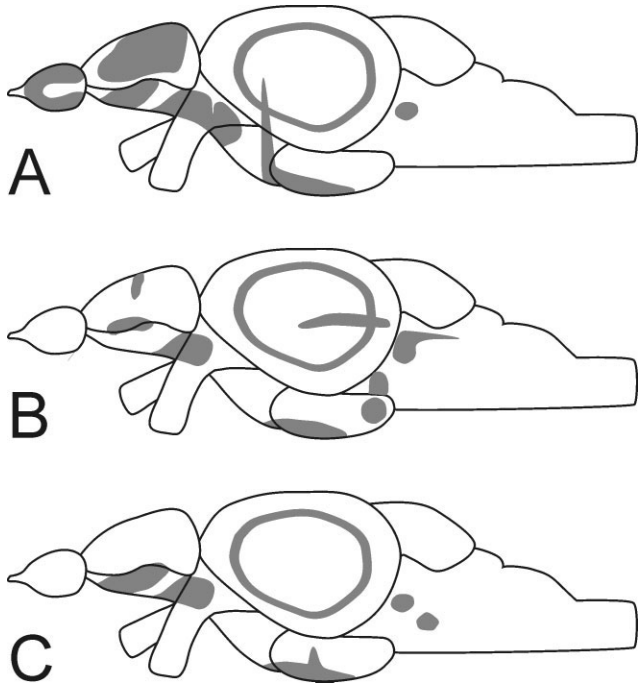

Fig. 1. Summary of regional gene expression for CRF (**A**), UI (**B**), and CRF-BP (**C**) in sagittal view of the zebrafish brain. Shading shows the approximate distribution of expression for each gene.

RESULTS

Serial sections of adult zebrafish brains were successfully hybridized to CRF-, UI-, or CRF-BP DIG-labelled antisense riboprobes, and all results using the complimentary sense riboprobes were negative. Overall, transcripts for each gene showed regionally specific distribution patterns with common and unique sites of expression (Fig. 1). The relative mRNA distribution of the three genes displayed two interesting trends. First, whereas there were a greater number of CRF-expressing regions in the brain than there were of UI, only 60% of the UI-containing regions also expressed CRF. Second, there was a tendency for CRF-BP to be regionally colocalized with CRF rather than UI. Among the 11 brain regions that consistently expressed CRF-BP, 91% also expressed CRF, but only 55% expressed UI. Furthermore, all of the regions that expressed CRF-BP and UI in tandem also contained CRF. Results are further summarized by gene and region below.

CRF

Forebrain. CRF was the only gene in this study to show expression in the olfactory bulbs, and the pattern of distribution tended toward the peripheral margins of the bulbs (Fig. 2A). Distribution of CRF was widespread throughout the dorsal telencephalon, including the dorsal (Dd) and central (Dc) zones (Fig. 2B). In the ventral telencephalon, CRF was expressed in the ventral (Vv; Fig. 2C) and supracommissural (Vs; Fig. 5D) nuclei and in two fish in the lateral nucleus (Vl; not shown). CRF mRNA

