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RESEARCH COMMUNICATION

Localisation and Physiological Regulation of Corticotrophin-Releasing Factor Receptor 1 mRNA in the *Xenopus laevis* Brain and Pituitary Gland

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In *Xenopus laevis*, corticotrophin-releasing factor (CRF) and urocortin 1 are present in the brain and they both are potent stimulators of α-melanophore stimulating hormone (MSH) secretion by melanotroph cells in the pituitary gland. Because both CRF and urocortin 1 bind with high affinity to CRF receptor type 1 (CRF_1) in mammals and *Xenopus laevis*, one of the purposes of the present study was to identify the sites of action of CRF and urocortin 1 in the *Xenopus* brain and pituitary gland. Moreover, we raised the hypothesis that the external light intensity is a physiological condition controlling CRF_1 expression in the pituitary melanotroph cells. By *in situ* hybridisation, the presence of CRF_1 mRNA is demonstrated in the olfactory bulb, amygdala, nucleus accumbens, preoptic area, ventral habenular nuclei, ventromedial thalamic area, suprachiasmatic nucleus, ventral hypothalamic area, posterior tuberculum, tectum mesencephali and cerebellum. In the pituitary gland, CRF_1 mRNA occurs in the intermediate and distal lobe. The optical density of the CRF_1 mRNA hybridisation signal in the intermediate lobe of the pituitary gland is 59.4% stronger in white-adapted animals than in black-adapted ones, supporting the hypothesis that the environmental light condition controls CRF_1 mRNA expression in melanotroph cells of *X. laevis*, a mechanism likely to be responsible for CRF- and/or urocortin 1-stimulated secretion of α-MSH.

Key words: corticotrophin-releasing factor receptor 1, *Xenopus laevis*, melanotroph cells, *in situ* hybridisation, α-melanophore-stimulating hormone, urocortin.

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In the mammalian central nervous system, corticotrophin-releasing factor (CRF) (1) and other members of the CRF-family of peptides, such as the urocortins 1, 2 and 3 (2–8), mediate various physiological, behavioural and immune responses to stressful challenges (9–18). The actions of CRF and urocortins are mediated by two G-protein-coupled receptors, designated CRF receptor 1 (CRF_1) and CRF receptor 2 (CRF_2) (19, 20), which have been localised in the brain by *in situ* hybridisation and receptor autoradiography (21–24). CRF_1 is most abundant in the pituitary intermediate and anterior lobe (22, 23), olfactory-related structures, amygdala, cerebral cortex, brainstem sensory relay nuclei and cerebellum, whereas CRF_2 has a more restricted distribution, being present in the posterior lobe of the pituitary, in subcortical structures and, most prominently, in the lateral septal nucleus, hypothalamus and amygdala (23).

The present study is concerned with the sites of action of CRF and CRF-like peptides in the amphibian brain and pituitary gland. Molecular cloning studies in the South African clawed toad *Xenopus laevis* showed a CRF-like gene, which encodes for a protein with 93% homology with rat and human CRF (25), whereas *Xenopus* urocortin 1 reveals approximately 70% homology with mammalian urocortin 1 (26). CRF is widely distributed in the *X. laevis* brain, with main CRF-immunoreactive (ir) sites in the nucleus accumbens, nucleus habenularis ventralis, magnocellular nucleus, paraventricular organ, tectum mesencephali, anterior

tegmental nucleus, locus ceruleus and nucleus motorius nervi trigemini (27, 28). By contrast to CRF, urocortin 1 shows a more limited distribution in the *X. laevis* brain, occurring in the magnocellular nucleus (Mg), Edinger–Westphal nucleus, nucleus posteroventralis tegmenti, central grey and nucleus motorius nervi trigemini (28). In the pituitary gland, CRF-ir fibres are scarce, whereas many strongly stained urocortin 1-ir fibres occur in the pars nervosa (28).

The functions of CRF and urocortin 1 in amphibians are less well investigated than in mammals, but CRF in the Mg, the amphibian homologue of the mammalian paraventricular and supraoptic nuclei, exerts its 'traditional' role as a controller of the hypothalamo-hypophyseal-adrenal axis (27, 29–31), and both CRF and urocortin 1 in the Mg may be involved in the control of feeding-related processes (26, 32, 33). Most interesting are the roles of CRF and urocortin 1 in the regulation of the neuroendocrine melanotroph cells in the intermediate lobe of the amphibian pituitary gland, a regulation unknown in mammals to date. Amphibian melanotrophs release α-melanophore stimulating hormone (MSH), a processing product of pro-opiomelanocortin (POMC), which is responsible for skin darkening during the process of background adaptation (34, 35). In *X. laevis*, CRF-like peptides, including *Xenopus* urocortin 1, stimulate the production of POMC and the secretion of α-MSH (28, 36, 37). The mechanism(s) by which amphibian CRF and urocortin 1 exert these actions on melanotroph cells are poorly understood with respect to the receptor(s) and pathways involved. In *X. laevis*, two types of CRF receptor, CRF_1 and CRF_2, have been identified in both the brain and the pituitary gland, using reverse transcription-polymerase chain reaction (RT-PCR). Both receptors share approximately 80% amino acid sequence homology with their mammalian counterparts. CRF_1 binds with high affinity to both CRF and urocortin 1, whereas CRF_2 has a higher affinity for urocortin 1 than for CRF (38–41).

