# Corticotropin-releasing factor receptor types 1 and 2 are differentially expressed in pre- and post-synaptic elements in the post-natal developing rat cerebellum

Swinny, JD; Kalicharan, D; Blaauw, EH; Ijkema-Paassen, J; Shi, F; Gramsbergen, A; van der Want, JJL

*Published in:*
European Journal of Neuroscience

*DOI:*
10.1046/j.1460-9568.2003.02776.x

*European Journal of Neuroscience, Vol. 18, pp. 549–562, 2003* 

# Corticotropin-releasing factor receptor types 1 and 2 are differentially expressed in pre- and post-synaptic elements in the post-natal developing rat cerebellum

J. D. Swinny,[1] D. Kalicharan,[1] E. H. Blaauw,[1] J. Ijkema-Paassen,[2] F. Shi,[3] A. Gramsbergen[2] and J. J. L. van der Want[1]
[1]Laboratory for Cell Biology and Electron Microscopy, Department of Cell Biology, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, the Netherlands
[2]Department of Medical Physiology, Graduate School of Behavioural and Cognitive Neuroscience University of Groningen, the Netherlands
[3]Department of Membrane Cell Biology, University of Groningen, the Netherlands

*Keywords*: climbing fibre, dendrite, neuromodulator, parallel fibre, Purkinje cell

## 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. The cerebellar expression of corticotropin-releasing factor-like ligands has been well documented, but their receptor localization has not. This is the first combination of a light microscopic and ultrastructural study to localize corticotropin-releasing factor receptors immunohistologically in the developing rat cerebellum. Both CRF-R1 and CRF-R2 were expressed in climbing fibres from early stages (post-natal day 3) to the adult, but CRF-R2 immmunoreactivity was only prominent throughout the molecular layer in the posterior cerebellar lobules. CRF-R1 immunoreactivity was concentrated in apical regions of Purkinje cell somata and later in primary dendrites exhibiting a diffuse cytoplasmic appearance. In Purkinje cells, CRF-R1 immunoreactivity was never membrane bound post-synaptically in dendritic spines while CRF-R2 immunoreactivity was found on plasmic membranes of Purkinje cells from post-natal day 15 onwards. We conclude that the localization of these receptors in cerebellar afferents implies their pre-synaptic control of the release of corticotropin-releasing factor-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 Purkinje cells 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.*, 2001) 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). The CRF in the cerebellum is restricted to the two major afferent systems, namely the mossy fibres and climbing fibres (Palkovits *et al.*, 1987). We have recently demonstrated that urocortin is localized within the afferents and within cerebellar neurons in the adult rat cerebellum (Swinny *et al.*, 2002). This disparate localization of CRF and urocortin suggests contrasting roles for these peptides in cerebellar function.

The 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) (Lovenberg *et al.*, 1995; Perrin *et al.*, 1995). Both receptors belong to the family of G-protein-coupled receptors and act by activating adenylate cyclase (Aguilera *et al.*, 1983; Bilezikjian & Vale, 1983; Giguere & Labrie, 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 localization 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 whether the receptors are localized in pre- or post-synaptic elements, or both. An ultrastructural examination at the electron microscopic level is imperative as the Purkinje cell is composed of functionally distinct regions due to the heterologous synaptic inputs it receives on different locations (see Voogd & Glickstein, 1998 for a review). Inhibitory interneurons, like basket cells and stellate cells, synapse on the Purkinje cell somata and dendritic shafts, respectively.

*Correspondence*: Dr J. D. Swinny, as above.
E-mail: j.d.swinny@med.rug.nl

*Received 15 November 2002, revised 10 April 2003, accepted 30 April 2003*

doi:10.1046/j.1460-9568.2003.02776.x

Climbing fibres and parallel fibres provide excitatory input at the proximal stubby dendritic spines and distal, thinner dendritic spines, respectively (Palay & Chan-Palay, 1974; Sotelo, 1978). This synaptic patterning and morphological development largely occurs within the first three post-natal weeks in the rat (Altman & 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 that CRF-like peptides serve, first in motor development and subsequently in motor learning.