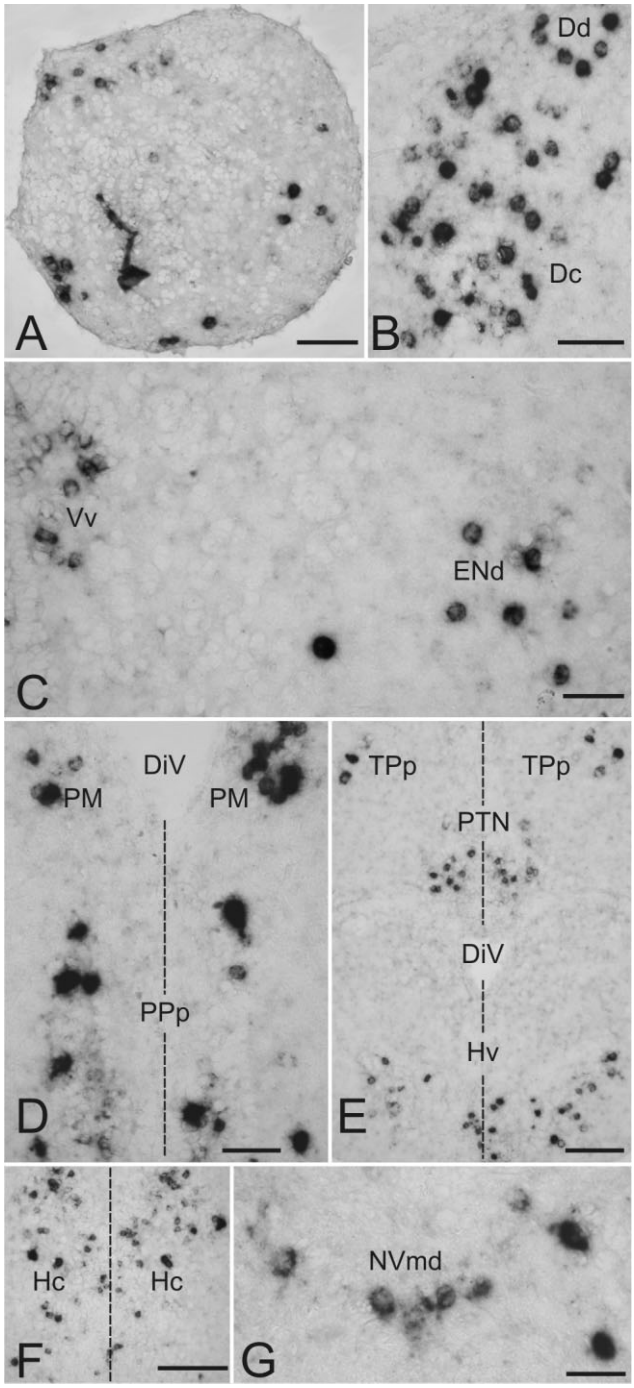

Fig. 2. Brightfield photomicrographs showing regional distribution of CRF gene expression in transverse sections of the zebrafish brain, as revealed by in situ hybridization. Brain region and the approximate level in the zebrafish brain atlas (Wullimann et al., 1996) are indicated. **A:** Olfactory bulb, level 31. **B:** Dorsal telencephalic area, level 85. **C:** Ventral telencephalic area, level 85. **D:** Posterior parvocellular and magnocellular preoptic area, level 114. **E:** Hypothalamus/thalamus, level 153. **F:** Hypothalamus, level 168. **G:** Rhombencephalon, level 213. For abbreviations see list. Vertical dashed lines indicate the midline of the section. Scale bars = 25 μm in B–D,F,G; 50 μm in A,E.

expression was also evident in the caudal extent of the dorsal endopeduncular nucleus (ENd; Fig. 2C).

Midbrain. A strong, positive hybridization signal was achieved throughout the preoptic area, including the anterior (PPa; see Fig. 5A) and posterior (PPp; Fig. 2D) portions of the parvocellular nucleus and the magnocellular nucleus (PM; Fig. 2D). In the more rostral portion of the midbrain, CRF expression was found in the suprachiasmatic nucleus and ventromedial thalamic nucleus (SC and VM, respectively; see Fig. 6E), and lined the diencephalic ventricle (DiV) from the ventral zone of the periventricular hypothalamus (Hv; Fig. 2E) to the dorsal posterior thalamic nucleus (DP; see Fig. 6F). Expression extended laterally from the central posterior thalamic nucleus (CP) toward and into the gray zone of the periventricular optic tectum (PGZ; Fig. 6F). In the torus semicircularis (TS), CRF-positive cells lined the tectal ventricle (TeV; Figs. 5D, 6G,H). CRF expression was present in all zones of the periventricular hypothalamus (Fig. 2E,F) and was continuous throughout the PGZ (Fig. 6F–I).

Hindbrain. Expression of CRF in the hindbrain was limited to the dorsal part of the trigeminal motor nucleus (NVmd; Fig. 2G).

UI

Forebrain. In the more rostral portion of the telencephalon, UI expression lined the dorsomedial edge of the dorsal telencephalon (Dm; Fig. 3A) but disappeared in the caudal portion of this zone. The reverse pattern was apparent in the dorsal nucleus of the dorsal and ventral telencephalon (Dd and Vd, respectively; Figs. 3B, 6C,D), where UI expression was seen in the caudal but not rostral extent of the nucleus. Toward the caudal limits of the telencephalon, UI expression was sparsely scattered in the dorsal telencephalon, often in close association with CRF (Fig. 6D,E).

Midbrain. UI expression in the preoptic region was restricted to the parvocellular region, with a stronger signal visible in the PPa (Fig. 5B) than in the PPp. As with CRF, UI was expressed throughout the periventricular hypothalamus (Figs. 5E, 6F,G) and the PGZ (Figs. 5E, 6F–I). In the TS, UI expression occurred only in a few cells relative to either CRF or CRF-BP (Fig. 3D–F). The strongest hybridization signal for UI was achieved along the TeV in the nucleus of the medial longitudinal fascicle (NMLF; Fig. 3C), dorsal tegmental nucleus, and nucleus lateralis valvulae (DTN and NLV, respectively; Fig. 3D). Further UI expression was evidenced in the nucleus interpeduncularis (NIn; Fig. 6H) of the mesencephalon and extensively throughout the corpus mamillare (CM; Fig. 3E) of the lateral hypothalamus.

Hindbrain. As with CRF, UI expression in the hindbrain was sparse. UI was expressed in the NVmd (Fig. 3F) and in one or two cells lining the rhombencephalic ventricle (RV) in the griseum centrale (GC; Fig. 3G).

CRF-BP

Forebrain. Telencephalic expression of CRF-BP was limited to the ventral telencephalon, in the ventral and supracommissural nuclei (Vv and Vs, Figs. 4A and 6D, respectively), often in close association with CRF-expressing cells (Fig. 6B,C).

