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Research report

Localisation and Physiological Regulation of CRF Receptor 1 mRNA in the *Xenopus laevis* Brain and Pituitary Gland

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Short title: CRF₁ in the *Xenopus laevis* brain and pituitary gland

Keywords: Corticotropin-releasing factor receptor 1; *Xenopus laevis*; Melanotrope cells; *In situ* hybridisation, α -Melanophore-stimulating hormone, urocortin

1. Abstract

In *Xenopus laevis*, corticotropin-releasing factor (CRF) and Urocortin 1 (Ucn1) are present in the brain and they both are potent stimulators of α -melanophore-stimulating hormone (α MSH) secretion by melanotrope cells in the pituitary gland. Since in mammals and *Xenopus laevis* both CRF and Ucn1 bind with high affinity to CRF receptor type 1 (CRF₁), one of the purposes of this study was to identify the sites of action of CRF and Ucn1 in the *Xenopus* brain and pituitary gland. Moreover, we raised the hypothesis that the external light intensity is a physiological condition controlling CRF₁ expression in the pituitary melanotrope cells. By *in situ* hybridisation we have demonstrated the presence of CRF₁ mRNA 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, CRF₁ mRNA occurs in the intermediate and distal lobe. The optical density of the CRF₁ 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 our hypothesis that the environmental light condition controls CRF₁ mRNA expression in melanotrope cells of *X. laevis*, a mechanism likely responsible for CRF- and/or Ucn1-stimulated secretion of α MSH.

2. Introduction

In the mammalian central nervous system (CNS), corticotropin-releasing factor (CRF) [1] and other members of the CRF-family of peptides, such as the urocortins (Ucn) 1, 2 and 3 [2-8] mediate various physiological, behavioural and immune responses to stressful challenges [e.g. 9-18]. The actions of CRF and Ucn are mediated by two G-protein-coupled receptors designated CRF-receptor 1 (CRF₁) and CRF-receptor 2 (CRF₂) [19,20], which have been localised in the brain by *in situ* hybridisation and receptor autoradiography [21-24]. CRF₁ 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 CRF₂ 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* Ucn1 reveals about 70% homology with mammalian Ucn1 [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]. In contrast to CRF, Ucn1 shows a more limited distribution in the *X. laevis* brain, occurring in the magnocellular nucleus (Mg), Edinger-Westphal nucleus (EW), nucleus posteroventralis tegmenti, central grey and nucleus motorius nervi trigemini [28]. In the pituitary gland, CRF-ir fibres are scarce, whereas many strongly stained Ucn1-ir fibres occur in the pars nervosa [28].

The functions of CRF and Ucn1 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 controller of the hypothalamo-hypophyseal-adrenal axis [27,29-31], and both CRF and Ucn1 in the Mg may be involved in

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4 the control of feeding-related processes [26,32,33]. Most interesting are the roles of CRF and
5
6 Ucn1 in the regulation of the neuroendocrine melanotrope cells in the intermediate lobe of
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8 the amphibian pituitary gland, a regulation up to now unknown in mammals. Amphibian
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10 melanotropes release α -melanophore-stimulating hormone (α MSH), a processing product of
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12 proopiomelanocortin (POMC), which is responsible for skin darkening during the process of
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14 background adaptation [*e.g.* 34,35]. In *X. laevis* CRF-like peptides, including *Xenopus* Ucn1,
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16 stimulate the production of POMC and the secretion of α MSH [28,36,37]. The mechanism(s)
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18 by which amphibian CRF and Ucn1 exert these actions are only fragmentary known. In *X.*
19
20 *laevis*, two types of CRF receptor, CRF₁ and CRF₂, have been identified in both the brain and
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22 the pituitary gland, using RT-PCR. Both receptors share ca. 80% amino acid sequence
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24 homology with their mammalian counterparts, CRF₁ binds with high affinity to both CRF and
25
26 Ucn1, whereas CRF₂ has a higher affinity for Ucn1 than for CRF [38-41].
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29 The present study focuses on CRF₁, and aims to identify the possible sites by which
30
31 CRF and Ucn1 act on this receptor in the brain and pituitary gland of *Xenopus laevis*.
32
33 Moreover, in view of the dependence of melanotrope secretory activity in this animal on the
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35 external light condition, we hypothesised that this light condition is a physiological factor
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37 controlling the expression of melanotrope cell CRF₁. We have assessed the presence of this
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39 receptor by *in situ* hybridisation of CRF₁ mRNA, and then we determined if this expression in
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41 the intermediate pituitary lobe depends on the state of the background light condition (black
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43 vs. white).
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45 46 47 **3. Materials and Methods** 48

49 50 51 **3.1. Animals** 52

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54 Ten young-adult specimens of *Xenopus laevis*, aged 6 months, with a body weight of
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56 28-32 g, were raised in our Nijmegen laboratory under constant illumination, at a water
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58 temperature of $22 \pm 1^\circ\text{C}$, and fed weekly on ground beef heart (Janssen, Nijmegen, The
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60 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,

