Ultrastructural localisation and functional implications of Corticotropin releasing factor, Urocortin and their receptors in cerebellar neuronal development

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2003

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 5

Corticotropin releasing factor receptor types 1 and 2 are differentially expressed in pre- and postsynaptic elements in the postnatal developing rat cerebellum

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European Journal of Neuroscience: (2003);18(3):549-562
ABSTRACT

Corticotropin releasing factor (CRF)-like proteins act via two G-Protein coupled receptors (CRF-R1 and CRF-R2) playing important neuromodulatory roles in stress responses and synaptic plasticity. Cerebellar expression of CRF-like ligands have been well documented, but not their receptor localisation. This is the first combination light microscopy ultrastructural study to localize CRF receptors immunohistologically (im) in the developing rat cerebellum. Both CRF-R1 and R2 were expressed in climbing fibres from early stages (postnatal day 3) to the adult but CRF-R2im was only prominent in throughout the molecular layer in the posterior cerebellar lobules. CRF-R1im was concentrated in apical regions of Purkinje cell (PC) somata and later in primary dendrites exhibiting a diffuse cytoplasmic appearance. In PC’s, CRF-R1im was never membrane bound postsynaptically in dendritic spines while CRF-R2im was found on plasmic membranes of PC from postnatal day 15 onwards. We conclude that the localisation of these receptors in cerebellar afferents implies their control of the presynaptic release of CRF-like ligands, impacting on the sensory information being transmitted from afferents. Furthermore, the fact that CRF-R2 is membrane bound at synapses, while CRF-R1 is not, suggests that ligands couple to CRF-R2 via synaptic transmission and to CRF-R1 via volume transmission. Finally, the distinct expression-profiles of receptors along structural domains of PC’s suggest that the role for these receptors is to modulate afferent inputs.
INTRODUCTION

Corticotropin releasing factor (CRF)-like peptides modulate neurite growth (Cibelli et al., 2000) and synaptic plasticity in the forms of long term depression (Miyata et al., 1999) and long term potentiation (Wang et al., 2000). Currently, this family of stress-related peptides consists of CRF (Vale et al., 1981), urocortin (Vaughan et al., 1995), stresscopin related peptide/urocortin II (Lewis et al., 2001) and stresscopin/urocortin III (Reyes et al., 2001). CRF in the cerebellum is restricted to the two major afferent systems, namely the mossy fibres and climbing fibres (Palkovitz et al., 1987). We have recently demonstrated that urocortin is localised within the afferents and within cerebellar neurons in the adult rat cerebellum (Swinny et al., 2002). This disparate localisation of CRF and urocortin suggests contrasting roles for these peptides in cerebellar function.

CRF-like peptides mediate their effects via two receptors, namely CRF receptor type one (CRF-R1) (Chang et al., 1993; Chen et al., 1993; Vita et al., 1993) and CRF receptor type two (CRF-R2) (Lovenburg et al., 1995; Perrin et al., 1995). Both receptors belong to the family of G-protein coupled receptors and act by activating adenylate cyclase (Bilizekjian and Vale, 1983; Giguere and Labrie, 1983; Aguilera et al., 1983). In the rat brain, the most prominent seat of expression of CRF-R1 is the cerebellum, occurring predominantly in Purkinje cells, from embryonic day 17 through to adulthood (Chang et al., 1993). In adulthood, Radulovic et al. (1998), van Pett et al. (2000), Chen et al. (2000) and Bishop et al. (2000) consistently observed, immunoreactivity in all cerebellar cortical layers and the deep cerebellar nuclei. However, reports on the immunocytochemical localisation of CRF-R2 are contradictory. Van Pett et al. (2000) reported no cerebellar CRF-R2 immunoreactivity whereas Bishop et al. (2000) showed CRF-R2 immunoreactivity in the cerebellar cortical layers.

The above light microscopical studies suggest that the Purkinje cell is one of the main sites of CRF receptor expression. However, they do not address such issues as to whether the receptors are localised in pre- or postsynaptic elements, or both. An ultrastructural examination at the electron microscopic level is imperative since the Purkinje cell is composed of functionally distinct regions due to the heterologous synaptic inputs it receives on different locations (see Voogd and Glickstein, 1998 for a review). Inhibitory interneurons, like basket cells and stellate cells synapse on the Purkinje cell somata and dendritic shafts respectively. Climbing fibres and parallel fibres, provide excitatory input at the proximal stubby dendritic spines and distal, thinner dendritic spines respectively (Palay and Chan-Palay, 1974; Sotelo, 1978). This synaptic patterning and morphological development largely occurs within the
first three postnatal weeks in the rat (Altman and Bayer, 1997). A comprehensive investigation of the developmental expression of CRF-R1 and CRF-R2 in different regions of the cerebellum, especially the Purkinje cell layer will help in the understanding of the roles of CRF-like peptides serve, first in motor development and subsequently in motor learning.