## Materials and methods

Black-hooded Lister rats were studied from post-natal day (PD) 3 to 25. The day of birth was considered PD 0. A total of twelve animals was used. Approval to conduct the study was obtained from the Ethics Committee on Animal Experimentation, University of Groningen. Care of the animals was according to EEC directive I6/609/EEC. All efforts were made to minimize the number of animals used. Animals were deeply anaesthetized with sodium pentobarbital (Nembutal®, i.p., 50 mg/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 (Towbin *et al.*, 1979; Shi *et al.*, 2001). Cerebella samples at different ages were prepared from fresh rat cerebellum lysed in sample buffer [3 mL/mg, 5% sodium dodecyl sulphate, 5% β-mercaptoethanol, 8 M urea, 6.25 mM Tris-HCl, pH 6.8, and 0.01% bromophenol blue]. Cerebellum samples (20 μL) were separated on 12.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA), blotted on a pure nitrocellulose membrane (Bio-Rad) and probed with goat anti-CRF-R1 or -CRF-R2 (1 : 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The CRF-R1 blot was followed by horseradish peroxidase-conjugated rabbit anti-goat antibody (1 : 5000; Sigma, Stinheim, Germany) and processed using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech) according to the manufacturer's instructions. 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 30 000) and 0.4% $NaNO_3$ in 0.1 M phosphate buffer (pH 7.4) and then with 100 mL of 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (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 cryoprotection and cryo-sectioned (30 μm thick) in the sagittal plane. Immunoreactivity was visualized by fluorescence. Briefly, free-floating sections were immersed for 2 h in a pre-incubation medium containing 1% normal rabbit serum and 1% bovine serum albumin in 0.1 M 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 Biotechnology), 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 2 h at room temperature. After further washing, the sections were mounted in Dako anti-fading mounting medium (Dako Corporation, CA, USA).

### *Imaging*

All sections were evaluated. However, selected sections were photographed with a digital camera (Olympus) mounted on a B50 fluorescence microscope (Olympus). 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. Sections (50 μm) were cut on a Vibratome in the sagittal plane. Immunoreactivity was visualized by the avidin-biotin-peroxidase complex (ABC) method. Briefly, free-floating sections were immersed for 2 h in a pre-incubation medium containing 1% normal rabbit serum, 0.025% Triton X-100 and 1% bovine serum albumin in 0.1 M 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 Biotechnology), 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 2 h at room temperature. After further washing, the sections were incubated with ABC (Vectastain Elite; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Immunoreactivity was visualized by incubation with 5 mg 3,3′-diaminobenzidine HCl and 0.03% hydrogen peroxide in 10 mL of 0.1 M PBS (pH 7.4) for 5–10 min 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 & Gorcs, 1986). Sections were osmicated in 1% $OsO_4$ and 1.5% potassium hexacyanoferrate in 0.1 M cacodylate buffer (pH 7.6) for 15 min, dehydrated in a graded series of ethanols 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 transmission electron microscope (CM 100; Philips).

#### *Post-embedding immunocytochemistry*

Post-embedding 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, post-fixed, 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 an EM CPC (Leica). Frozen tissue was immersed in 1.5% uranyl acetate in methanol at −90 °C in an AFS freeze-substitution instrument (Leica), infiltrated with Lowicryl HM 20 resin at −45 °C and polymerized with u.v. light (−45 to 0 °C). Thin sections were cut on an ultramicrotome (Reichert Ultracut S; Leica), placed on nickel grids (Electron Microscopy Sciences, Fort Washington, USA), incubated in 0.1% sodium borohydride plus 50 mM glycine in Tris-buffered saline/0.1% Triton X-100 for 10 min and in blocking serum in Tris-buffered saline/0.1% Triton X-100 for 10 min (10% normal goat serum). 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, USA). Following washing, the immunogold particles were further enhanced for 10 min in the dark according to a modified protocol of Yi *et al.* (2001). Concentrations of primary antibodies were selected to minimize 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 Biotechnology). 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-RI 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, 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 was assessed by immunoblotting rat cerebellar homogenates. As 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 recognized 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 (Grigoriadis & De Souza, 1989; Chang *et al.*, 1993; Vita *et al.*, 1993). Weak indistinct bands were evident in immunoblots from homogenates of PD 1–4, with signal intensity increasing from PD 8 onwards through to PD 12. A slight tapering off was observed in adult homogenates (Fig. 1A). Pre-adsorption 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 pre-treated with the antigenic peptide (data not shown). For CRF-R2, the antiserum recognized a protein in the 40–50 kDa range, also in close approximation of previous data (Lovenberg *et al.*, 1995; Vita *et al.*, 1993; Perrin *et al.*, 1995) (Fig. 1B). 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 PD 4–12 with a slight decrease at PD 25. Like CRF-R1, pre-adsorption of the primary antiserum with a blocking peptide consisting of the immunogenic epitope (100 μg/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 urocortin peptides resulted in no discernable decrease in the intensity of immunoreactivity, confirming that there the antisera are not cross-reactive with these peptides.