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4 for three weeks. Animal treatment was in agreement with the Declaration of Helsinki and the
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6 Dutch law concerning animal welfare, as tested by the ethical committee for animal
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8 experimentation of Radboud University Nijmegen.
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10 11 12 3.2. Tissue preparation for *in situ* hybridisation 13

14 Toads were deeply anaesthetised by immersion in 0.1% tricaine methane sulfonate
15 (MS222; Novartis, Basel, Switzerland) in tap water, and transcardially perfused with ice-cold
16 0.6% sodium chloride, for 5 min. Then they were perfused with 250 ml ice-cold Bouin's
17 0.6% sodium chloride, for 5 min. Then they were perfused with 250 ml ice-cold Bouin's
18 fixative, for 15 min. After decapitation, the brain and pituitary gland were quickly dissected
19 and postfixed in the same fixative, for 16 h at 4°C, washed in 70% ethanol for 24 h, to
20 eliminate excess of picric acid, dehydrated in a graded ethanol series, and embedded in
21 paraffin. Coronal serial sections (7 µm) were mounted on poly-L-lysine-coated slides (Sigma
22 Chemical, St Louis, MO, USA) and allowed to air-dry, for 16 h at 45°C, deparaffinised and
23 rehydrated. Brain sections of black-adapted (BA) and white-adapted (WA) animals were
24 processed in parallel for *in situ* hybridisation for *Xenopus* CRF₁ mRNA.
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37 3.3. *In situ* hybridisation 38

39 A Greenstar labelled probe was used (GeneDetect, Auckland, New Zealand) and a 3'-
40 digoxigenin (DIG)-labelled antisense oligonucleotide probe was synthesised to hybridise with
41 the *Xenopus* CRF₁. The CRF₁ probe was a synthetic antisense oligonucleotide corresponding
42 to bases 264-311
43 (TCCTGGCATTGAGCGTAGTCTCCTCTCCCAGCCCAGCTGCCGTTTCAGG, 48 mer) of
44 the cDNA sequence of *Xenopus* CRF₁ [38] (Genbank accession no. Y14036). Starting from
45 the same bases, a sense oligonucleotide probe
46 (CCTGAACGGCAGCTGGGCTGGGAGAGGAGACTACGCTCAATGCCAGGA, 48 mer)
47 was synthesised for specificity control tests with *in situ* hybridisation. Computer analysis
48 with the basic local alignment search tool (BLAST) [42] did not reveal any homology or
49 similarity of our probes with other *X. laevis* genes. After deparaffination, sections were rinsed
50 in autoclaved 0.1 M sodium phosphate-buffered saline (PBS; pH 7.4) for 10 min, and
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4 postfixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.4), for 5 min
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6 at 20°C. After rinsing the sections in PBS for 10 min, acetylation was performed with 0.25%
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8 acetic acid anhydride in 0.1 M tri-ethanolamine buffer (pH 8.0), for 10 min, followed by
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10 rinsing in 2 times concentrated (X2) standard saline citrate buffer (SSC; pH 7.0), for 3 min.
11
12 Sections were then treated with 0.1% pepsin (Sigma Chemical) in 0.2 N HCl, at 37°C for 15
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14 min, followed by 2 x 5 min wash in PBS and subsequently incubated in hybridisation mixture
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16 containing 4 ml X20 SSC, 10 ml deionised formamide, Denhardt's solution, 10% dextran
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18 sulfate, 0.25 mg/ml tRNA, 2 ml 1M DTT and 2 ml autoclaved MQ, for 2 h at 37°C. After
19
20 rinsing in X2 SSC for 5 min, sections were incubated in hybridisation mixture with the 3'-
21
22 DIG-labelled antisense/sense oligonucleotide probe (ca. 100 ng/ml), for 16 h at 37°C,
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24 followed by a quick rinse in SSC containing 1M DTT, at room temperature. Then, sections
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26 were stringently washed twice in X1 and X0.5 SSC containing 1M DTT (Roche, Basel,
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28 Switzerland), for 15 min at 55°C, and in SSC, for 10 min at 20°C. DIG label was visualised
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30 by the alkaline phosphatase (AP) method with nitro-bluetetrazoliumchloride/5-bromo-4-
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32 chloro-3-indolyl phosphate-toluidine salt (NBT/BCIP; Roche) as a substrate. In brief, after 3
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34 x 5 min rinses in 0.1 M Tris-buffered saline (TBS; pH 7.5) sections were washed in TBS
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36 containing 1% of blocking agent (TBS-BA; Roche), for 30 min, followed by incubation in
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38 sheep-anti-DIG-AP (1:200; Roche) in TBS-BA, for 4 h at 20°C. Sections were then rinsed 3
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40 x 5 min in TBS followed by a 5 min wash in TBS buffer containing 0.05M MgCl₂ (TBS-M;
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42 pH 9.5), and after 48 h incubation hybridisation was visualised in TBS-M containing
43
44 NBT/BCIP. The reaction was stopped by several rinses in tap water followed by rinses in
45
46 distilled water. Sections were then mounted in Kaiser's glycerol gelatine (Merck, Darmstadt,
47
48 Germany) and examined using a Leica DMRBE microscope (Leica Microsystems,
49
50 Heerbrugg, Switzerland).
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55 3.4. Morphometry and statistical analysis