**MATERIAL AND METHODS**

Black-hooded Lister rats were studied from postnatal day (PD) 3 to 25. The day of birth was considered postnatal day (PD) 0. Twelve animals in total were used. Approval to conduct the study was obtained from the Ethics Committee on Animal Experimentation, University of Groningen. All efforts were made to minimise the number of animals used and their suffering. Animals were deeply anaesthetised with sodium pentobarbital (Nembutal®, i.p., 50mg/kg).

*Western blotting*

Levels of CRF receptors in rat cerebellum and the specificity of the binding of the antisera used, were evaluated by Western immunoblot (Shi et al., 2001; Towbin et al., 1979). Cerebella samples at different ages were prepared from fresh rat cerebellum lysed in sample buffer (3ml/mg, 5%SDS, 5% a-mercaptoethanol, 8M urea, 6.25mM Tris-HCl, pH 6.8, and 0.01% bromophenol blue). Twenty microliter cerebellum samples were separated on 12.5% SDS-PAGE (Bio-Rad, Hercules, CA), blotted on a pure nitrocellulose membrane (Bio-Rad, Hercules, CA) and probed with goat anti-CRF-R1 or CRF-R2 (1:500; Santa Cruz). The CRF-R1 blot was followed by horseradish peroxidase-conjugated rabbit anti-goat antibody (1:5000, Sigma, Stinheim, Germany), and processed using ECL (Amersham Pharmacia Biotech) according to the manufacturer’s instruction. The CRF-R2 blot was followed by alkaline phosphatase-conjugated rabbit anti-goat antibody (1:3000, Sigma) and colour processed by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl (Sigma).

*Immunocytochemistry*

Following anaesthesia, animals were perfused transcardially first with 10 ml of a solution containing 2% polyvinyl pyrrolidone (molecular weight 30000), 0.4% NaNO₃ in 0.1 M phosphate buffer (PB) (pH 7.4), and then with 100ml of 4% paraformaldehyde and 0.2% picric acid in 0.1M PB (pH 7.4). The brains were removed and stored overnight in the fixative solution at 4°C.

For light microscopy, brains were stored overnight at 4°C in 30% sucrose for
corticotropic releasing factor receptor types 1 and 2

cryoprotection and cryo-sectioned (30µm thick) in the sagittal plane. Immuno-reactivity was visualised by fluorescence. Briefly, free-floating sections were immersed for two hours in a pre-incubation medium containing 1% normal rabbit serum and 1% bovine serum albumin in 0.1M phosphate buffered saline (PBS) (pH 7.4). The sections were incubated overnight at 4°C with affinity purified goat anti-CRF-R1 IgG or goat anti-CRF-R2 IgG (Santa Cruz Biotechnologies, Santa Cruz, CA), diluted 1:400 in PBS. After washing with PBS, the sections were incubated with Alexa Fluor® 488 rabbit anti-goat IgG, diluted 1:200 in PBS, for two hours at room temperature. After further washing, the sections were mounted in Dako anti-fading mounting medium (Dako Corporation, Ca).

**Imaging**

All sections were evaluated. However, selected sections were photographed on an Olympus Digital camera, mounted on an Olympus B50 fluorescence microscope. When necessary, these images were adjusted to enhance the contrast and brightness and saved at 300 dpi.

**Electron microscopy**

**Pre-embedding immunocytochemistry**

Animals were perfusion fixed as for light microscopy. Fifty micrometer sections were cut on a Vibratome in the sagittal plane. Immunoreactivity was visualised by the avidin-biotin-peroxidase complex (ABC) method. Briefly, free-floating sections were immersed for two hours in a pre-incubation medium containing 1% normal rabbit serum, 0.025% Triton X-100 and 1% bovine serum albumin in 0.1M phosphate buffered saline (PBS) (pH 7.4). The sections were incubated overnight at 4°C with either affinity purified goat anti-CRF-R1 IgG or goat anti-CRF-R2 IgG (Santa Cruz Biotechnologies, Santa Cruz, CA), diluted 1:400 in PBS. After washing with PBS, the sections were incubated with biotinylated rabbit anti-goat IgG, diluted 1:200 in PBS, for two hours at room temperature. After further washing, the sections were incubated with ABC (Vectastain Elite, Vector Labs, Burlingame, CA) for one hour at room temperature. Immunoreactivity was visualised by incubation with 5 mg 3,3 diaminobenzidine HCl (DAB) and 0.03% hydrogen peroxide in 10 ml of 0.1 M PBS (pH 7.4) for 5 to 10 minutes at room temperature. The reaction was stopped by washing the sections in cold PBS. Immunoreactivity was enhanced according to the gold substituted silver peroxidase method (van den Pol and Gorcs, 1986). Sections were osmicated in 1% OsO₄, 1.5% potassium hexacyanoferrate in 0.1M cacodylate buffer (pH 7.6) for 15 minutes, dehydrated in a graded series of ethanol and embedded in Epon. Semithin sections (1 µm) were cut on an LKB Ultratome, stained with
toluidine blue and used for orientation purposes. Ultrathin sections were cut, counterstained with uranyl acetate and lead citrate and examined with a Philips CM 100 transmission electron microscope.