The present study focuses on CRF_1, and aims to identify the possible sites by which CRF and urocortin 1 act on this receptor in the brain and pituitary gland of *X. laevis*. Moreover, in view of the dependence of melanotroph secretory activity in this animal on the external light condition, we hypothesised that this light condition is a physiological factor controlling the expression of melanotroph cell CRF_1. We assessed the presence of this receptor by *in situ* hybridisation of CRF_1 mRNA, and then determined whether this expression in the intermediate pituitary lobe depends on the state of the background light condition (black versus white).

Materials and methods

Animals

Ten young-adult specimens of *X. laevis*, aged 6 months, with a body weight of 28–32 g, were raised in our Nijmegen laboratory under constant illumination, at a water temperature of 22 ± 1 °C, and fed weekly on ground beef heart (Janssen, Nijmegen, The Netherlands) and Trouvit trout pellets (Trouvit, Trouw, Putten, The Netherlands). Before the experiments, animals had been adapted to either a black ($n = 5$) or a white ($n = 5$) background, for 3 weeks. Animal treatment was in agreement with the Declaration of Helsinki and the Dutch law concerning animal welfare, as tested by the ethical committee for animal experimentation of Radboud University Nijmegen.

Tissue preparation for *in situ* hybridisation

Toads were deeply anaesthetised by immersion in 0.1% tricaine methane sulphonate (MS222; Novartis, Basel, Switzerland) in tap water, and transcardially perfused with ice-cold 0.6% sodium chloride, for 5 min. Then they were perfused with 250 ml of ice-cold Bouin's fixative, for 15 min. After decapitation, the brain and pituitary gland were quickly dissected and post-fixed in the same fixative, for 16 h at 4 °C, washed in 70% ethanol for 24 h to eliminate excess of picric acid, dehydrated in a graded ethanol series, and embedded in paraffin. Sagittal serial sections (7 μm) were mounted on poly L-lysine-coated slides (Sigma Chemical, St Louis, MO, USA) and allowed to dry in air, for 16 h at 45 °C, deparaffinised and rehydrated. Brain sections of black-adapted (BA) and white-adapted (WA) animals were processed in parallel for *in situ* hybridisation for *Xenopus* CRF_1 mRNA.

In situ hybridisation

A Greenstar labelled probe was used (GeneDetect, Auckland, New Zealand) and a 3′-digoxigenin (DIG)-labelled antisense oligonucleotide probe was synthesised to hybridise with the *Xenopus* CRF_1. The CRF_1 probe was a synthetic antisense oligonucleotide corresponding to bases 264–311 (TCCTGGCATTGAGCGTAGTCTCCTCTCCCAGCCCAGCTGCCGTTCAGG, 48 mer) of the cDNA sequence of *Xenopus* CRF_1 (38) (GenBank accession no. Y14036). Starting from the same bases, a sense oligonucleotide probe (CCTGAACGGCAGCTGGGCTGGGAGAGGAGACTACGCTCAATGCCAGGA, 48 mer) was synthesised for specificity control tests with *in situ* hybridisation. Computer analysis with the basic local alignment search tool (BLAST) (42) did not reveal any homology or similarity of our probes with other *X. laevis* genes. After deparaffination, sections were rinsed in autoclaved 0.1 M sodium phosphate-buffered saline (PBS; pH 7.4) for 10 min, and postfixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4), for 5 min at 20 °C. After rinsing the sections in PBS for 10 min, acetylation was performed with 0.25% acetic acid anhydride in 0.1 M tri-ethanolamine buffer (pH 8.0), for 10 min, followed by rinsing in twice concentrated (× 2) standard saline citrate buffer (SSC; pH 7.0), for 3 min. Sections were then treated with 0.1% pepsin (Sigma Chemical) in 0.2 N HCl, at 37 °C for 15 min, followed by 2 × 5-min wash in PBS and subsequently incubated in hybridisation mixture containing 20% 20 × SSC, 50% deionised formamide, 0.2 ml of 50 × Denhardt's solution for 20 ml of hybridisation mixture, 10% dextran sulphate, 0.25 mg/ml tRNA, 10% of 1 M DTT and 10% of autoclaved MQ, for 2 h at 37 °C. After rinsing in 2 × SSC for 5 min, sections were incubated in hybridisation mixture with the 3′-DIG-labelled antisense/sense oligonucleotide probe (approximately 100 ng/ml), for 16 h at 37 °C, followed by a quick rinse in SSC containing 1 M DTT, at room temperature. Then, sections were stringently washed twice in 1 × and 0.5 × SSC containing 1 M DTT (Roche, Basel, Switzerland), for 15 min at 55 °C, and in SSC, for 10 min at 20 °C. DIG label was visualised by the alkaline phosphatase (AP) method with nitro-bluetetrazoliumchloride/5-bromo-4-chloro-3-indolyl phosphate-toluidine salt (NBT/BCIP; Roche) as a substrate. In brief, after 3 × 5-min rinses in 0.1 M Tris-buffered saline (TBS; pH 7.5) sections were washed in TBS containing 1% of blocking agent (TBS-BA; Roche), for 30 min, followed by incubation in sheep-anti-DIG-AP (dilution 1 : 200; Roche) in TBS-BA, for 4 h at 20 °C. Sections were then rinsed 3 × 5-min in TBS followed by a 5-min wash in TBS buffer containing 0.05 M $MgCl_2$ (TBS-M; pH 9.5), and hybridisation was visualised after 48 h of incubation in TBS-M containing NBT/BCIP. The reaction was stopped by several rinses in tap water followed by rinses in distilled water. Sections were then mounted in Kaiser's glycerol gelatine (Merck, Darmstadt, Germany) and examined using a Leica DMRBE microscope (Leica Microsystems, Heerbrugg, Switzerland).