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57 Digital images of 5 WA and 5 BA toads were taken using the Leica DMRBE optical
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59 system with a Leica DC 500 digital camera (Leica Microsystems) and a x20 objective lens,
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and analysed with Scion Image software (version 3.0b; NIH, Bethesda, MD, USA). *In situ*

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4 hybridisation staining intensity was determined in three 7 μm sagittal sections per animal, in
5
6 the middle of (a) the internal granule cell layer of the olfactory bulb, (b) the intermediate
7
8 pituitary and (c) the distal pituitary lobe. Values were expressed as means of the optical
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10 density (OD) \pm standard error of the mean (SEM). Means per adaptation state were analysed
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12 with analysis of variance ($\alpha=5\%$), using Statistica (StatSoft, Tulsa, OK, USA), after testing
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14 for homogeneity of variance (Bartlett's test) [43] and normality [44].
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16 17 18 **4. Results**

19 20 21 22 *4.1. Distribution of CRF₁ mRNA in the brain and pituitary gland*

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24 With the anti-sense mRNA probe, CRF₁ mRNA hybridisation signals were seen
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26 throughout the brain in the neuronal perikarya, with variable staining intensities, whereas the
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28 neuronal cell nucleus, axons, axon terminals and other elements in the brain like glial cells,
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30 and blood vessels are devoid of signal (Fig. 1a). The absence of any background staining and
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32 of staining with the sense probe (control) (Fig. 1b), indicates that the signal specifically
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34 reveals the presence of CRF₁ mRNA. The occurrence of the CRF₁ mRNA hybridisation
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36 signal in a brain structure was further studied in serial sagittal sections (Fig. 2) and the
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38 different hybridisation intensities, assessed by visual observation and semiquantitatively
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40 expressed as 'low', 'moderate' and 'strong', are summarised in Table 1. The detailed
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42 distribution of CRF₁ mRNA is as follows.
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46 In the telencephalon, the most rostral perikarya expressing CRF₁ mRNA are situated in
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48 the olfactory bulb (Fig. 2), where the internal granule cell layer surrounding the lateral
49
50 ventricle shows a strong signal (Fig. 3a). In the latero-dorsal and latero-ventral pallium, cells
51
52 are moderately stained, whereas in the mitral cell layer and post-olfactory eminence no signal
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54 was observed. In the nucleus accumbens a moderate expression signal is present (Fig. 3b),
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56 whereas in the medial and lateral part of the amygdala scattered cells were found with low
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58 expression levels of CRF₁ mRNA (Fig. 3c).

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60 In the diencephalon, a rostral group of neurones showing moderate hybridisation signals
is situated in the anterior preoptic area of the hypothalamus (Figs. 2,3d). A low expression

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4 signal occurs in small cell group in the dorsal and ventral habenular nuclei. No signal was
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6 detected in the Mg. More caudally, moderate expression of CRF₁ mRNA occurs in cells
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8 scattered in the suprachiasmatic nucleus (Fig. 3d) and in the parallel cell layers of the
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10 ventromedial thalamic nucleus (Fig. 3e). In the posterior tubercle some slightly stained cells
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12 are present, whereas in the infundibular area, strong CRF₁ mRNA staining is shown by the
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14 ventral hypothalamic nucleus (Fig. 3f).
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17 In the dorsal mesencephalon, several piriform cell layers of the optic tectum reveal
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19 hybridisation signals with varying intensities, ranging from moderate in the internal layers to
20
21 strong in the external layers 5 and 6 (Fig. 3g).
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24 In the rhombencephalon, strong CRF₁ mRNA expression was encountered in the
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26 cerebellum (Fig. 3h).
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29 *Pituitary gland.* In the pars nervosa of the pituitary lobe no hybridisation signal was
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31 observed (Figs. 2,4a), but strong staining is present in the melanotrope cells of the
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33 intermediate lobe (Fig. 4b,d). In the distal lobe, a moderate to strong CRF₁ mRNA expression
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35 signal occurs in a rostral cluster of endocrine cells. In view of their position, they most likely
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37 are corticotrope cells (Fig. 4e).
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40 Differences between CRF₁ mRNA expression as a result of different adaptation states
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42 are dealt with in the next paragraph.
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44 4.2 Effect of background adaptation on the expression of CRF₁ mRNA

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46 In order to determine whether and to what extent the expression of CRF₁ mRNA in
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48 pituitary melanotrope cells is physiologically regulated by the background light condition, we
49
50 measured mRNA hybridisation intensities in WA (n=5) and BA (n=5) *X. laevis*, and
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52 expressed them as optical density (OD) in arbitrary units. As controls we studied two other
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54 structures of which we had found (see above, Fig. 2, and Table 1) that they are rich in CRF₁
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56 mRNA but which are not known to be involved in the process of background light
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58 adaptation: the internal granule cell layer of the olfactory bulb (igl) and the presumed
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60 corticotrope cells in the distal lobe.