Midbrain. In the parvocellular preoptic nucleus, CRF-BP was consistently expressed in the PPa (Fig. 5C) and in one fish in the PPp (not shown). The periventricu-

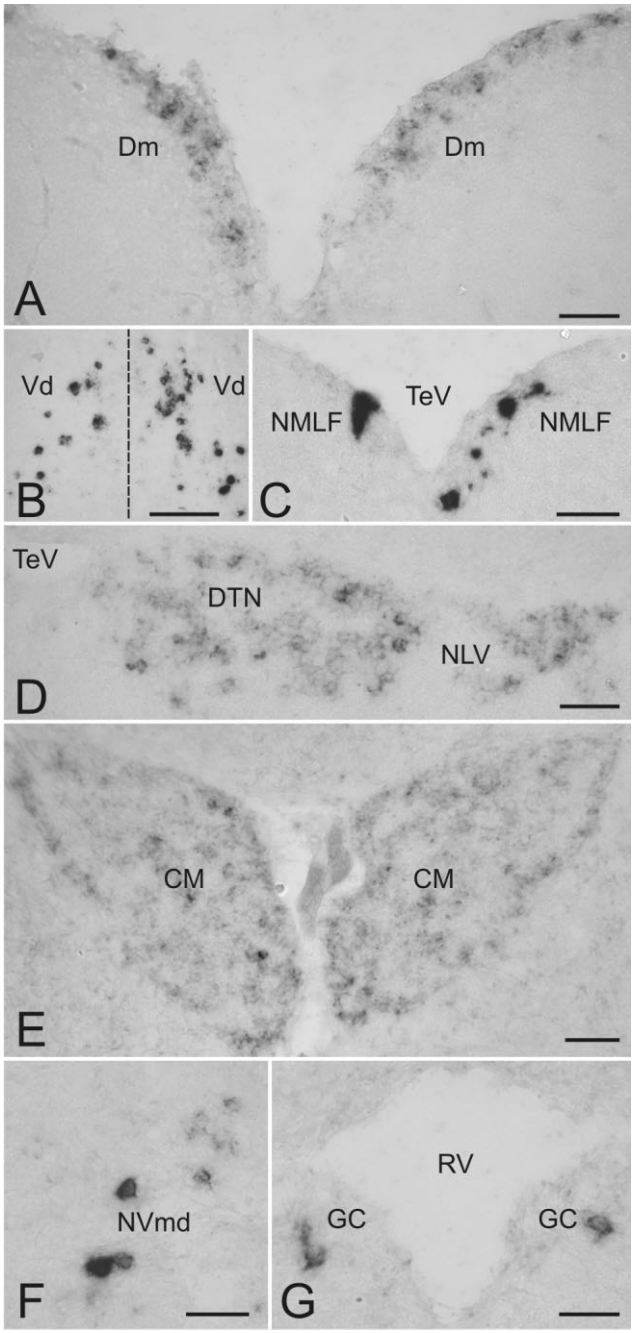

Fig. 3. Brightfield photomicrographs showing regional distribution of UI gene expression in transverse sections of the zebrafish brain, as revealed by in situ hybridization. Brain region and the approximate level in the zebrafish brain atlas (Wullimann et al., 1996) are indicated. **A:** Dorsal telencephalic area, level 71. **B:** Ventral telencephalic area, level 85. **C:** Thalamus, level 168. **D:** Thalamus, level 185. **E:** Inferior lobes of hypothalamus, level 185. **F,G:** Rhombencephalon, level 213. For abbreviations see list. Scale bars = 25 μm in A–F; 50 μm in G.

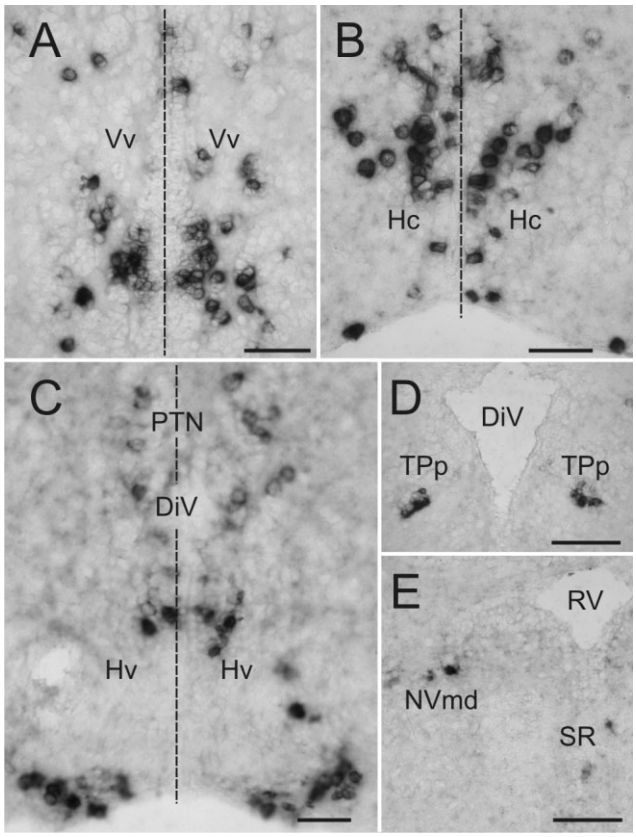

Fig. 4. Brightfield photomicrographs showing regional distribution of CRF-BP gene expression in transverse sections of the zebrafish brain, as revealed by in situ hybridization. Brain region and the approximate level in the zebrafish brain atlas (Wullimann et al., 1996) are indicated. **A:** Ventral telencephalic area, level 71. **B:** Hypothalamus, level 168. **C:** Hypothalamus/thalamus, level 149. **D:** Thalamus, level 158. **E:** Rhombencephalon, level 208. For abbreviations see list. Vertical dashed lines indicate the midline of the section. Scale bars = 25 μm.

lar hypothalamus contained many CRF-BP-expressing cells (Fig. 4B,C), and the ventral zone of this region (Hv) showed CRF-BP in close association with CRF- and UI-expressing cells (Fig. 5G,H). CRF-BP mRNA was also evident in the posterior tuberal nucleus (PTN; Fig. 4C) and the periventricular nucleus of the posterior tuberculum (TPp; Fig. 4D). As with CRF and UI, CRF-BP was expressed throughout the PGZ of the optic tectum and the TS (Figs. 5D–F, 6F–I).