Morphometry and statistical analysis

Digital images of five WA and five BA toads were taken using the Leica DMRBE optical system with a Leica DC 500 digital camera (Leica Microsystems) and a ×20 objective lens, and analysed with Scion Image software (version 3.0b; NIH, Bethesda, MD, USA). *In situ* hybridisation staining intensity was determined in three 7-μm sagittal sections per animal, in the middle of: (i) the internal granule cell layer of the olfactory bulb; (ii) the intermediate pituitary; and (iii) the distal pituitary lobe. Values were expressed as means ± SEM of the optical density (OD). Means per adaptation state were analysed with analysis of variance ($\alpha = 5\%$), using Statistica (StatSoft, Tulsa, OK, USA), after testing for homogeneity of variance (Bartlett's test) (43) and normality (44).

Results

Distribution of CRF_1 mRNA in the brain and pituitary gland

With the antisense mRNA probe, CRF_1 mRNA hybridisation signals were seen throughout the brain in the neuronal perikarya, with variable staining intensities, whereas the neuronal cell nucleus, axons, axon terminals and other elements in the brain, like glial cells and blood vessels, are devoid of signal (Fig. 1A). The absence of any background staining and of staining with the sense probe (control) (Fig. 1B), indicates that the signal specifically reveals the presence of CRF_1 mRNA. The occurrence of the CRF_1 mRNA hybridisation signal in a brain structure was further studied in serial sagittal sections (Fig. 2) and the different hybridisation intensities, assessed by visual observation and semiquantitatively expressed as 'low', 'moderate' and 'strong', are summarised in Table 1.

Distribution of CRF_1 mRNA

In the telencephalon, the most rostral perikarya expressing CRF_1 mRNA are situated in the olfactory bulb (Fig. 2), where the internal granule cell layer surrounding the lateral ventricle shows a strong signal (Fig. 3A). In the latero-dorsal and latero-ventral pallium, cells are moderately stained, whereas no signal was observed in the mitral cell layer and postolfactory eminence. In the nucleus accumbens a moderate expression signal is present (Fig. 3B). In the medial and lateral part of the amygdala, scattered cells were found with low expression levels of CRF_1 mRNA (Fig. 3C).

In the diencephalon, a rostral group of neurones showing moderate hybridisation signals is situated in the anterior preoptic area of the hypothalamus (Figs 2 and 3D). A low expression signal occurs in the small cell group in the dorsal and ventral habenular nuclei. No signal was detected in the Mg. More caudally, moderate expression of CRF_1 mRNA occurs in cells scattered in the suprachiasmatic nucleus (Fig. 3D) and in the parallel cell layers of the ventromedial thalamic nucleus (Fig. 3E). In the posterior tubercle, some slightly stained cells are present, whereas in the infundibular area, strong CRF_1 mRNA staining is shown by the ventral hypothalamic nucleus (Fig. 3F).

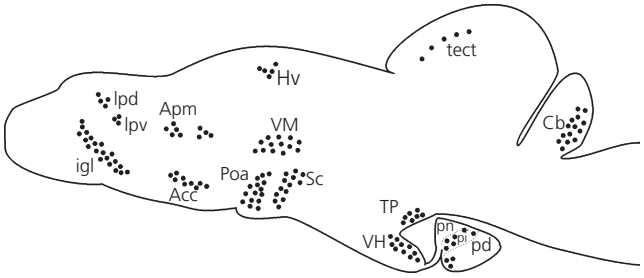

Fig. 2. Schematic sagittal view of the brain of *Xenopus laevis*. The locations of corticotrophin-releasing factor receptor type 1 mRNA-positive neurones as visualised by *in situ* hybridisation are indicated by black dots. Acc, Nucleus accumbens; Apl, amygdala, pars lateralis; Apm, amygdala, pars medialis; Cb, cerebellum; Hv, nucleus habenularis ventralis; igl, internal granule cells of the olfactory bulb; lpd, latero-dorsal pallium; lpv, latero-ventral pallium; pd, pituitary gland, pars distalis; pi, pituitary gland, pars intermedia; pn, pituitary gland, pars nervosa; Poa, preoptic area; SC, suprachiasmatic nucleus; tect, mesencephalic tectum; TP, posterior tubercle; VH, ventral hypothalamic nucleus; VM, ventromedial thalamic nucleus.