As can be readily seen at low magnification (Fig. 4a,b) the intermediate lobe of the

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4 pituitary gland in BA animals is much larger than in WA toads, which is due to hyperplasia
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6 of the melanotrope cells, which are the only endocrine cells in the intermediate lobe [45]. On
7
8 the other hand, the intensity of the CRF₁ mRNA hybridisation signal in the intermediate lobe
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10 of WA animals is much higher than in BA animals (Fig. 4b,d). A special observation is that
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12 in WA animals, the intensity of the hybridisation signal differs among individual melanotrope
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14 cells, being moderate in some cells, but strong or even very strong in others. In BA animals
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16 all cells are stained with the same, rather weak intensity (Fig. 4a,c). No effect of background
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18 light intensity on the expression of CRF₁ in the brain was noted.

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21 This stimulatory effect of white background adaptation on, specifically, the expression
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23 of CRF₁ mRNA in the melanotrope cells, clearly appears from the morphometric study. In
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25 both the igl and in the distal lobe, the OD of the CRF₁ mRNA hybridisation signal does not
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27 differ between BA and WA animals (Fig. 5) but the melanotrope cells in the intermediate
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29 pituitary lobe of WA animals show a 59.4% higher OD than melanotropes of BA animals
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31 ($P < 0.05$; Fig. 5).

32 33 34 35 36 37 **5. Discussion**

38 39 40 *5.1. Technical considerations*

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42 With *in situ* hybridisation, using a DIG-labelled oligonucleotide probe, we here
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44 describe for the first time the detailed distribution of mRNA encoding CRF₁ in the brain and
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46 in the pituitary gland of a non-mammalian vertebrate, the South African clawed toad *X.*
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48 *laevis*. The reasons for using an oligonucleotide probe instead of a ribonucleotide probe are:
49
50 1) the higher stability (resistance to RNase degradation) when compared to a RNA probe, 2)
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52 the small molecular size of the oligonucleotide, which provides better tissue penetration, and
53
54 3) the fact that oligonucleotides do not self-hybridise.
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57 58 *5.2. Comparison of CRF₁ mRNA distribution in the brain of amphibians with mammals*

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60 The general existence of CRF₁ 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

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4 expressing this receptor. This is the first report describing the presence of CRF₁ mRNA, and
5 hence, most probably, of CRF₁, in the telencephalon, diencephalon and brainstem of *X.*
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8 *laevis*.

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10 The distribution of the CRF₁ mRNA in the *Xenopus* brain appears to be substantially
11 less extensive than that of CRF₁ mRNA in the mammalian brain. For example, in the rat
12 telencephalon, CRF₁ mRNA occurs in the periglomerular layer, mitral layer and olfactory
13 tubercle as well as in the hippocampus, diagonal band of Broca and bed nucleus of the stria
14 terminalis [23], all regions that in *X. laevis* are devoid of CRF₁ mRNA. In the rat
15 diencephalon CRF₁ mRNA is present in many thalamic areas such as the latero-dorsal and
16 latero-posterior nucleus and the lateral geniculate nucleus, whereas in *Xenopus* only the
17 ventromedial thalamic nucleus shows CRF₁ mRNA expression. CRF₁ mRNA has been
18 observed furthermore in the rat brainstem, viz. in many sensory relay structures, whereas in
19 the toad only the tectum mesencephali and the cerebellum reveal CRF₁ mRNA hybridisation.
20 The wider distribution of this receptor mRNA in the rat suggests that its ligands CRF and
21 Ucn1 have acquired novel central functions during evolution.
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35 On the other hand, the distributions of CRF₁ mRNA in the brain of *X. laevis* and of
36 mammals show some clear similarities. In *X. laevis*, going from rostral to caudal, strong
37 CRF₁ hybridisation signals occur in the internal granule cell layer of the olfactory bulb, the
38 ventral hypothalamic area, the tectum mesencephali and the cerebellum. The homologous
39 nuclei in the rat brain, viz. the granule cell layer of the olfactory region, the arcuate nucleus,
40 the superior colliculus and the granule layer of the cerebellum, respectively, also express
41 CRF₁ signals [23]. Furthermore, nor in the Mg of *X. laevis* nor in its rat homologue, the PVN,
42 any CRF₁ mRNA signal has been observed [23]. These similarities between CRF₁ locations
43 in *Xenopus* and rat indicate that the presence/absence and functions of this receptor type are
44 strongly evolutionary conserved.
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57 5.3. Relation between CRF₁ mRNA and CRF/Ucn1 peptide distributions