### Light microscopy

#### *CRF-R1*

Post-natal day 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 in Purkinje cell immunoreactivity. Interneurons in the molecular layer also exhibited immunoreactivity (Fig. 2A and B). Electron microscopic evidence showed conclusively that both basket and stellate cells are immunoreactive (see Fig. 6).

Between the stages of PD 9–12, labelling in Purkinje cells was preferentially localized 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 (Fig. 2C and D). Also evident at this stage were varicose fibre-like immunoreactive profiles at the level of the Purkinje cell somata (Fig. 2E). 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 Fig. 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 subset 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. (Fig. 2G and H).

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

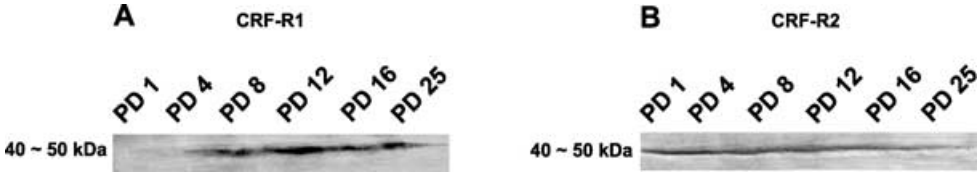



FIG. 1. Western blot analysis of post-natal cerebellar homogenates of post-natal day (PD) 1, 4, 8, 12 and 25 showing the specificity and degree of binding of corticotropin-releasing factor (CRF) receptor antisera. (A) CRF receptor type one (CRF-R1) antiserum recognized a single protein of approximately 40 kDa. Weak banding was observed between the stages of PD 1–4. Expression became more intense between the stages of PD 4–8 with PD 12 showing the most intense banding pattern. (B) CRF receptor type two (CRF-R2) antiserum consistently recognized a single protein of approximately 50 kDa. 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 4–12.