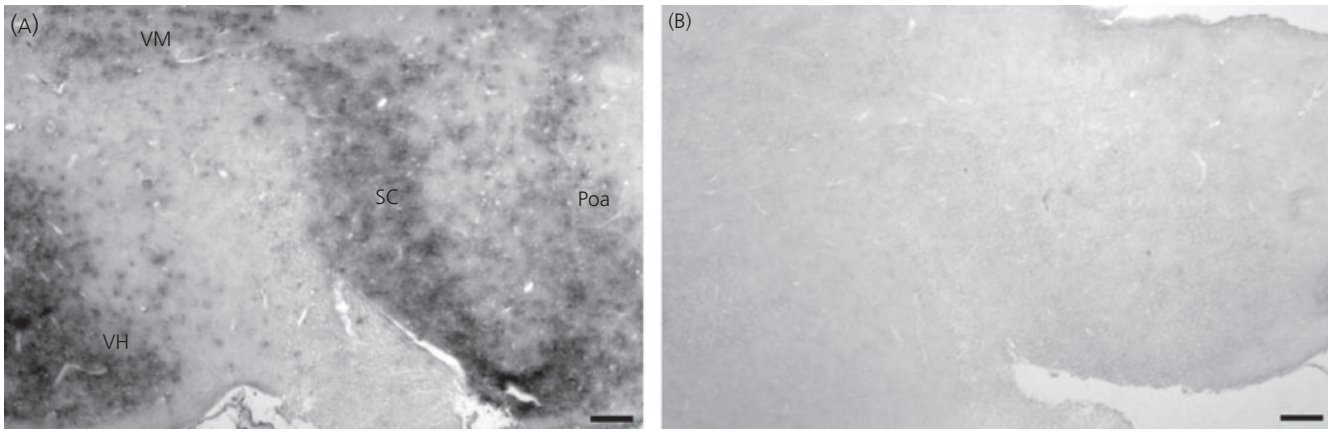

Fig. 1. Overview of *in situ* hybridisation of corticotrophin-releasing factor receptor type 1 mRNA in the brain of *Xenopus laevis*, at the level of the diencephalon (sagittal section), with various positive regions such as the preoptic area (Poa), suprachiasmatic nucleus (SC), ventromedial thalamic nucleus (VM) and ventral hypothalamic area (VH), with an antisense oligonucleotide probe (A), and lack of hybridisation signal with the complementary sense probe (B). Scale bar = 100 μm.

Table 1. Distribution of Corticotrophin-Releasing Factor Receptor Type 1 (CRF_1) mRNA, CRF- and Urocortin 1-Immunoreactive (ir) Cells and Fibres in the *Xenopus laevis* Brain and Pituitary Gland, as Visualised by *In Situ* Hybridisation and Immunocytochemistry (27, 28).

		CRF-ir (27, 28)		Urocortin 1-ir (28)	
Brain region	CRF_1 mRNA	Cells	Fibres	Cells	Fibres
Telencephalon					
Olfactory bulb, internal granule cells (igl)	+++	+	+	–	+
Mitral cell layer (ml)	–	++	+	–	–
Medial olfactory tract (mot)	–	–	–	–	+
Post-olfactory eminence (pe)	–	++	–	–	–
Medial septum (ms)	–	–	+	–	+
Lateral septum (ls)	–	–	++	–	–
Latero-dorsal/ventral pallium (lpd, lpv)	++	–	+	–	–
Dorsal pallium (dp)	–	–	+	–	+
Medial pallium (mp)	–	–	+	–	–
Striatum (Str)	–	+	–	–	+
Accumbens (Acc)	++	+++	+	–	+
Diagonal band of Broca (DB)	–	–	+	–	+
Amygdala pars medialis (Apm)	+	+	+	–	–
Amygdala pars lateralis (Apl)	+	++	+	–	–
Anterior commissure (ac)	–	–	+	–	–
Diencephalon					
Preoptic area (Poa)	++	++	+	–	–
Nucleus habenularis ventralis (Hv)	++	+++	–	–	–
Ventromedial thalamic nucleus (VM)	++	+	–	–	–
Anterior thalamic nucleus (A)	–	+	–	–	+
Central thalamic nucleus (C)	–	–	–	–	+
Posterior thalamic nucleus (P)	–	–	–	–	+
Magnocellular nucleus, medial part (Mgm)	–	++	+	++	+
Magnocellular nucleus, ventral part (Mgv)	–	++	+	+++	+
Magnocellular nucleus, dorsal part (Mgd)	–	–	–	–	–
Suprachiasmatic nucleus (SC)	++	+	+	–	–
Paraventricular organ (NPv)	–	++	+	–	–
Posterior tubercle (TP)	+	+	+	–	+
Ventral hypothalamic nucleus (VH)	+++	+	+	–	–
Median eminence zona interna (zi)	–	–	+	–	+
Median eminence zona externa (ze)	–	–	+	–	+
Pituitary pars nervosa (pn)	–	–	+	–	+
Pituitary pars intermedia (pi)	s.d.	–	–	–	–
Pituitary pars distalis (pd)	++	–	–	–	–
Mesencephalon					
Tectum mesencephali (tect)	++	+++	+	–	+
Tegmentum mesencephali (tegm)	–	–	+	–	+
Edinger–Westphal nucleus (EW-N)	–	–	+	+++	+
Anterior tegmental nucleus (Avenue, Ad)	–	++	+	–	–
Posterior commissure (pc)	–	–	+	–	–
Nucleus posteroventralis tegmenti (pv)	–	+	–	++	–
Torus semicircularis (Tor)	–	–	+	–	+
Rhombencephalon	–	–	–	–	–
Cerebellum (Cb)	+++	–	+	–	–
Locus coeruleus (Lc)	–	++	+	–	–
Central grey (cg)	–	–	–	++	–
Nucleus reticularis medius (Rm)	–	–	–	+	–
Cochlear nucleus (LL)	–	–	–	–	+
Nucleus motorius nervi trigemini (Vm)	–	++	+	++	–
Nucleus motorius of the facial and glossopharingeal nerve (IX)	–	–	–	++	+
Nucleus motorius nervi vagi (Xm)	–	–	–	++	–

For *in situ* hybridisation, ratings reflect the intensity of positively labelled cells as visually observed in five white- and five black-adapted animals (+, low; ++, moderate; and +++, strong intensity of staining); for immunocytochemistry, the number of positive cells is indicated as follows: one to four cell bodies (+), five to eight cell bodies (++) and eight to 20 cell bodies (+++). Immunoreactive fibres are indicated by (+). (–) indicates a complete lack of labelling; sd, stimulus-dependent staining.