58 The *in situ* hybridisation signal for CRF₁ mRNA is a strong indication for the local
59 existence of CRF₁ protein. Because *in vitro* studies showed that both *Xenopus* CRF and Ucn1
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4 bind with high affinity to *Xenopus* CRF₁ [41], it is of functional interest to assess if the CRF₁
5 mRNA expressions described in this study match with the descriptions of CRF- and Ucn1-ir
6 fibres and perikarya [27,28]. Table 1 lists our present description of CRF₁ mRNA distribution
7 in *Xenopus*, as well as literature data on the distribution of CRF and Ucn1 peptides in this
8 animal [27,28]. As the table shows, both CRF- and Ucn1-ir elements occur in the olfactory
9 bulb, nucleus accumbens, posterior tubercle and tectum mesencephali, suggesting that in
10 these four areas both CRF and Ucn1 can act on CRF₁. However, in most brain structures
11 CRF₁ mRNA coexists with CRF-ir fibres only, which fact supports the notion that CRF is the
12 endogenous ligand for CRF₁, and that Ucn1 binds preferably to CRF₂ [26,38]. In the nucleus
13 habenularis ventralis, CRF₁ mRNA expression occurs in CRF-ir perikarya. Here,
14 ultrastructural studies may be helpful to determine if the same cells both release CRF and
15 possess CRF₁, which would point to an autocrine action of CRF.
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29 These data together, support the idea that CRF and Ucn1 act on CRF₁ receptors in the
30 *Xenopus* brain. Whether the absence of CRF₁ mRNA in CRF- and/or Ucn1-peptide
31 containing brain structures points to actions of these ligands on CRF₂, or on an action of
32 these ligands in other areas of the brain that are reached by volume transmission or via axons,
33 that cannot be revealed at the light microscope level, remains to be established.
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41 5.4. Physiological regulation of CRF₁ mRNA expression in the amphibian intermediate 42 pituitary lobe 43 44

45 We here demonstrate for the first time that CRF₁ occurs in the intermediate lobe of
46 the pituitary gland of a non-mammalian species. The presence of CRF₁ in the intermediate
47 lobe of *X. laevis* extends the previous general demonstration by RT-PCR of this receptor type
48 in the total pituitary gland [38], as it shows that CRF₁ is restricted to the melanotrope cells of
49 the intermediate lobe. Recently, we showed the presence of CRF and Ucn1 peptides in the
50 median eminence and in the pars nervosa of the pituitary lobe of *Xenopus* and revealed by *in*
51 *vitro* superfusion studies that both peptides stimulate the release of α MSH from melanotrope
52 cells [28,37]. We now show an upregulation of CRF₁ mRNA in WA toads, as compared to
53 BA animals. This receptor upregulation is specific for the melanotrope cells, as no effect of
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4 the background illumination state was visible in the igl or in the distal pituitary lobe. Our
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6 demonstration of CRF₁ mRNA in the intermediate lobe fits in with the fact that the Mg,
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8 projecting to neurohemal areas in the median eminence and the pituitary pars nervosa, is
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10 controlling melanotrope cell secretory activity via the release of CRF and Ucn1, both
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12 peptides stimulating α -MSH release *in vitro* [28,37]. Interestingly, in WA the strength of the
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14 CRF₁ mRNA signal is not homogenous throughout the gland, suggesting that some cells are
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16 producing more receptors than others. This result supports the notion that the intermediate
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18 lobe of the pituitary gland is composed of a heterogeneous cell population as to size [45] and
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20 POMC production [46].
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22
23 Up to now, it is not known what factors control the release of CRF and Ucn1 release
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25 from the pituitary pars nervosa. In the present study we show that adaptation of *Xenopus* to a
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27 white background upregulates the expression of CRF₁ mRNA. This finding suggests that in
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29 these WA animals, melanotropes become highly sensitive to CRF and Ucn1 so that when
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31 placed on a black background, their high receptor density enables the cells to rapidly mobilise
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33 stored α -MSH to the secretory process. In this way, the animals can turn black quickly,
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35 permitting fast camouflage. Indeed, in line with these results, Verburg-van Kemenade et al.
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37 [37] reported that ovine CRF evokes stronger *in vitro* α MSH release from neurointermediate
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39 lobes of WA than of BA toads. For *X. laevis* melanotropes a similar situation has been
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41 described with respect to thyrotropin-releasing hormone-receptor type 3 mRNA, and for NPY
42
43 Y1 receptor mRNA, which are both upregulated in WA animals [47,48]. Like CRF and
44
45 Ucn1, TRH simulates α -MSH release, whereas NPY inhibits this release [49,50].
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48 Further studies on the dynamics of the Mg and the *in vivo* conditions by which the
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50 activity of this nucleus is regulated, as well as on the possible presence and regulation of
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52 CRF₂ receptors in the pituitary gland, may elucidate the plastic interactions between CRF and
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54 Ucn1 and their receptors that are crucial to the functioning of the neuroendocrine reflex
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56 mechanism by which melanotrope cells in amphibians control the process of skin colour
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58 adaptation to background illumination. We propose that this mechanism is an example of
59
60 neuroendocrine plasticity crucial to adaptation processes in general and to adaptation to
stressful environmental events in particular.