Post-embedding immunocytochemistry
Postembedding immunogold labelling was performed according to a modified protocol of Petralia et al. (1998). Briefly, animals were fixed as for light microscopy. The cerebella were removed, postfixed, washed, and 100 μm sagittal sections were cut with a Vibratome. In the last two steps, tissue was kept in phosphate buffer (0.1 M with 4% glucose). Tissue was cryoprotected in a series of 10, 20, and 30% glycerol (last step overnight) in 0.1 M phosphate buffer, and was plunge-frozen in liquid propane in a Leica EM CPC. Frozen tissue was immersed in 1.5% uranyl acetate in methanol at -90°C in a Leica AFS freeze-substitution instrument, infiltrated with Lowicryl HM 20 resin at -45°C, and polymerized with UV light (-45 to 0°C). Thin sections were cut on a Leica Reichert Ultracut S ultramicrotome, placed on nickel grids (Electron Microscopy Sciences, Fort Washington), incubated in 0.1% sodium borohydride plus 50 mM glycine in Tris-buffered saline/0.1% Triton X-100 (TBST) for 10 min, and in blocking serum in TBST for 10 min (10% normal goat serum (NGS). Sections on the grids were incubated in primary antibody (goat anti-CRF-R1 or R2, diluted 1.25 in PBS) overnight at 4°C. After washing in PBS, the grids were incubated in 1:50 immunogold (0.8 nm rabbit anti-goat immunogold; Amersham: Arlington Heights). Following washing, the immunogold particles were further enhanced for 10 minutes in the dark according to a modified protocol of Yi et al. (2001). Concentrations of primary antibodies were selected to minimise background immunogold labelling. Such background artefactual staining was examined in control sections, (processed with the omission of the primary antibodies) and, within the experimental sections. Immunogold labelled sections were considered acceptable if they showed little or no labelling inside the mitochondria and nucleus.

Controls
To assess antibody specificity, both primary antibodies were separately pre-incubated with their specific blocking peptides (Santa Cruz Biotechnologies, Santa Cruz, CA). Cross-reactivity between the two antisera was tested by applying the blocking peptide for CRF-R2 in the pre-incubation medium with anti-CRF-R1 and vice versa. To assess cross-reactivity with CRF or urocortin, the receptor antibodies were pre-incubated with either synthetic CRF or urocortin peptides. Sections were incubated with the individual cocktails overnight, and processed further for western blotting or light microscopy.
Antisera
The CRF-R1 antiserum used in this study was raised against a peptide mapping within an internal region of CRF-R1 of human origin (sc 12381), specific for CRF-R1 of mouse, rat and human origin and does not cross-react with CRF-R2. The CRF-R2 antiserum used in this study was raised against a peptide mapping at the amino terminus of CRF-R2 of mouse origin and is specific for CRF-R2 and does not cross-react with CRF-R1.

RESULTS

Western blotting
The specificity of the anti-CRF-R1 and CRF-R2 antisera were assessed by immunoblotting rat cerebellar homogenates. Since only single immunoreactive bands were evident for both antisera, only the regions of the blots containing the immunoreactive bands are shown. For CRF-R1, the antiserum recognised a protein in the 40-45 kDa range, which is in accordance with previous observations of the molecular weight of rat cerebellar CRF-R1 protein (Chang et al., 1993; Grigoriadis and DeSouza, 1989; Vita et al., 1993). Weak indistinct bands were evident in immunoblots from homogenates of PD 1 to PD 4, with signal intensity increasing from PD 8 onwards through to PD 12. A slight tapering off was observed in adult homogenates (fig 1 A). Preadsorption of the primary antiserum with a blocking peptide consisting of the immunogenic epitope (100 µg/ml) completely abolished the CRF-R1 band (data not shown). The specificity of CRF-R1 immunoreactivity was also demonstrated by the absence of labelling in cerebellar sections that were stained using antiserum pretreated with the antigenic peptide (data not shown). For CRF-

![Figure 1](image_url)