In the dorsal mesencephalon, several piriform cell layers of the optic tectum reveal hybridisation signals with varying intensities, ranging from moderate in the internal layers to strong in the external layers 5 and 6 (Fig. 3G).

In the rhombencephalon, strong CRF_1 mRNA expression was encountered in the cerebellum (Fig. 3H).

In the pars nervosa of the pituitary lobe, no hybridisation signal was observed (Figs 2 and 4A), but strong staining is present in the melanotroph cells of the intermediate lobe (Fig. 4B,D). In the distal lobe, a moderate to strong CRF_1 mRNA expression signal occured in a rostral cluster of endocrine cells (Fig. 4E). In view of their position, it is possible that these cells are corticotrophs, but this idea awaits confirmation.

Differences between CRF_1 mRNA expression as a result of different adaptation states

To determine whether and to what extent the expression of CRF_1 mRNA in pituitary melanotroph cells is physiologically regulated by the background light condition, we measured mRNA hybridisation intensities in WA ($n = 5$) and BA ($n = 5$) *X. laevis*, and expressed them as optical density (OD) in arbitrary units. As controls, we studied two other structures (Fig. 2, Table 1) rich in CRF_1 mRNA but not known to be involved in the process of background light adaptation: (i) the internal granule cell layer of the olfactory bulb (igl) and (ii) the presumed corticotroph cells in the distal lobe.

As can be readily seen at low magnification (Fig. 4A,B), the intermediate lobe of the pituitary gland in BA animals is much larger than in WA toads due to hyperplasia of the melanotroph cells, which are the only endocrine cells in the intermediate lobe (45). On the other hand, the intensity of the CRF_1 mRNA hybridisation signal