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Legends

Fig. 1. Overview of *in situ* hybridisation of CRF₁ mRNA in the brain of *Xenopus laevis*, at the level of the diencephalon, with various positive regions, with an antisense oligonucleotide probe (a), and lack of hybridisation signal with the complementary sense probe (b). Bar = 100 μ m.

Fig. 2. Schematic sagittal view of the brain of *X. laevis*. The locations of CRF₁ mRNA-positive neurones as visualised by *in situ* 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. 3. Sagittal sections of the brain and pituitary gland of *X. laevis*, showing CRF₁ 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. Bar a,d = 50 μ m; b,c,e-h = 20 μ m.

Fig. 4. CRF₁ mRNA in the pituitary gland of *X. laevis*. In the melanotrope 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). Bar a,b = 50 μ m; bar c = 30; bar d-f = 20 μ m.

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4 Fig. 5. Effect of background adaptation on the OD expressed as arbitrary units (a.u.) of CRF₁
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6 *in situ* hybridisation signals in internal granule cell layer of the olfactory bulb (igl), distal
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8 (pd) and intermediate lobe (pi) of the pituitary gland. White bars indicate white-adapted and
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10 black bars black-adapted *Xenopus*. Values are expressed as means \pm standard error of the
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12 mean. Asterisk indicates significant difference between the two adaptation states (* $P < 0.05$).
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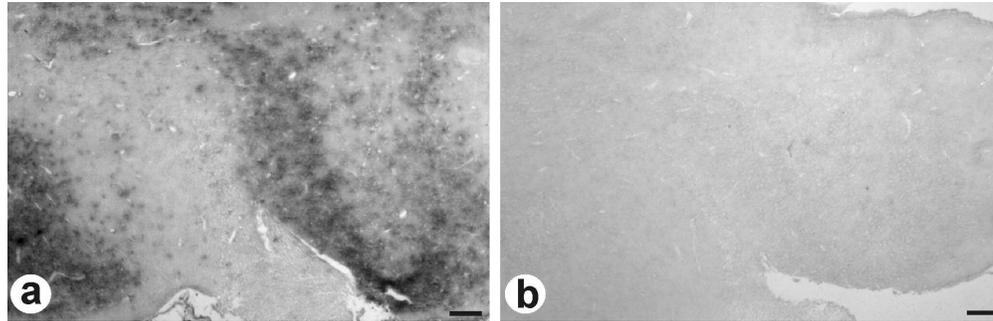
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4 Table 1. Distribution of α CRF₁ mRNA, CRF- and Ucn1-ir cells and fibres in the *X. laevis*
5 brain and pituitary gland, as visualised by *in situ* hybridisation and immunocytochemistry,
6 according to Yao [27] and Calle [28]. For the *in situ* hybridisation, ratings reflect the intensity
7 of positively labelled cells, with (+) indicating a low, (++) moderate and (+++) strong
8 intensity of the staining; for immunocytochemistry the number of positive cells is indicated
9 as follows: (+) rare, (++) some and (+++) numerous. Immunoreactive fibres are indicated by
10 (+), (-) indicates a complete lack of labelling; s.d. stimulus-dependent staining.
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BRAIN REGION	CRF ₁ mRNA	CRF-ir refs [27,28]		Ucn1-ir ref. [27]	
		Cells	Fibres	Cells	Fibres
<i>Telencephalon</i>					
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)	-	-	+	-	-
<i>Diencephalon</i>					
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)	++	-	-	-	-
<i>Mesencephalon</i>					
Tectum mesencephali (tect)	++	+++	+	-	+
Tegmentum mesencephali (tegm)	-	-	+	-	+
Edinger-Westphal nucleus (EW-N)	-	-	+	+++	+
Anterior tegmental nucleus (Av, Ad)	-	++	+	-	-
Posterior commissure (pc)	-	-	+	-	-
Nucleus posteroventralis tegmenti (pv)	-	+	-	++	-
Torus semicircularis (Tor)	-	-	+	-	+
<i>Rhombencephalon</i>					
Cerebellum (Cb)	+++	-	+	-	-
Locus coeruleus (Lc)	-	++	+	-	-
Central gray (cg)	-	-	-	++	-
Nucleus reticularis medius (Rm)	-	-	-	+	-
Cochlear nucleus (LL)	-	-	-	-	+
Nucleus motorius nervi trigemini (Vm)	-	++	+	++	-
Nucleus motorius of the facial and glossopharyngeal nerve (IX)	-	-	-	++	+
Nucleus motorius nervi vagi (Xm)	-	-	-	++	-