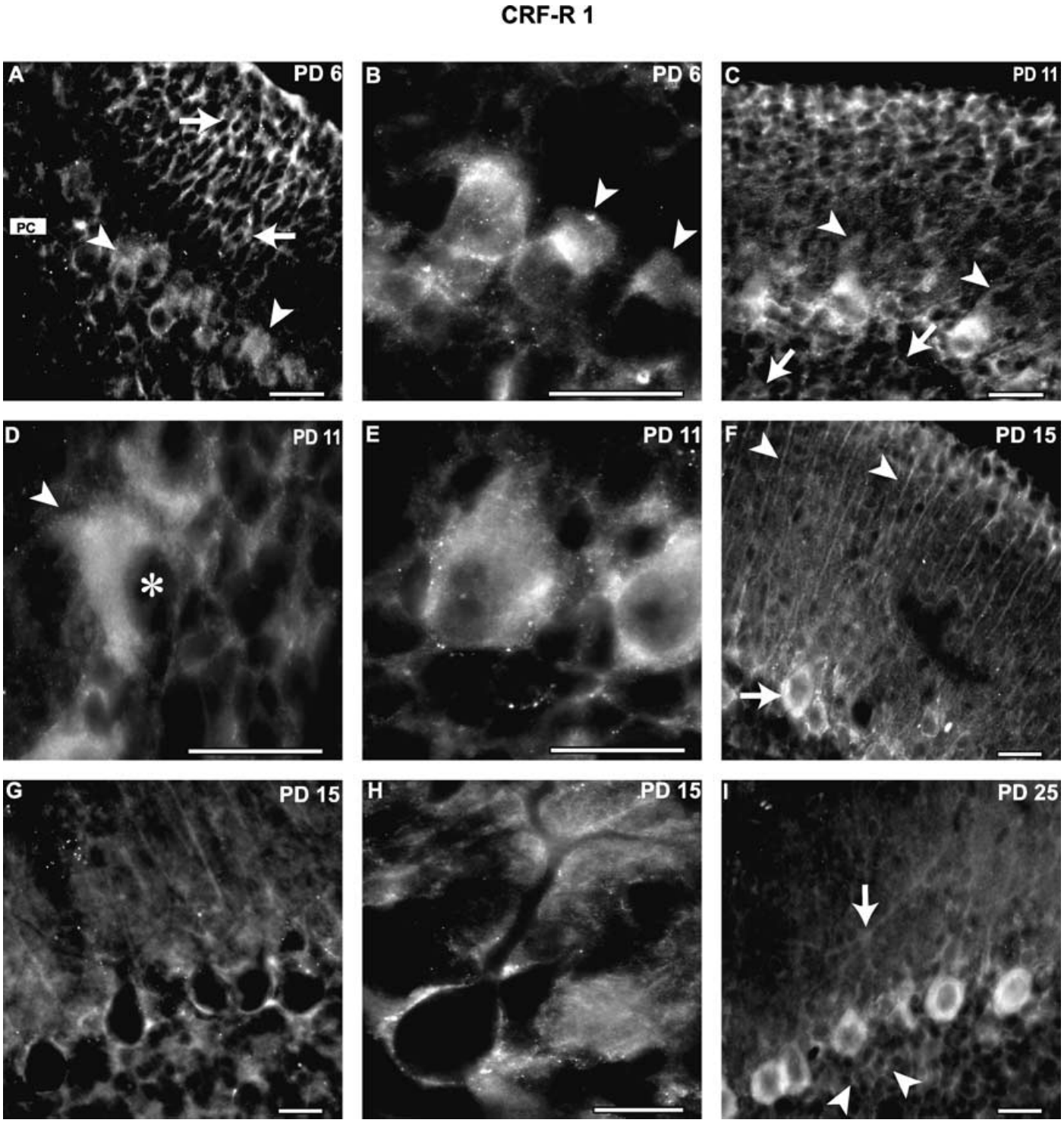



FIG. 2. Immunofluorescence of corticotropin-releasing factor receptor type one (CRF-R1) at different developmental stages. (A) An overview of the cerebellar cortex at post-natal day (PD) 6, showing immunoreactivity in the Purkinje cell (PC) layer, especially in the apical caps of Purkinje cells (arrowheads). 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 localized 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, 20 μm (A–E); 25 μm (F–I).