Hindbrain. In the NVmd, CRF-BP was expressed in a few cells (Fig. 4E). CRF-BP was the only transcript of the three genes studied to be expressed in the superior raphe nucleus (SR; Fig. 4E).

DISCUSSION

The broad distribution of CRF, UI, and CRF-BP mRNAs in the brain of healthy, unstressed adult zebrafish supports a complex and diverse functional role of the CRF system in teleost fish. Although coexpression within individual cells was not confirmed in this study, the expression patterns of the three genes appeared to overlap at least in some cells of the PGZ, Hc, Hv, TS, and NVmd. The two ligands, CRF and UI, displayed an interesting combination of shared and unique regional mRNA localization, suggesting both overlapping and specific signaling capac-

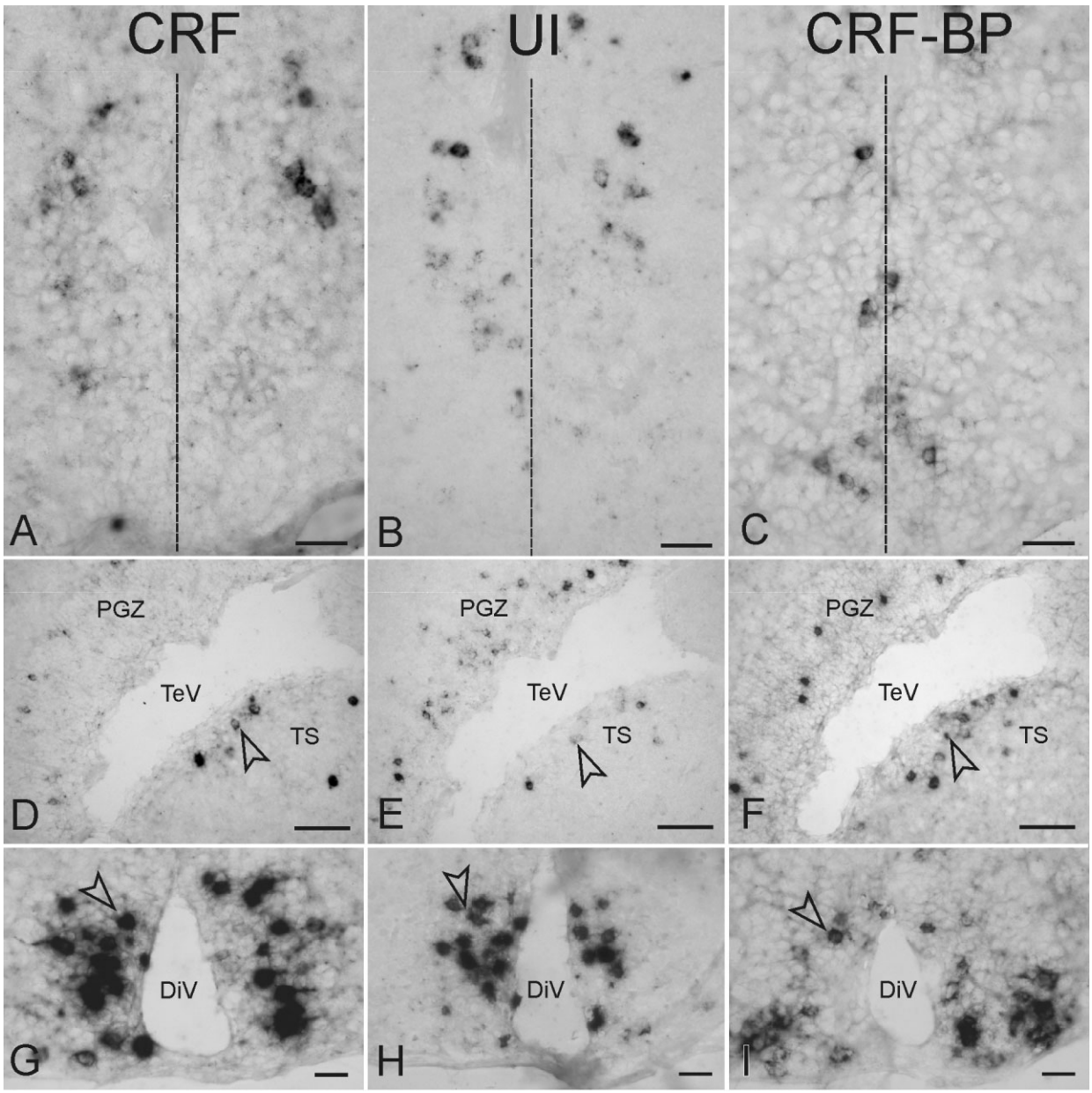

Fig. 5. Brightfield photomicrographs showing mRNA expression of CRF (**A,D,G**), UI (**B,E,H**), and CRF-BP (**C,F,I**) in matched transverse sections of the zebrafish brain, as revealed by in situ hybridization. A–C: All three transcripts were located in the PPa (approximately level 92 in Wullimann et al., 1996). D–F: All three genes were expressed throughout the PGZ of the optic tectum and lining the TeV in the TS. G–I: At the base of the hypothalamus, all three genes were expressed in the Hv lining the Div (approximately level 149 in Wullimann et al., 1996). For abbreviations see list. Vertical dashed lines in A–C indicate the midline of the section. Arrowheads in D–F and G–I indicate what is likely the same cell expressing all three transcripts of interest. Scale bars = 25 μm.

ities for each. The CRF-BP may have differential functional roles within the brain, based on its position relative to CRF and UI. The presence of all three peptides in the preoptic and tuberal nuclei of the hypothalamus supports a role for the CRF system in regulating hypophysial secretion, whereas the additional distribution in regions of the hindbrain and forebrain suggests involvement in the autonomic and behavioral functions more recently attributed to CRF-related peptides.

Relative ligand distribution

Both CRF and UI showed a remarkably widespread mRNA distribution pattern in the brain of the zebrafish, with many examples of unique and common expression sites. The most divergent expression profile for these two genes occurred throughout the forebrain and in two planes of the midbrain.

We found no evidence of UI mRNA in the olfactory bulbs, but a strong hybridization signal for CRF was evi-

dent. This is consistent with data from Bernier et al. (1999), who reported a high level of CRF mRNA in the goldfish olfactory bulbs and no UI, and is further supported by data from Pepels et al. (2002), who located CRF-ir cells in the olfactory bulb granular cell layer of *Oreochromis mossambicus*. We are unaware of any studies to examine UI-ir in the olfactory bulbs of fish. Distinct regions of telencephalic expression were also observed in the zebrafish. For the dorsal telencephalon, we identified extensive and diffuse CRF expression, whereas UI was restricted to isolated areas in the rostral- and caudal-most