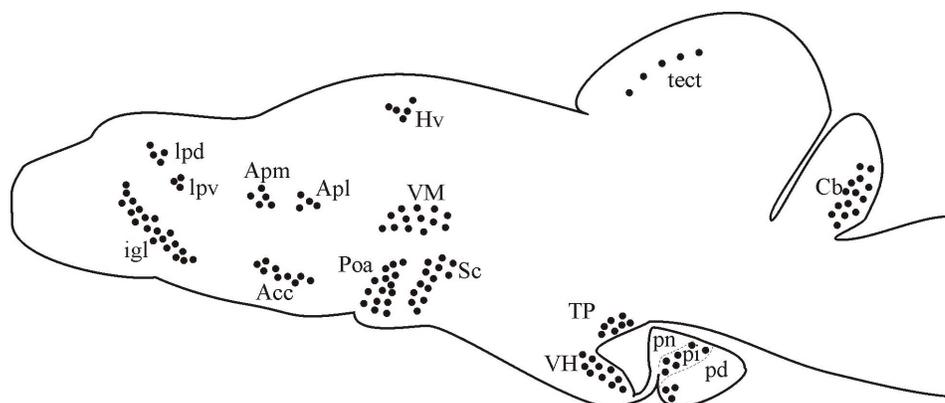
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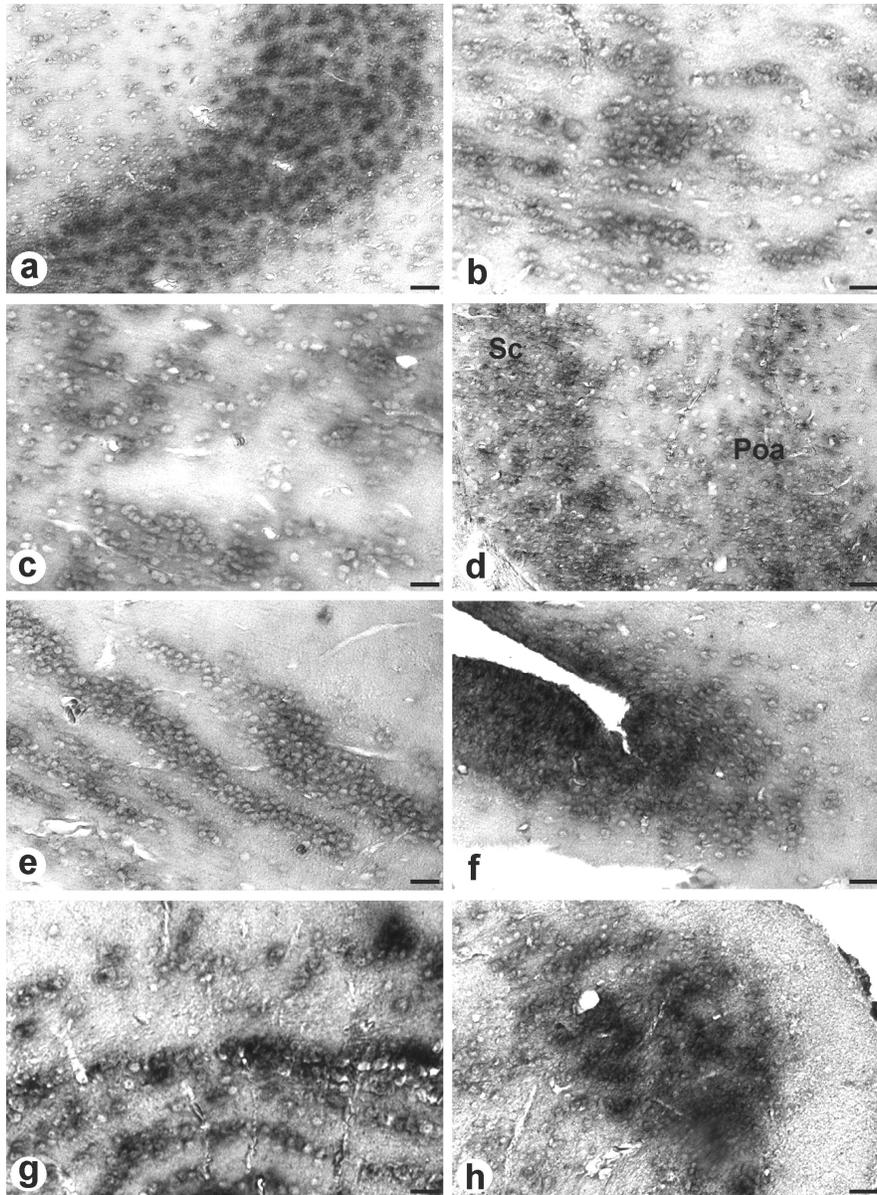
Overview of in situ hybridisation of CRF₁ mRNA in the brain of *Xenopus laevis*, at the level of the diencephalon, with various positive regions, with an antisense oligonucleotide probe (a), and lack of hybridisation signal with the complementary sense probe (b). Bar = 100 μ m.