Figure 1. Western blot analysis of postnatal cerebellar homogenates of PDs 1,4,8,12 and 25, showing the specificity and degree of binding of CRF receptor antisera. (A) CRF-R1 antiserum recognised a single protein of approximately 40kDa. Weak banding was observed between the stages of PD 1 to PD 4. Expression became more intense between the stages of PD 4 to PD 8 with PD 12 showing the most intense banding pattern. (B) CRF-R2 antiserum consistently recognised a single protein of approximately 50kDa. In contrast to CRF-R1, banding was more intense and was expressed as early as PD 1 through to young adulthood. However, most intense immunoblotting was observed between the stages of PD 4 through to PD 12.
Figure 2. Immunofluorescence of CRF-R1 at different developmental stages. (A) an overview of the cerebellar cortex at PD 6, showing immunoreactivity in the Purkinje cell layer, especially in the apical caps of Purkinje cells (arrowheads). Neurons in the external granular (upper arrow) and developing molecular layers (lower arrow) also exhibited immunoreactivity. Based on electron microscopic evidence, these labelled neurons represent a mixture of migrating basket, stellate cells. (B) a higher magnification of Purkinje cells at PD 6 showing CRF-R1 immunoreactivity that is predominantly cytoplasmic and concentrated in the apical cap regions of the somata (arrowheads). (C) an overview of the cerebellar cortex at PD 11. CRF-R1 immunoreactivity was present in the Purkinje cell primary dendrites (arrowheads). Immunoreactivity in cell bodies in the granular layer is also present (arrows). (D) higher magnification showing immunoreactivity in some Purkinje cells that was exclusively localised in the apical caps (arrowhead) whereas the basal parts of the somata are devoid of immunoreactivity. The asterisk indicates the nucleus. (E) enlargement of the Purkinje cell layer showing immunoreactive fibre-like profiles encircling the somata. It is evident that bouton or fibre-like immunoreactivity is more intense than that in the somata. Electron microscopic data confirm that these profiles are climbing fibres. (F) an overview of the molecular layer at PD 15, immunoreactive pallisades of Bergmann glia were conspicuous (arrowheads). In some Purkinje cells, immunoreactivity was distributed within the soma (arrow). G and H in some Purkinje cells, immunoreactivity appeared less distinct being either membrane bound to their somata and dendrites, or expressed within glia, known to ensheath Purkinje cells. (I) an overview of the cerebellar cortex at PD 25 showing CRF-R1 immunoreactivity in Purkinje cell dendrite (arrow) and in the granular layer (arrowheads). Scale bar: A-E, 20 \( \mu \text{m} \); F-I, 25\( \mu \text{m} \).
Figure 3. CRF-R2 immunofluorescence in the cerebellar cortex at different postnatal stages. (A) an overview from lobule III showing immunoreactivity predominating in Purkinje cell somata (arrowheads). The molecular and granular layers show indistinct labelling. (B) an overview of lobule IX showing strong immunostaining in Purkinje cell somata (arrows) and primary dendrites (arrowhead). (C) a detail of the Purkinje cell layer. The immunoreactivity is localised in the cytoplasm and extends into the initial axonal segments (arrowheads). (D) an overview and (E), a higher magnification of the Purkinje cell layer at PD 11, showing that immunoreactivity within Purkinje cells is more restricted in comparison with earlier stages. Reactivity is restricted to cytoplasmic membranes, especially in the basal regions and initial axonal segments (arrowhead). (F) at PD 11, immunoreactive fibre-like profile (arrowheads), extending from the granular layer into the molecular layer were evident. Such bouton or fibre-like profiles were more intense than similar profiles expressing CRF-R2 immunoreactivity. These are probably a combination of climbing fibres, Purkinje cell axons or recurrent axonal collaterals based on electron microscopic data (see figure 7 B). (G to I) from PD 15 through to adulthood, Purkinje cell immunoreactivity is clearly condensed and membrane bound to their somata with the cytoplasm being relatively free of labelling. Scale bars: A-F, 20µm; G-I, 25 µm
R2, the antiserum recognised a protein in the 40~50 kDa range, also in close approximation of previous data (Lovenberg et al., 1995; Perrin et al., 1995; Vita et al., 1993) (fig 1 B). More intense banding, in comparison to CRF-R1, was evident from homogenates taken at PD 1. Banding appeared to be equally intense between the stages of PDs 4 to 12 with a slight decrease at PD 25. Like CRF-R1, preadsorption of the primary antiserum with a blocking peptide consisting of the immunogenic epitope (100 g/ml) completely abolished the CRF-R2 band (data not shown). To assess cross-reactivity between the two receptor antisera, pre-incubation with the alternate blocking peptides did not reduce immunoreactivity (data not shown). Similarly, pre-incubation of the two antisera with CRF or urocin peptides resulted in no discernable decrease in the intensity of immunoreactivity, confirming that the antisera are not cross-reactive with these peptides.