regions. Although there is as yet no evidence of immunoreactive cells in the dorsal telencephalon of teleosts for either peptide, extensive fiber networks have been reported for both CRF and UI (Lederis et al., 1985; Pepels et al., 2002). In the ventral telencephalon, each gene was localized to discrete nuclei. Our observation of UI mRNA in the dorsal nucleus (Vd) concurs with immunopositive UI cells in this region of *C. commersoni* (Yulis and Lederis, 1986), whereas CRF mRNA in the ventral, lateral, and supracommisural nuclei (Vv, Vl, Vs) concurs with immunopositive cells and fibers in these regions of *O. mossambicus* (Pepels et al., 2002) and *Apteronotus leptorhynchus* (Zupanc et al., 1999).

In the more rostral portion of the midbrain, approximately between the preoptic area and the inferior hypothalamic lobes, abundant CRF expression was observed in the periventricular nuclei along the diencephalic ventricle, including the tuberal nucleus, whereas UI expression was scarce and limited to the ventral zone of the periventricular hypothalamus (Hv). Conversely, at the level of the caudal portion of the inferior hypothalamic lobes, UI was highly expressed along the tectal ventricle and within the inferior lobes (CM), whereas CRF expression was absent except in the lateral thalamic region and in the TS. Previously, both CRF and UI ir-cells have been reported in the tuberal nucleus (Yulis and Lederis, 1986; Pepels et al., 2002), and the NMLF is proposed as a principal site of UI production in fish (Lovejoy and Balment, 1999).

Despite the differences outlined above, there were many examples of regional overlap in CRF and UI expression. In the preoptic area, both peptides were produced in the parvocellular nucleus, although hybridization of the CRF riboprobe consistently yielded a stronger and more extensive signal throughout the preoptic nucleus, including its magnocellular division. There appears to be a near-ubiquitous presence of CRF in the preoptic nucleus of teleosts (discussed by Pepels et al., 2002); however, this is the first study to report the additional presence of UI. This discrepancy may be indicative of inter-species differences, however a low affinity of the available UI antibodies or low sensitivity detection methods could also explain why our expression map indicates a much broader distribution of UI in the teleost brain than has previously been reported from immunohistochemical evidence (Lederis et al., 1985; Yulis and Lederis, 1986). Alternatively, association with CRF-BP in situ could mask the antigenicity of CRF/UI, if these proteins are indeed located within the same cells. Both CRF and UI were also evident in the periventricular optic tectum and the dorsal part of the trigeminal motor nucleus, and a subset of cells within each of these nuclei appeared to transcribe both ligands. Although CRF and UI may perform distinct signaling functions even if co-expressed, it is also possible that the apparent overlapping distribution in discrete brain regions is indicative of built-in redundancy within the CRF system.

Relative binding-protein distribution.