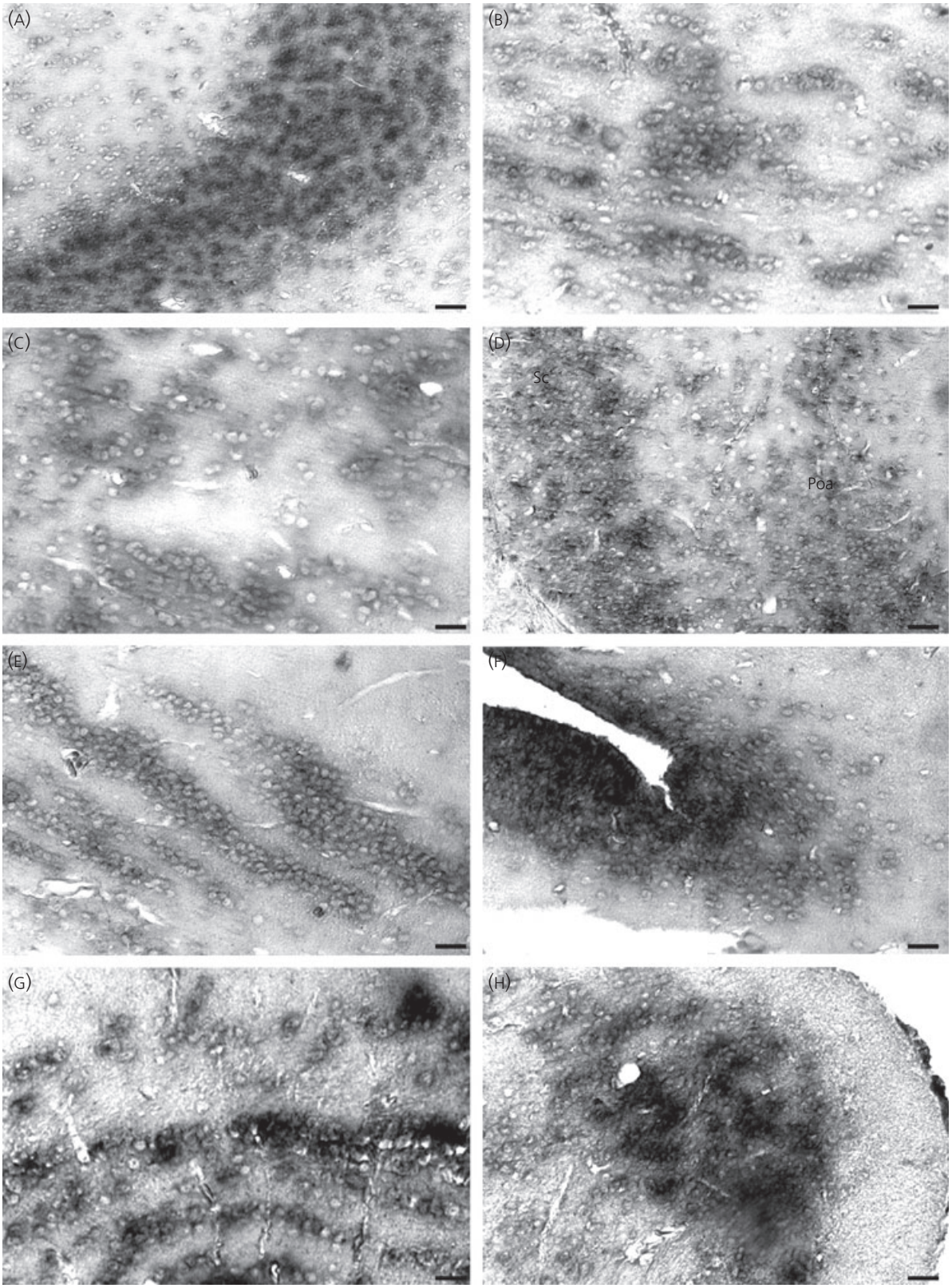

Fig. 3. Sagittal sections of the brain and pituitary gland of *Xenopus laevis*, showing corticotrophin-releasing factor receptor type 1 mRNA hybridisation in (A) internal granule cell layer of the olfactory bulb, (B) nucleus accumbens, (C) medial part of the amygdala, (D) preoptic area of the hypothalamus (Poa), suprachiasmatic nucleus (Sc), (E) ventromedial thalamic nucleus, (F) ventral hypothalamic area, (G) tectum mesencephali and (H) cerebellum. Scale bar = 50 μm in (A,D) and 20 μm in (B,C,E–H).

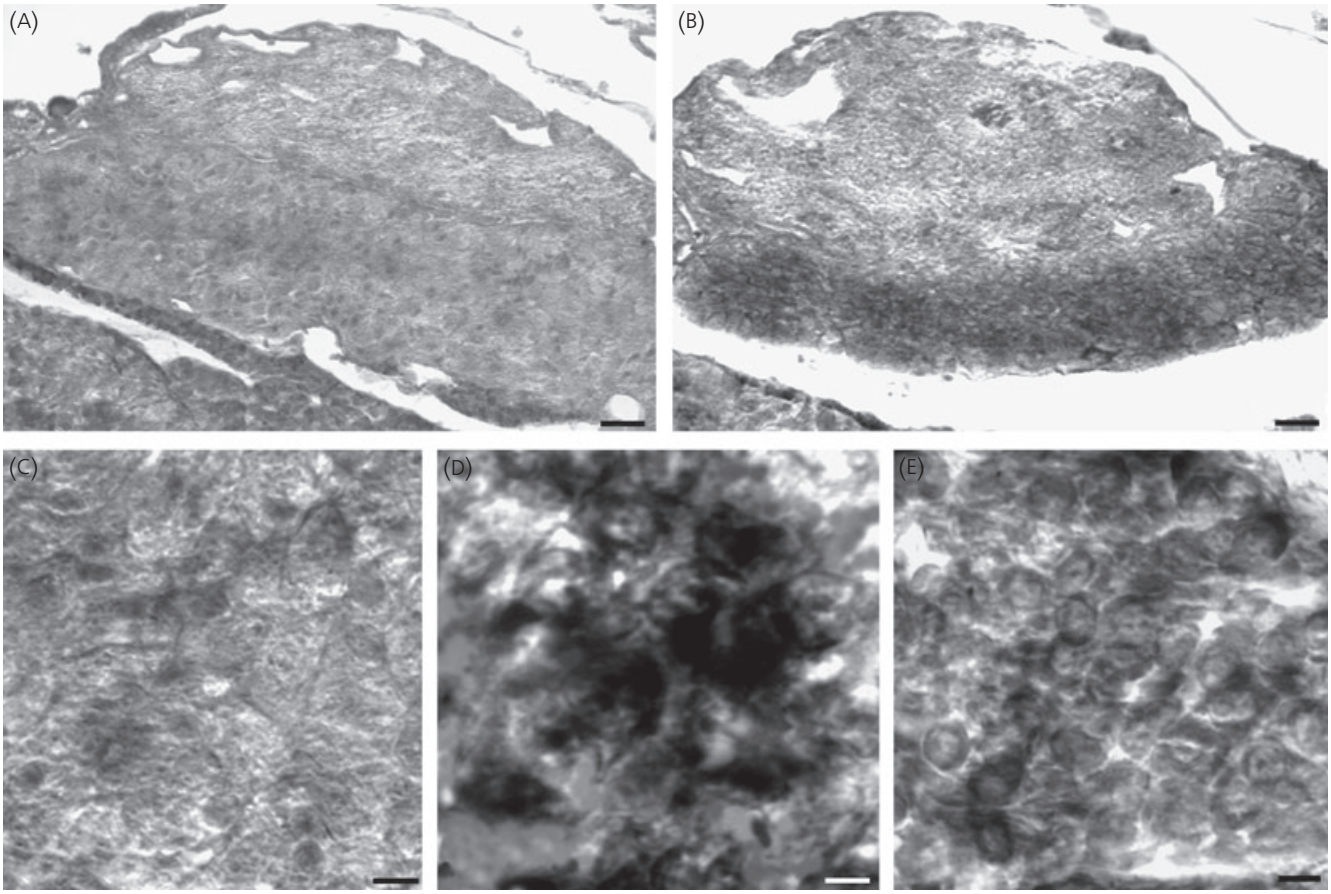

Fig. 4. Corticotrophin-releasing factor receptor type 1 mRNA in the pituitary gland (sagittal section) of *Xenopus laevis*. In the melanotroph cells in the pars intermedia (pi), hybridisation is weak in a black-adapted animal (A, detail in C) but strong in a white-adapted one (B, detail in D). In the pars nervosa (pn), no signal is present (A). Only some cells of the pars distalis (pd) show a positive hybridisation signal (E). Scale bar = 50 μm in (A,B); 30 μm in (C); and 20 μm in (D–F).

in the intermediate lobe of WA animals is much higher than in BA animals (Fig. 4B,D). Worthy of note, in WA animals, the intensity of the hybridisation signal differs among individual melanotroph cells, being moderate in some cells, but strong or even very strong in others. In BA animals, all cells are stained with the same, rather weak intensity (Fig. 4A,C). No effect of background light intensity on the expression of CRF_1 in the brain was noted.