Light microscopy

CRF-R1

PD 3 was the first stage to be examined, showing rather weak labelling. However, from PD 6 onwards, immunoreactivity increased appreciably in all layers of the cerebellar cortex, in fibre tracts in the cerebellar white matter and in the deep cerebellar nuclei. The reaction product was most abundant in Purkinje cells, the molecular layer and the external granular layer. In Purkinje cells, immunoreactivity was first concentrated in the apical caps and basal region of Purkinje cell cytoplasm. There were no apparent regional differences between Purkinje cell immunoreactivity. Interneurons in the molecular layer also exhibited immunoreactivity (figs 2 A and B). Electron microscopic evidence showed conclusively that both basket and stellate cells are immunoreactive (see figure 6).

Between the stages of PDs 9 to 12, labelling in Purkinje cells was preferentially localised in the apical cap of somata and in the proximal regions of the primary dendrites. This pattern of labelling was not uniform in all Purkinje cells, with some cells exhibiting labelling in the basal parts of the somata (figs 2 C and D). Also evident at this stage were varicose fibre-like immunoreactive profiles at the level of the Purkinje cell somata (fig 2 E). It was apparent that for both CRF-R1 and CRF-R2, fibre-like or bouton immunoreactivity was more intense than that of the labelling in cell bodies. At the electron microscopical level, these profiles were shown to be climbing fibres (see figure 5). At PD 15, Purkinje cell dendritic labelling had become more pronounced. Labelling within the somata of Purkinje cells was diffuse, not restricted to any specific regions as in the earlier stages. Immunoreactivity within the palisades of Bergmann glia was fully evident especially in the central and posterior lobules (fig 2F). A subset of Purkinje cells showed immunoreactivity only around
their somata and dendrites. This subsets of Purkinje cells appeared randomly throughout the cerebellum and could not be related to their position within an anterior posterior plane or a particular region within a lobule. (figs 2 G and H).

In adulthood, reaction product was localised in somata and in primary dendrites. On the whole, CRF-R1 immunoreactivity occurred dispersed throughout the cytoplasm
was not bound to any cell membranes (fig 2 I). Furthermore, there was no sagittal banding pattern with respect to CRF-R1 immunoreactivity (data not shown).

**Figure 5.** Electron micrographs of climbing fibres showing dense and selective CRF-R1 immunoreactivity. (A) shows an immunoreactive climbing fibre terminal making synaptic contacts on spines of Purkinje cell dendritic spine at PD 15. (B) shows similar profiles at PD 25. Note that the dendritic spines are always free of reaction product whilst the dendritic shaft contains reaction product (arrowheads). Images are taken of pre-embedding immunostaining. Cf, climbing fibre; Pden, Purkinje cell dendrite; Sp, Purkinje cell spine. Scale bars, 1 µm.
In the first postnatal week, CRF-R2 immunoreactivity was weak and rather indistinct. At PD 6, immunoreactivity was evident in Purkinje cells and in the granular layer. In the anterior lobules, labelling was sparse (fig 3 A). However, in the posterior lobules, labelling in the molecular layer was more intense. Purkinje cell immunoreactivity

**Figure 6.** Electron micrographs of CRF-R1 immunoreactivity within interneurons in the molecular layer. (A) an overview of a stellate cell at PD 3 identified by its pale nucleus (in comparison to basket cells) showing reaction product within its soma (arrowheads). (B) a detail of an interneuron in the upper molecular layer expressing prominent somatic immunoreactivity. (C), shows the dendrite of the soma in (B) containing prominent reaction product. (D) shows an interneuron in the molecular layer containing membrane bound immunogold particles (arrowheads) with the insert showing gold particles membrane bound to dendritic profiles also in the molecular layer. Note that figures A to C are of pre-embedding immunocytochemistry and figure D of post-embedding immunocytochemistry. Scale bars: A, C 1 µm; B, D, 2 µm

**CRF-R2**

In the first postnatal week, CRF-R2 immunoreactivity was weak and rather indistinct. At PD 6, immunoreactivity was evident in Purkinje cells and in the granular layer. In the anterior lobules, labelling was sparse (fig 3 A). However, in the posterior lobules, labelling in the molecular layer was more intense. Purkinje cell immunoreactivity
was prominently expressed in apical caps. It was difficult to positively ascertain whether immunoreactivity in the basal regions was present within the initial axonal segments of Purkinje cells or in climbing fibres since immunoelectron microscopy revealed reaction product in both profiles. In certain Purkinje cells, labelling was evident in the primary dendrites (fig 3 B and C). Between the stages of PDs 9 to 12, Purkinje cell immunoreactivity was localised mainly in the basal regions of their somata and was apposed to the cell membranes (fig 3 D and E). Immunoreactive fibre-like profiles originating from the granular layer, encircling Purkinje cell somata and encroaching on their proximal primary dendrites were observed at this stage. These are presumably climbing fibres, primary Purkinje cell axons or recurrent axonal collaterals (fig 3 F). CRF-R2 immunoreactive boutons were in greater abundance in comparison to CRF-R1 (see fig. 2 E). Between PDs 15 to 25, Purkinje cell immunoreactivity increased, mainly localised on somatic membranes and near the initial axonal segment. Some Purkinje cells exhibited immunoreactivity in the proximal dendritic regions (figs 3 G to I).