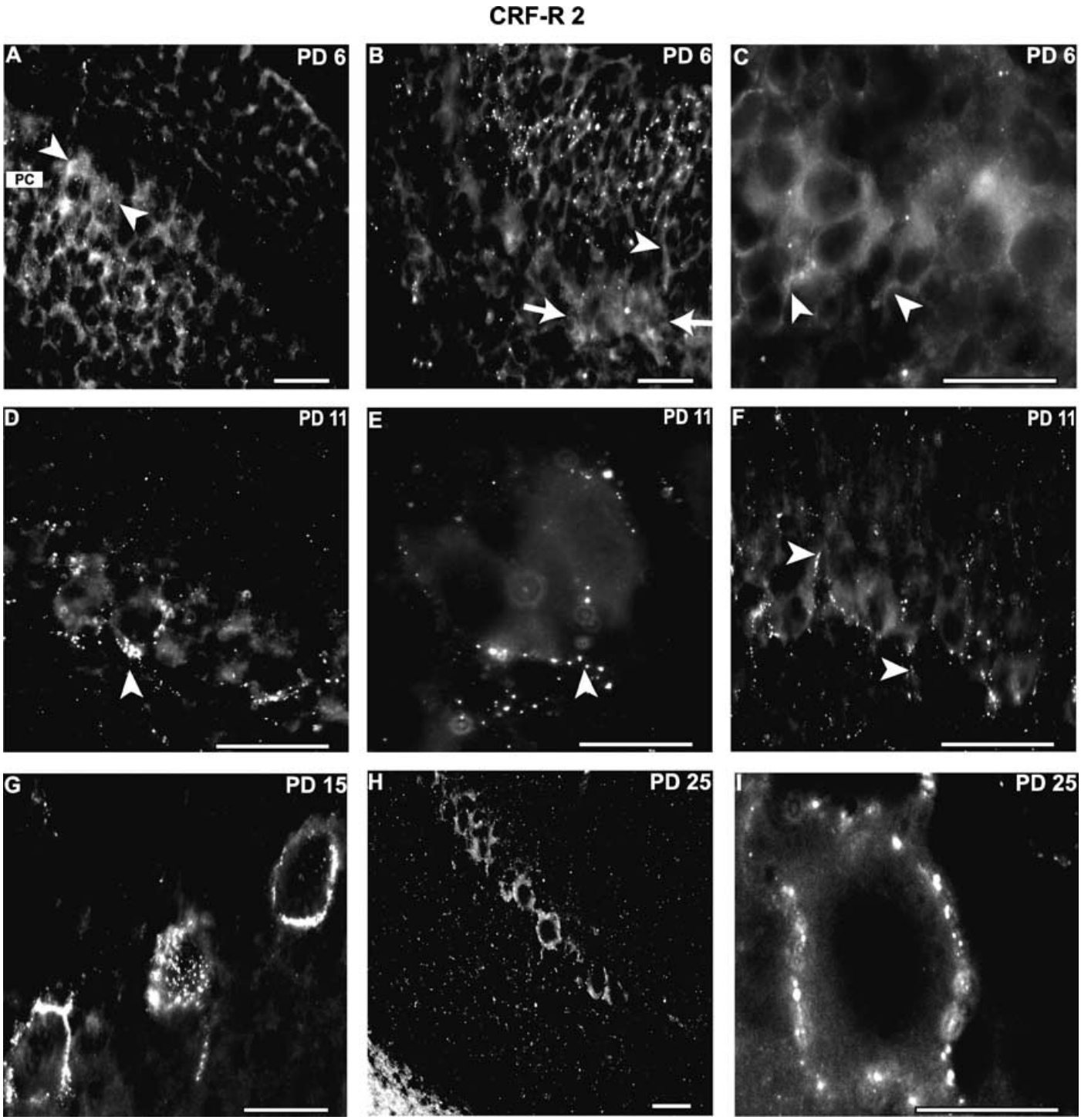



FIG. 3. Corticotropin-releasing factor receptor type two (CRF-R2) immunofluorescence in the cerebellar cortex at different post-natal stages. (A) An overview from lobule III showing immunoreactivity predominating in Purkinje cell (PC) somata (arrowheads). The molecular and granular layers show indistinct 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 localized 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 post-natal day (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 profiles (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 Fig. 7B). (G–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, 20 μm (A–F); 25 μm (G–I).

*CRF-R2*

In the first post-natal 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. 3A). 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