This stimulatory effect of white background adaptation on, specifically, the expression of CRF_1 mRNA in the melanotroph cells is apparent in the morphometric study. In both the igl and in the distal lobe, the OD of the CRF_1 mRNA hybridisation signal does not differ between BA and WA animals (Fig. 5) but the melanotroph cells in the intermediate pituitary lobe of WA animals show a 59.4% higher OD than melanotrophs of BA animals ($P < 0.05$; Fig. 5).

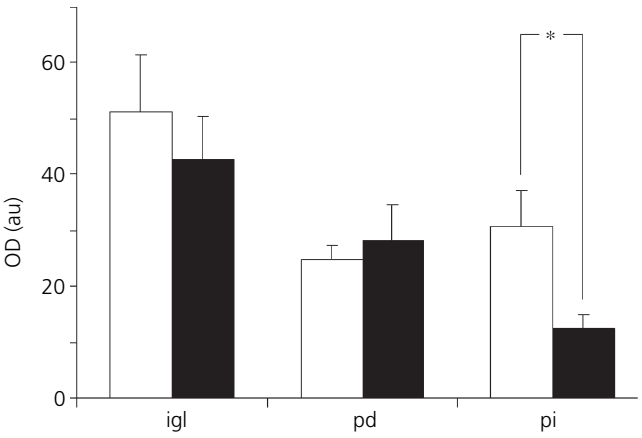

Fig. 5. Effect of background adaptation on the optical density (OD) expressed as arbitrary units (au) of corticotrophin-releasing factor receptor type 1 *in situ* hybridisation signals in internal granule cell layer of the olfactory bulb (igl), and distal (pd) and intermediate lobe (pi) of the pituitary gland. White bars indicate white-adapted and black bars indicate black-adapted *Xenopus*, respectively. Values are expressed as means ± SEM. *Significant difference between the two adaptation states ($P < 0.05$).

Discussion

Technical considerations

With *in situ* hybridisation using a DIG-labelled oligonucleotide probe, we describe, for the first time, the detailed distribution of

mRNA encoding CRF_1 in the brain and in the pituitary gland of a nonmammalian vertebrate, the South African clawed toad *X. laevis*. The reasons for using an oligonucleotide probe instead of a ribonucleotide probe are: (i) the higher stability (resistance to RNAse degradation) compared to an RNA probe; (ii) the small molecular size of the oligonucleotide, which provides better tissue penetration, and (iii) the fact that oligonucleotides do not self-hybridise.

Comparison of CRF_1 mRNA distribution in the brain of amphibians with mammals

The general existence of CRF_1 in the brain of *X. laevis* was previously assumed on the basis of RT-PCR (38, 41), but no information was available regarding the brain structures expressing this receptor. This is the first report to describe the presence of CRF_1 mRNA and hence, most probably, of CRF_1, in the telencephalon, diencephalon and brainstem of *X. laevis*.

The distribution of the CRF_1 mRNA in the *Xenopus* brain, as detected by using a DIG-labelled oligonucleotide probe, appears to be substantially less extensive than that of CRF_1 mRNA in the mammalian brain (23). For example, in the rat telencephalon, CRF_1 mRNA occurs in the periglomerular layer, mitral layer and olfactory tubercle, as well as in the hippocampus, diagonal band of Broca and bed nucleus of the stria terminalis (23), which are all regions that are devoid of CRF_1 mRNA in *X. laevis*. In the rat diencephalon, CRF_1 mRNA is present in many thalamic areas such as the laterodorsal and latero-posterior nucleus and the lateral geniculate nucleus whereas, in *Xenopus*, only the ventromedial thalamic nucleus shows CRF_1 mRNA expression. Furthermore, CRF_1 mRNA has been observed in the rat brainstem in many sensory relay structures whereas, in the toad, only the tectum mesencephali and the cerebellum reveal CRF_1 mRNA hybridisation. The wider distribution of this receptor mRNA in the rat suggests that its ligands CRF and urocortin 1 have acquired novel central functions during evolution. It might be argued that our DIG-labelling technique could be less sensitive than radioactive *in situ* hybridisation methods, which might be the cause of the different expression patterns of CRF_1 mRNA between rat and toad. Although our preliminary results on CRF_1 receptor studies in the mouse do not support this idea, because either method yielded essentially the same results (A. Korosi, unpublished data), only future quantitative comparisons with both methods between *Xenopus* and rat, will definitively solve this issue.