**Electron microscopy**

**CRF-R1**

Early CRF-R1 labelling was observed in Purkinje cell somata and in climbing fibre terminals making synaptic contact with somatic spines of Purkinje cells. In Purkinje cells, the reaction product was localised either within the trans Golgi network, in small vesicles and in multivesicular bodies (figs 4 A and B). Importantly, somatic spines of Purkinje cells were always free of label, whereas the climbing fibre terminals making synaptic contact with spines expressed strong CRF-R1 immunoreactivity. From PDs 6 to 11 onwards, Purkinje cell labelling increased in density and was concentrated within the apical caps showing a clear gradient between the apical and basal regions, evident at PD 12 (fig 4 C). Dendritic labelling became pronounced from PD 15 onwards, with reaction product in the primary dendrites and their branches. Dendritic spines were always free of label. Indeed, using pre- and postembedding techniques, during the entire period of ontogeny, CRF-R1im in the Purkinje cell was never found on or close to synaptic membrane specialisations (fig 4 D). Climbing fibre terminals show intense immunoreactivity from PD 3 onwards. At this stage, they made synaptic contacts on somatic spines on Purkinje cells (see fig 4 A). Climbing fibre immunoreactivity persisted through to adulthood, when the translocation of the somatic terminals to the stubby spines of Purkinje cell dendrites had been established. Postembedding labelling showed that the reaction product was localised in the cytoplasm and was not related to membranes (data not shown) (fig 5 A and B).
Figure 7. Electron micrographs of CRF-R2 immunoreactivity in Purkinje cells at different postnatal stages. (A) an overview of the soma of a Purkinje cell at PD 6 showing immunogold particles closely associated with rough endoplasmic reticulum (arrowheads). (B) shows part of a soma of a Purkinje cell at PD 12. Evident are numerous immunoreactive climbing fibre terminals making synaptic contacts on somatic spines. (C) shows a region of a soma of a Purkinje cell at PD 15. Immunogold particles are cytoplasmically localised and membrane bound (arrowheads). (D) shows a Purkinje cell soma at PD 25 with membrane bound immunogold particles (arrowheads). (E) is a magnified view of the rectangular boxed area in (D) showing an immunogold particle in the perisynaptic region of a basket cell-Purkinje cell synapse (arrowheads). (F) is a magnified view of the square boxed area in (D) showing an immunogold particle that is closely attached to the presynaptic membrane specialisation. Bt, basket cell terminal; Cf, climbing fibre; Pnuc, Purkinje cell nucleus; Sp, Purkinje cell somatic spine. Scale bars: A-D, 2µm; E and F, 200nm.
At early developmental stages, the external granular layer and the molecular layer exhibited immunoreactivity in various cellular elements. From PD 6 differentiating neurons resembling basket or stellate cells expressed CRF-R1im. Reaction product was mainly localised in somata and dendrites and not in terminals. The pattern of somatic and dendritic expression persisted into adulthood, with postembedding labelling showing reaction product often membrane bound in the somata and dendrites (fig 6).

**Figure 8.** Electron micrographs of CRF-R2 at Purkinje cell-parallel fibre contacts at PDs 12 and 25. (A) an overview showing parallel fibres making synaptic contact with immunoreactive dendritic spines at PD 12. Note the reaction product contained within the dendritic branch as well. (B) a magnified view of the boxed area in (A), showing scattered reaction product within the spine and in the region of the postsynaptic density (arrowheads). (C) an overview of parallel fibre-Purkinje cell contacts at PD 25 showing immunoreactivity selectively localised in parallel fibre terminals with the insert being a magnified view of the boxed area. (D) a magnified view of a similar contact showing that were either in the lumen of the parallel fibre terminal or (E), membrane bound in the perisynaptic region (arrowhead). Figures A to C are of pre-embedding immunostaining with D and E of post-embedding immunostaining. Pden, Purkinje cell dendrite; Pf, parallel fibre; Sp, Purkinje cell spine. Scale bars: A, C, 1 μm; B, D, E 200 nm.
CRF-R2

Within the first postnatal week, pre- and postembedding labelling showed that Purkinje cell immunoreactivity was purely cytoplasmic, particularly in the region of the rough endoplasmic reticulum. Membrane bound reaction product at this early stage was not evident (fig 7 A). At PD 12, the reaction product was evident in somata, while climbing fibre terminals making synaptic contact on somatic spines were also immunoreactive (fig 7 B). Using postembedding labelling, PD 15 was shown to be the first stage at which reaction product was localised bound to plasma membranes (fig 7 C). At later stages, immunogold particles were found in close proximity to terminals contacting Purkinje cell somata. These were either basket cell terminals or recurrent axonal collaterals of Purkinje cells. The reaction product was always perisynaptic. Membrane bound reaction product was also associated with vacuolisation, presumably due to endocytotic receptor internalisation (figs 7 D – F).