It remains to be shown whether fish CRF-BPs bind any or all of their native CRF-related peptides, but the general conservation of the CRF system throughout the vertebrate lineage (Seasholtz et al., 2002) and the ability of mammalian CRF-BPs to bind fish UI (Sutton et al., 1995) suggest a functional role for CRF-BP within the fish CRF system. We observed that CRF-BP was rarely expressed in regions independently of its potential ligands. Expression of all three transcripts occurred in the parvocellular division of the preoptic nucleus, the periventricular optic tectum, the torus semicircularis, the periventricular hypothalamus, and the dorsal part of the trigeminal motor nucleus. Furthermore, CRF-BP mRNA was more often associated with CRF than with UI, a situation also observed with CRF and UCN at the protein level in the rat hypothalamus (Henry et al., 2005).

Research on fish CRF-BPs is limited. Doyon and colleagues (2005) cloned a CRF-BP from the preoptic area of *Oncorhynchus mykiss* and showed temporal- and stressor-specific up-regulation of its mRNA in the pituitary. For *Cyprinus carpio*, Huising and colleagues (2004) cloned two CRF-BP genes, reported relatively high expression levels in the hypothalamus, and showed coimmunoreactivity of CRF and CRF-BP within individual nerve fibers in the pituitary. While no overlapping immunoreactivity of CRF and CRF-BP was observed in the carp hypothalamus (Huising et al., 2004), we observed several instances of regional overlap in CRF and CRF-BP mRNA in the zebrafish hypothalamus. Although interspecies differences are likely, there may also be tight translational regulation of either or both of these genes that precludes protein distribution analyses in unstressed fish.

Traditionally, CRF-BP has been labeled as a bioactive regulator of CRF, owing to its ability to reduce the ACTH-releasing activity of CRF in vitro (Potter et al., 1991; Cortright et al., 1995). Other functions of hormone-binding proteins in general include protection of ligands from clearance and subsequent degradation, and specific signaling capacities with or without bound ligand (Kemp et al., 1998; Seasholtz et al., 2002). Evidence for direct involvement of CRF-BP in certain signaling pathways mediated by CRF-related peptides supports a diverse functional role for this binding protein (Chan et al., 2000; Ungless et al., 2003). Moreover, differential affinities for (Sutton et al., 1995) and dissociation rates from (Henriot et al., 1999) CRF-BP between the CRF-related peptides further adds to the complexity by which CRF-BP can regulate the signaling activities of the CRF system.

Expression of CRF-BP in the rat brain occurs in both neuronal and astrocytic cell types (Behan et al., 1995), and its subcellular localization varies between brain regions (Peto et al., 1999). For example, in the raphe nucleus and in pituitary corticotropes, CRF-BP is located in the lysosomal system and not in secretory vesicles, suggesting that CRF-BP may be involved in recycling ligand–receptor complexes at target sites of CRF signaling (Peto et al., 1999). We observed CRF-BP expression in the raphe nucleus of zebrafish (this study) and trout (unpublished observation), independent of either CRF or UI. Although more evidence is needed, it may be that the location of CRF-BP at target sites of CRF-related peptides is indica-