On the other hand, the distributions of CRF_1 mRNA in the brain of *X. laevis* and of mammals show some clear similarities. In *X. laevis*, going from rostral to caudal, strong CRF_1 hybridisation signals occur in the internal granule cell layer of the olfactory bulb, the ventral hypothalamic area, the tectum mesencephali and the cerebellum. The homologous nuclei in the rat brain (i.e. the granule cell layer of the olfactory region, the arcuate nucleus, the superior colliculus and the granule layer of the cerebellum) also express CRF_1 signals (23). Furthermore, no CRF_1 mRNA signal has been observed either in the Mg of *X. laevis* or in its rat homologue, the PVN (23). These similarities between CRF_1 locations in *Xenopus* and the rat indicate that the presence/absence and functions of this receptor type are strongly evolutionary conserved.

Relationship between CRF_1 mRNA and CRF/urocortin 1 peptide distributions

The *in situ* hybridisation signal for CRF_1 mRNA is a strong indication for the local existence of CRF_1 protein. Because *in vitro* studies have shown that both *Xenopus* CRF and urocortin 1 bind with high affinity to *Xenopus* CRF_1 (41), it is of functional interest to assess whether the CRF_1 mRNA expressions described in the present study match the descriptions of CRF- and urocortin 1-ir fibres and perikarya (27, 28). Table 1 shows our description of the distribution of CRF_1 mRNA in *Xenopus*, as well as data from the literature on the distribution of CRF and urocortin 1 peptides in this animal (27, 28). As can be seen, both CRF- and urocortin 1-ir elements occur in the olfactory bulb, nucleus accumbens, posterior tubercle and tectum mesencephali, suggesting that, in these four areas, both CRF and urocortin 1 can act on CRF_1. However, taking a more general view, in most brain structures, CRF_1 mRNA coexists with CRF-ir fibres (but not with urocortin 1-ir fibres), supporting the notion that CRF is the endogenous ligand for CRF_1, and that urocortin 1 binds preferably to CRF_2 (26, 38). In the nucleus habenularis ventralis, CRF_1 mRNA expression occurs in CRF-ir perikarya. Here, ultrastructural studies may be helpful to determine whether the same cells both release CRF and possess CRF_1, which would indicate an autocrine action of CRF.

These data taken together, support the idea that CRF and urocortin 1 act on CRF_1 receptors in the *Xenopus* brain. Whether the absence of CRF_1 mRNA in CRF- and/or urocortin 1-peptide-containing brain structures points to actions of these ligands on CRF_2, or to an action of these ligands in other areas of the brain that are reached by volume transmission or via axons that cannot be revealed at the light microscope level, remains to be established.

Physiological regulation of CRF_1 mRNA expression in the amphibian intermediate pituitary lobe

The present study demonstrates for the first time that CRF_1 occurs in the intermediate lobe of the pituitary gland of a nonmammalian species. The presence of CRF_1 in the intermediate lobe of *X. laevis* extends the previous general demonstration by RT-PCR of this receptor type in the total pituitary gland (38) because it reveals that CRF_1 is restricted to the melanotroph cells of the intermediate lobe. Recently, we showed the presence of CRF and urocortin 1 peptides in the median eminence and in the pars nervosa of the pituitary lobe of *Xenopus* and revealed, by *in vitro* superfusion studies, that both peptides stimulate the release of α-MSH from melanotroph cells (28, 37). We now show an up-regulation of CRF_1 mRNA in WA toads compared to BA animals. This receptor up-regulation is specific for the melanotroph cells because no effect of the background illumination state was visible in the igl or in the distal pituitary lobe. Our demonstration of CRF_1 mRNA in the intermediate lobe fits in with the fact that the Mg, projecting to neurohemal areas in the median eminence and the pituitary pars

nervosa, is controlling melanotroph cell secretory activity via the release of CRF and urocortin 1, both peptides stimulating α-MSH release *in vitro* (28, 37). Interestingly, in WA, the strength of the CRF_1 mRNA signal is not homogeneous throughout the gland, suggesting that some cells produce more receptors than others. This result supports the notion that the intermediate lobe of the pituitary gland is composed of a heterogeneous cell population with respect to size (45) and POMC production (46).

To date, the factors controlling the release of CRF and urocortin 1 release from the pituitary pars nervosa are not known. In the present study, we show that adaptation of *Xenopus* to a white background up-regulates the expression of CRF_1 mRNA. This finding suggests that, in these WA animals, melanotrophs become highly sensitive to CRF and urocortin 1 such that, when placed on a black background, their high receptor density enables cells to rapidly mobilise stored α-MSH to the secretory process. In this way, the animals can turn black quickly, permitting fast camouflage. Indeed, in line with these results, Verburg-van Kemenade *et al.* (37) reported that ovine CRF evokes stronger *in vitro* α-MSH release from neurointermediate lobes of WA than BA toads. For *X. laevis* melanotrophs, a similar situation has been described with respect to thyrotrophin-releasing hormone-receptor type 3 mRNA, and for neuropeptide Y (NPY) Y1 receptor mRNA, which are both up-regulated in WA animals (47, 48). Similar to CRF and urocortin 1, TRH simulates α-MSH release, whereas NPY inhibits this release (49, 50).

Further studies on the dynamics of the Mg and the *in vivo* conditions regulating the activity of this nucleus, as well as on the possible presence and regulation of CRF_2 receptors in the pituitary gland, may elucidate the plastic interactions between CRF and urocortin 1 and the receptors crucial to the functioning of the neuroendocrine reflex mechanism by which melanotroph cells in amphibians control the process of skin colour adaptation to background illumination. We propose that this mechanism is an example of neuroendocrine plasticity crucial to adaptation processes in general and to adaptation to stressful environmental events in particular.

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