In the molecular layer, CRF-R2im was present in Purkinje cell dendritic spines contacted by parallel fibres. Labelling in spines was only expressed from PD 9 through

Figure 9. Electron micrographs of CRF-R2 immunoreactivity in climbing fibre profiles. (A) shows two characteristic climbing fibre profiles making synaptic contacts on spines of Purkinje cells at PD 15 with (B) showing similar immunoreactive profiles at PD 25. Cf, climbing fibre; Sp, Purkinje cell dendritic spine. Scale bars: 1µm.
to PD 15. During this period, parallel fibre terminals were always free of immunoreactivity (figs 8 A and B). Labelling in parallel fibre terminals was evident at later stages. In the presynaptic terminal, immunogold particles were visualised, close to the synaptic membrane specialisation (figs 8 C – E).

CRF-R2im was expressed in climbing fibres from early stages (see fig 7 B) through to adulthood. It could be documented using pre- and postembedding labelling that reaction product was often associated with vesicles and was rarely bound to presynaptic membranes (fig 9).

DISCUSSION

The current study is the first to provide a detailed account of the localisation of CRF-R1 and CRF-R2 at different developmental stages at the subcellular level. We have largely concentrated on the developing Purkinje cells since they develop their dendritic tree and afferent input during the first three postnatal weeks (Sotelo, 1978) and are also considered the organising centre of the cerebellum (Hatten, 1999). The present study addresses probing questions posed by Bishop et al. (2000) that can only be addressed by using immunoelectron microscopy. Firstly, are these receptors localised presynaptically or postsynaptically? Secondly, do cerebellar afferents express CRF receptors? Such data is crucial for an integrative analysis of light microscopic observations on CRF and urocortin localisation, an approach essential in the elucidation of the role of the CRF system in cerebellar development and in motor learning (Miyata et al., 1999).

Technical considerations

We have used three different immunocytochemical techniques to provide complementary information on the ultrastructural localisation of CRF receptors. We used antisera which, based on our controls, appear to offer a high degree of specificity for the CRF receptors. Light microscopy was used to confirm the earlier findings (Bishop et al., 2000) and to extend those findings to the developing cerebellum. Two independent methods of immunocytochemistry at the electron microscopic level were used to assess consistency of our results and the precise subcellular distribution of the receptors. Pre-embedding immunocytochemistry using a peroxidase reaction is a good indicator of the immunoreactivity of a profile (Lopez-Bendito et al., 2001). However, the diffuse nature of the reaction product precludes the precise localisation of receptors, especially G-protein coupled receptors which are expected to be predominantly membrane bound. We have used postembedding immunocytochemistry using immunogold particles since this is regarded as the method to
provide the most accurate localisation (Lopez-Bendito et al., 2001). However, the trade-off, compared to the peroxidase method, is a decreased intensity on labelling and this is evident in this study.

**CRF receptors in cerebellar afferents**

From PDs 3-4, CRF-R1 and CRF-R2 are expressed in climbing fibres and mossy fibre rosettes while only parallel fibres express CRF-R2. CRF and urocortin bind with equal affinity to CRF-R1, however, urocortin exhibits a 40-fold greater affinity for CRF-R2, suggesting that urocortin is the natural ligand for CRF-R2 (Vaughan et al., 1995). In a previous study, we showed that both CRF and urocortin are expressed in climbing fibres and mossy fibre rosettes whereas only urocortin is present in parallel fibres (Swinny et al., 2002). Hence, CRF and urocortin are capable of coupling to CRF-R1 and CRF-R2 respectively presynaptically. The presynaptic localisation of these receptors is in accordance with physiological data from other brain regions (Lawrence et al., 2002; Lewis et al., 2002). The reason and consequences for this presynaptic interaction is unclear. However, presynaptic localisation of receptors, acting as autoreceptors (Merighi, 2002), is not uncommon for neuropeptides. For example, both CGRP and its receptors are localised in climbing fibres (A. Rosina, personal communication). The presynaptic coupling to receptors, usually in concert with other factors such as receptor activity modifying proteins (Born et al., 2002) usually serves to control the release of the neuropeptide and/or other co-stored modulators (Malcangio and Bowery, 1999; Khakh and Henderson, 2000). The presynaptic localisation of CRF receptors suggests that they might play a role in the release of CRF-like ligands.