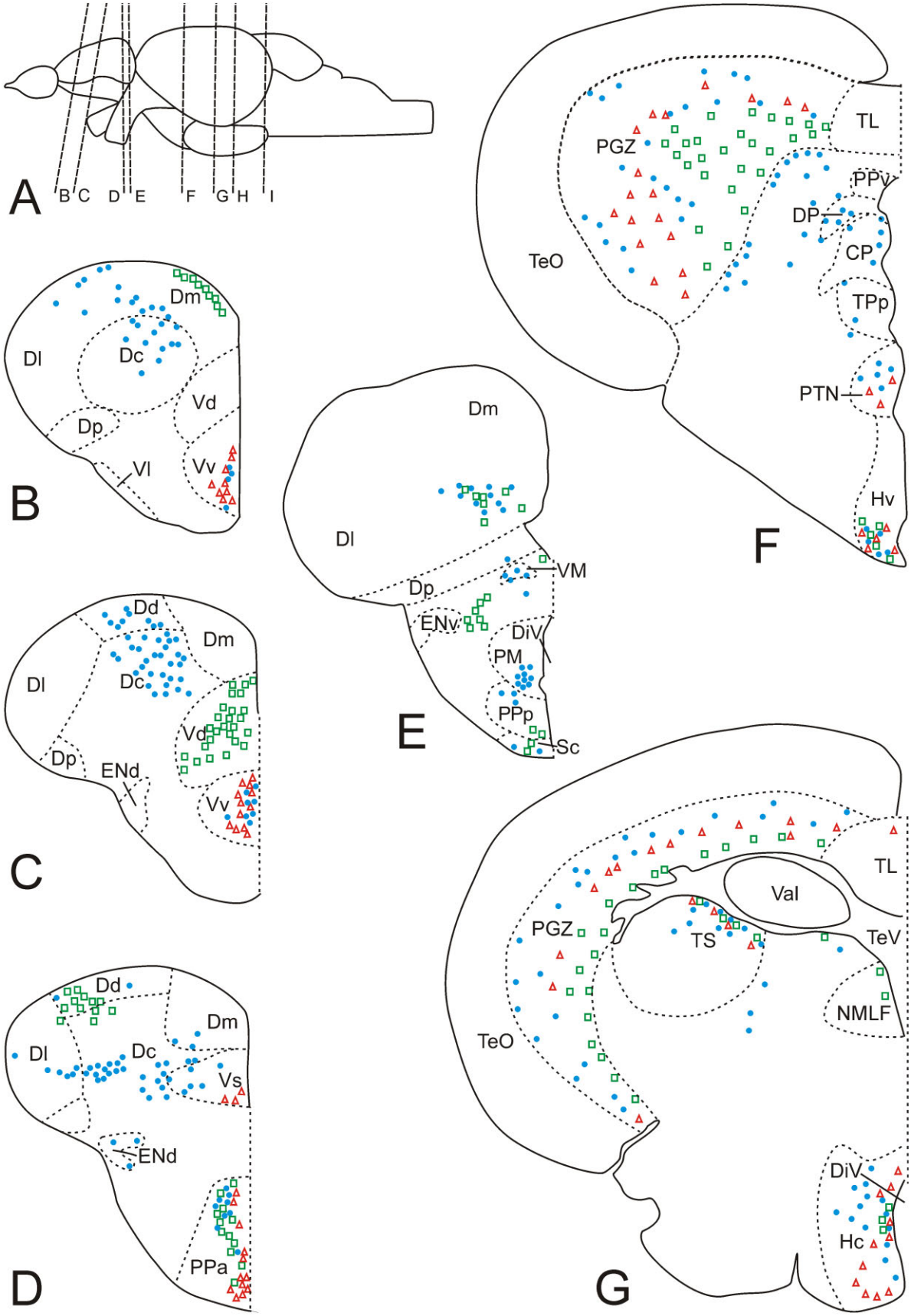

Fig. 6. Composite line drawings showing regional distribution of CRF (blue circles), UI (green squares), and CRF-BP (red triangles) gene expression in transverse hemisections of the zebrafish brain. Original photomicrographs were traced then overlaid on serial sections to display the relative mRNA distribution of each gene. **A:** Sagittal view of a whole zebrafish brain showing the approximate plane of transverse sectioning for B–I. Approximate levels in the zebrafish brain atlas (Wullimann et al., 1996) are: level 71 (**B**), level 85 (**C**), level 92 (**D**), level 114 (**E**), level 149 (**F**), level 168 (**G**), level 185 (**H**), level 208–213 (**I**). For abbreviations see list. Scale bar = 200 μm for B–I.

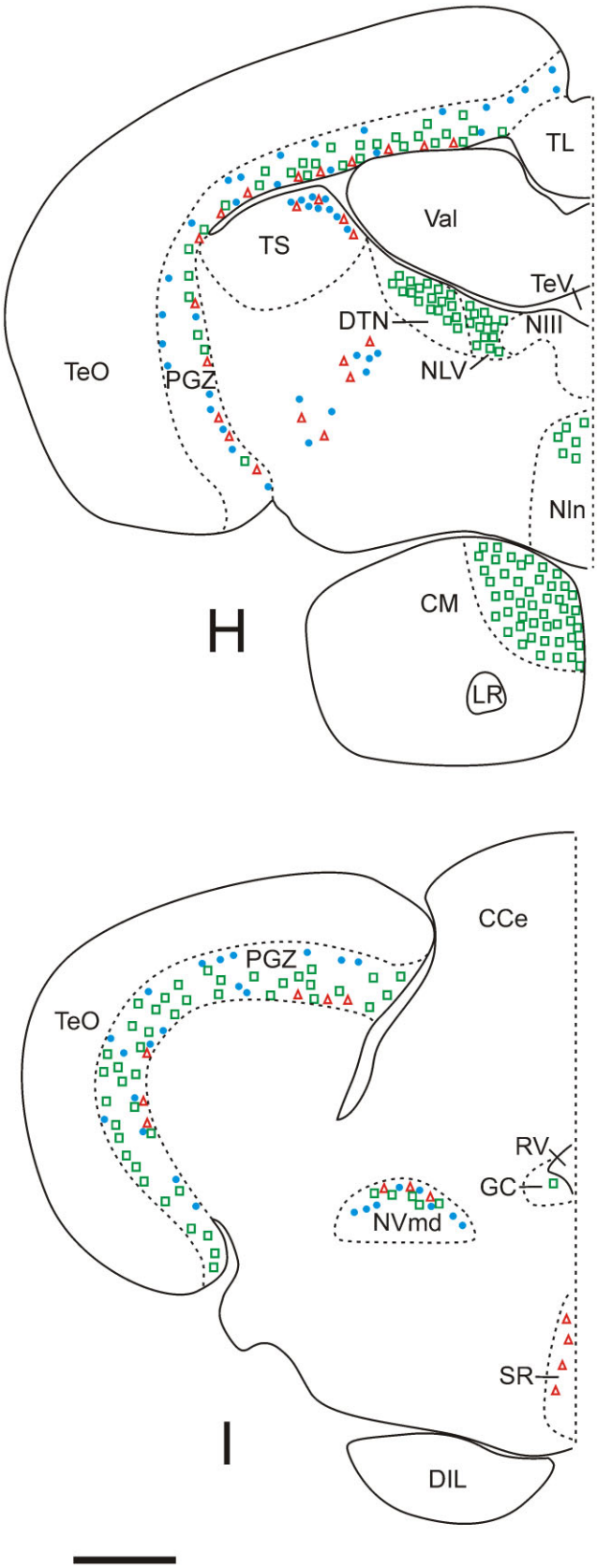

Figure 6 (Continued)

tive of a functional role in intercellular postreceptor events, whereas its regional colocalization with CRF-related peptides allows synaptic or parasynaptic modulation of signaling activities.