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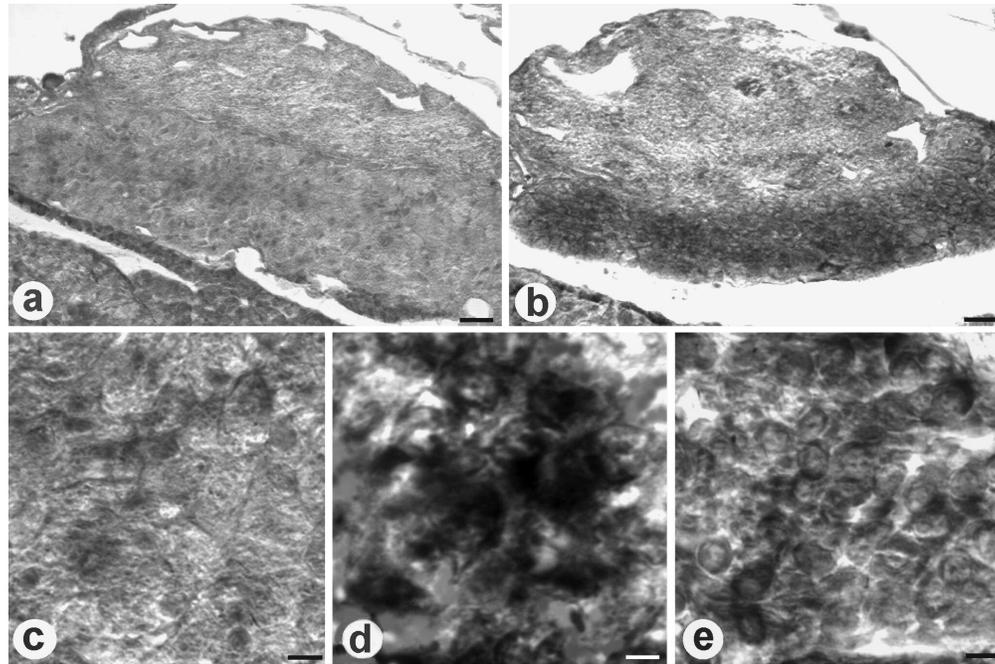


Schematic sagittal view of the brain of *X. laevis*. The locations of CRF₁ mRNA-positive neurones as visualised by in situ are indicated by black dots. Acc, nucleus accumbens; Apl, amygdala, pars lateralis; Apm, amygdala, pars medialis; Cb, cerebellum; Hv, nucleus habenularis ventralis; iql, internal 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.

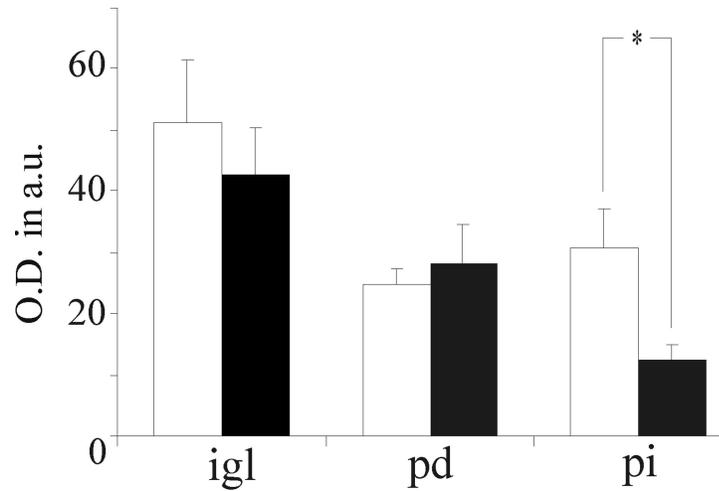
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Sagittal sections of the brain and pituitary gland of *X. laevis*, showing CRF₁ mRNA hybridisation in (a) internal granule cell layer of the olfactory bulb, (b) nucleus accumbens, (c) medial part of the amygdala, (d) preoptic area of the hypothalamus (Poa), suprachiasmatic nucleus (Sc), (e) ventromedial thalamic nucleus, (f) ventral hypothalamic area, (g) tectum mesencephali, and (h) cerebellum. Bar a,d = 50 μ m; b,c,e-h = 20 μ m.



CRF₁ mRNA in the pituitary gland of *X. laevis*. In the melanotrope 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). Bar a,b = 50 μm; bar c = 30; bar d-f = 20 μm.



Effect of background adaptation on the OD expressed as arbitrary units (a.u.) of CRF₁ in situ hybridisation signals in internal granule cell layer of the olfactory bulb (igl), distal (pd) and intermediate lobe (pi) of the pituitary gland. White bars indicate white-adapted and black bars black-adapted *Xenopus*. Values are expressed as means \pm standard error of the mean. Asterisk indicates significant difference between the two adaptation states (*P<0.05).