In climbing fibres, CRF and urocortin probably adopt the roles of neuromodulators, as opposed to the roles of classic neurotransmitters. While receptors of classical neurotransmitters are generally concentrated directly beneath or in close proximity of the postsynaptic membrane (Merighi, 2002) CRF-R1 or CRF-R2 were never visualised on postsynaptic sites of climbing fibre contacts, namely the proximal stubby spines. CRF-R1 was consistently visualised only in primary dendrites and their branches. This potential neuromodulator role of CRF, as opposed to a neurotransmitter function, is validated by studies of Bishop, (1990) who showed that CRF itself had no excitatory action on Purkinje cell activity. Instead, CRF caused Purkinje cell excitation by potentiating the effects of endogenous neurotransmitters like glutamate. Cross-talk between different receptors is not uncommon (Bloch et al., 1999; Satake et al., 2000). The interaction between the climbing fibre CRF and glutamatergic systems would appear to be crucial since both CRF receptors (Miyata et al., 1999) and metabotropic glutamate receptors (mGluR-1) (Ichise T et al., 2000) are essential
for LTD. mGlu-R1 are also necessary for the elimination of supernumery climbing fiber (Ichise et al., 2000), processes deemed crucial in the correct wiring and functioning of the cerebellar circuitry.

**CRF receptors in Purkinje neuron**

The Purkinje cell showed significant CRF-R1 and CRF-R2 immunoreactivity from early developmental stages through to adulthood. CRF-R1 immunoreactivity showed a more dynamic developmental profile than CRF-R2, occurring at early stages in the Purkinje cell somata, but shifting first into the apical caps, the precursor regions for dendritic outgrowth (Altman and Bayer, 1997) and later into the primary dendrites and dendritic branches. Also, CRF-R1 was not evidently bound to membranes, however, it was always occurring in the cytoplasm of somata or dendrites. These observations are rather unexpected since CRF-R1 belongs to the family of G-protein coupled receptors which are usually membrane bound. However, this cytoplasmic localisation is consonant with the report by Radulovic et al. (1998), who showed that Purkinje neurons are the only cell type in the brain without membrane bound reaction product. The scarcity of membrane bound CRF-R1 expression could be a reflection of the degree of ligand-receptor coupling and subsequent cytoplasmic internalisation that this receptor undergoes, so as to limit the effects of the ligands binding to it (Bloch et al., 1999).

In contrast, CRF-R2 immunoreactivity resided predominantly in Purkinje cell somata, particularly in the basal regions and axon hillocks. Only during the developmental stages of PD 9 to 15 was reaction product evident in dendritic spines contacted by parallel fibres. This is a critical period of Purkinje cell dendritic development thought to be heavily influenced by parallel fibre activity (Altman and Bayer, 1997). It is speculative whether urocortin contained in parallel fibres (Swinny et al., 2002) acts directly on CRF-R2 contained in the spines during this time, perhaps playing a collaborative role with other agents implicated synapse formation and dendritic maturation. An additional piece of evidence for CRF-R2 being more active at the synaptic level, in comparison to CRF-R1, is that it appears to be membrane bound, especially in the Purkinje cell somata. The presence of CRF-R2, membrane bound on the Purkinje cell soma, a region contacted by basket cell terminals suggests that CRF-R2 could be coupled postsynaptically to urocortin released from basket cell terminals or recurrent axonal collaterals of Purkinje cells.

The translocation of CRF-R1 into the developing dendrite infers an active role of the receptor in the early outgrowth of Purkinje cells dendrites. Dendritic development is governed by intrinsic patterns (Threadgill et al., 1997) and extrinsic cues (Whitford et al., 2002). CRF, by coupling to CRF-R1 has been shown to mediate neurite
outgrowth in catecholaminergic immortalized neuron (Cibelli et al., 2001). Further investigations are needed to determine whether CRF, or particularly urocortin are implicated in the early intrinsic pattern of Purkinje cell dendritic development. Since CRF-R1 was mainly in the dendritic shafts and CRF-R2 in dendritic spines, these two receptors, in concert, could play significant roles in modulating the development of the functionally different domains of Purkinje cell dendrites.

In conclusion, the presynaptic expression of CRF receptors, together with co-localised CRF and urocortin could play a role in differentially modulating the transmission of sensory information contained within the cerebellar afferent systems. Secondly, in light of the established developmental role of CRF receptors, their localisation within different regions of the Purkinje cell dendritic tree alludes to their participation in the intrinsic program regulating the outgrowth and maturation of Purkinje cell dendrites. Functional studies are underway to test the above hypotheses.

REFERENCES


corticotropin releasing factor receptor types 1 and 2