Functional implications of distribution.

Our observation of CRF in the preoptic and tuberal nuclei of the hypothalamus supports the well-established neuroendocrine functions of the CRF system in vertebrates (Lovejoy and Balment, 1999). CRF-related peptides are potent secretagogues of ACTH, α-melanocyte-stimulating hormone, and TSH in many species, including fish (Tran et al., 1990; Larsen et al., 1998), and these hypophysiotropic effects stem from neurons in both the preoptic and tuberal nuclei of the hypothalamus (Lederis et al., 1994). The presence of UI and CRF-BP in these nuclei of the zebrafish may be indicative of a hypophysiotropic role for these peptides as well, but there is as yet no evidence that these UI- and CRF-BP-expressing neurons reach the pituitary. The physiological outcomes associated with this hypothalamus-adenohypophysis connection can range from stress-induced increases in circulating glucocorticoids (Peter and Fryer, 1983) to modification of developmental timing (Boorse and Denver, 2002).

Stress-induced autonomic responses attributed to the CRF system include altered locomotor activity (Lovejoy and Balment, 1999) and reduced food intake (Bernier, 2006). CRF can stimulate firing of serotonergic neurons to affect stress-related locomotory behaviors in rats (Kirby et al., 2000; Lowry et al., 2000; Summers et al., 2003), amphibians (Lowry et al., 1996), and fish (Clements et al., 2003). Further locomotor modulation by the CRF system could be invoked via the optic tectum, the NMLF, and/or the NVmd. CRF in the anuran optic tectum is implicated in the visuomotor pathways of feeding behavior and predator avoidance (Carr, 2006). In the common carp (*C. carpio*), electrical stimulation of the NMLF resulted in rhythmic tail movements (Uematsu and Todo, 1997), and van den Burg and colleagues (2006) identified the trigeminal system and the NMLF in a sensorimotor pathway involved in perceiving and escaping large fluctuations in ambient temperature. Furthermore, the NMLF may be partially homologous to the mammalian Edinger-Westphal nucleus (Lovejoy and Balment, 1999), a principal site of UCN production that is recruited in a variety of adrenal-independent stress responses (Gaszner et al., 2004), suggesting a functional conservation for these UI-expressing neurons in fish.

As in mammals, central injections of CRF-related peptides evoke a reduction in food intake (de Pedro et al., 1993; Bernier and Peter, 2001) and may act through a combination of descending brainstem pathways and the so-called hypothalamic feeding center (Bernier, 2006). The candidate nuclei responsible for stress-induced anorexia in fish are the preoptic area, tuberal nuclei, and nucleus recessus (Bernier, 2006). Thus the expression profiles of CRF, UI, and CRF-BP outlined in this study are in line with the autonomic actions of the CRF system in fish.

Forebrain expression of the CRF system suggests a functional role in the integration of external and internal afferent sensory information. In rats, icv injection of α-helical CRF_{9-41}, a CRF receptor antagonist, reduces defensive-withdrawal behavior associated with exposure to odor from stressed conspecifics (Takahashi, 2004). Fear-like behavior is also evoked by olfactory cues in the cru-

cian carp (*Carassius carassius*; Höglund et al., 2005), thus the localization of CRF in the olfactory bulbs of zebrafish (this study), tilapia (Pepels et al., 2002), and goldfish (Bernier et al., 1999) may indicate a conserved functional role for CRF in relaying olfaction stress signals in Cyprinids and other teleosts.

The CRF system is also strongly implicated in the mammalian limbic system. Specifically, CRF localized in the rat amygdala plays a role in a variety of stress-related behaviors, including acoustic startle responses and exploratory behavior (Koob and Heinrichs, 1999). The teleostean medial pallium (Dm) has been identified as a structure homologous to the mammalian amygdala (Wullimann and Mueller, 2004) and is involved in memory processes such as delay and trace avoidance conditioning (Broglio et al., 2005). Our observation of UI expression in the zebrafish Dm and CRF expression in surrounding regions of the dorsal telencephalic area may represent an evolutionary conservation of the CRF system in coordinating behavioral responses to stressors.

This is the first study to investigate and compare the in situ mRNA distribution patterns of three important components of the CRF system in a teleost CNS. We have demonstrated widespread expression of CRF, UI, and CRF-BP in the brain of adult zebrafish that suggest diverse functional roles for the CRF system in endocrine, autonomic, and behavioral responses to stress. Although some regional overlap was observed in CRF and UI mRNA distribution, the relatively unique distribution patterns of these related peptides imply that some divergence of function has occurred in teleosts, a subject that may be partially addressed by further investigations into receptor subtype affinity and distribution analyses. The observation that CRF-BP mRNA is often found in brain regions that also transcribe its ligands encourages further investigations into the in vivo functional role of this protein in CRF-related peptide signaling. Finally, this work will aid in identifying the specific neuronal pathways involved in mediating the variety of physiological responses to stress.

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