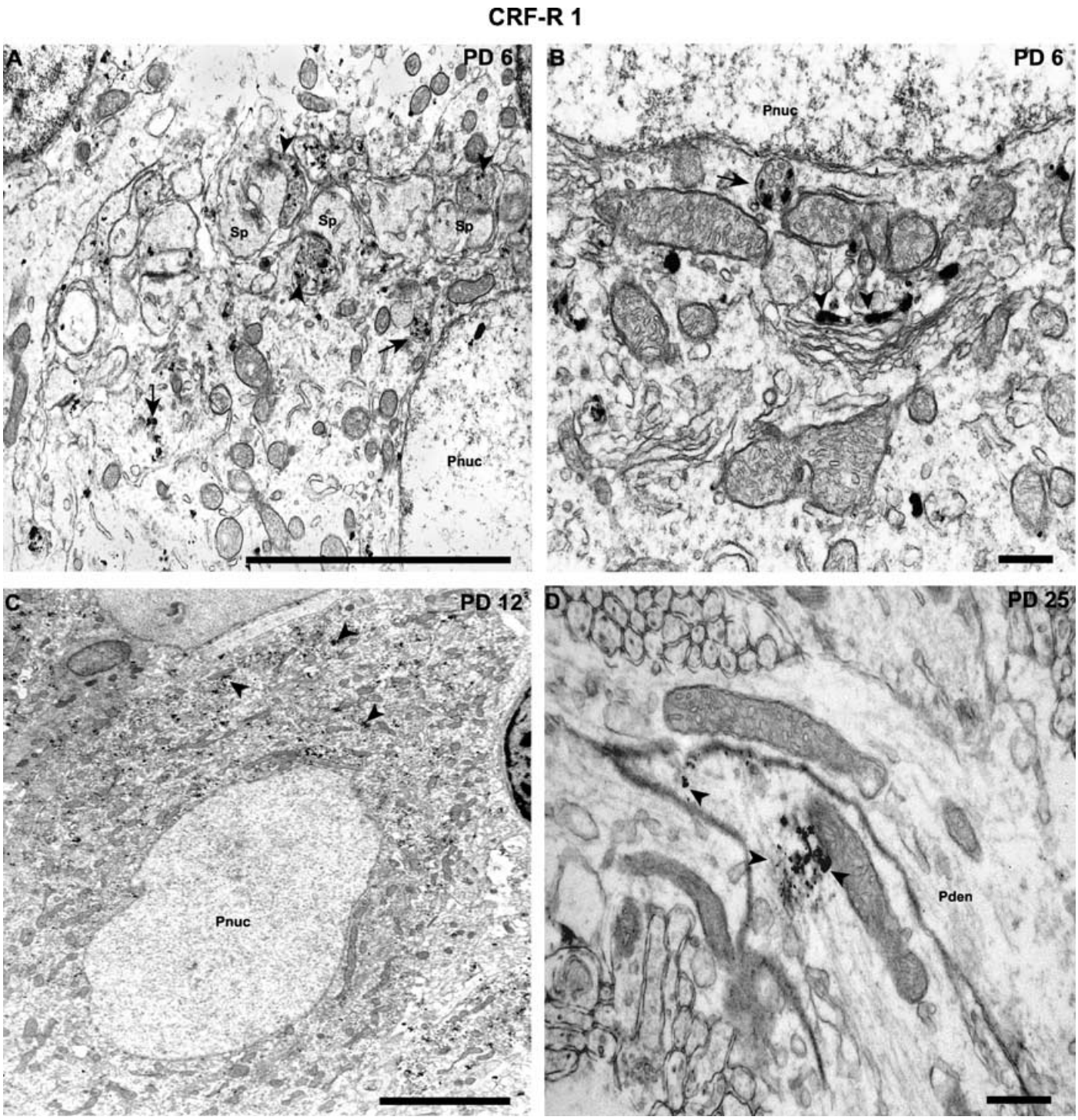



FIG. 4. Electron micrographs of corticotropin-releasing factor receptor type one (CRF-R1) immunoreactivity in the Purkinje cell at different developmental stages. (A) Overview of a Purkinje cell at post-natal day (PD) 6. Reaction product is contained within the somata (arrows) and also in multiple climbing fibre terminals making synaptic contacts on somatic spines (arrowheads). (B) A high magnification showing the expression of receptor protein within the soma of a Purkinje cell. The reaction product is present within the Golgi lamellae (arrowhead) and a multivesicular body (arrow). (C) An overview of a Purkinje cell at PD 12 showing the reaction product concentrated in the apical region of the soma (arrowheads) with the basal region relatively free of immunoreactivity. (D) At PD 25, the reaction product was diffusely spread throughout the Purkinje cell dendrite. Note that images are taken from pre-embedding immunostaining. Pden, Purkinje cell dendrite; Pnuc, Purkinje cell nucleus; Sp, Purkinje cell spine. Scale bars: (A and C), 5 μm; (B), 1 μm; (D), 500 nm.

climbing fibres as immunoelectron microscopy revealed reaction product in both profiles. In certain Purkinje cells, labelling was evident in the primary dendrites (Fig. 3B and C). Between the stages of PD 9–12, Purkinje cell immunoreactivity was localized mainly in the basal regions of their somata and was apposed to the cell membranes (Fig. 3D and E). Immunoreactive fibre-like profiles originating from the granular layer, encircling Purkinje cell somata and encroaching on their proximal primary dendrites were observed

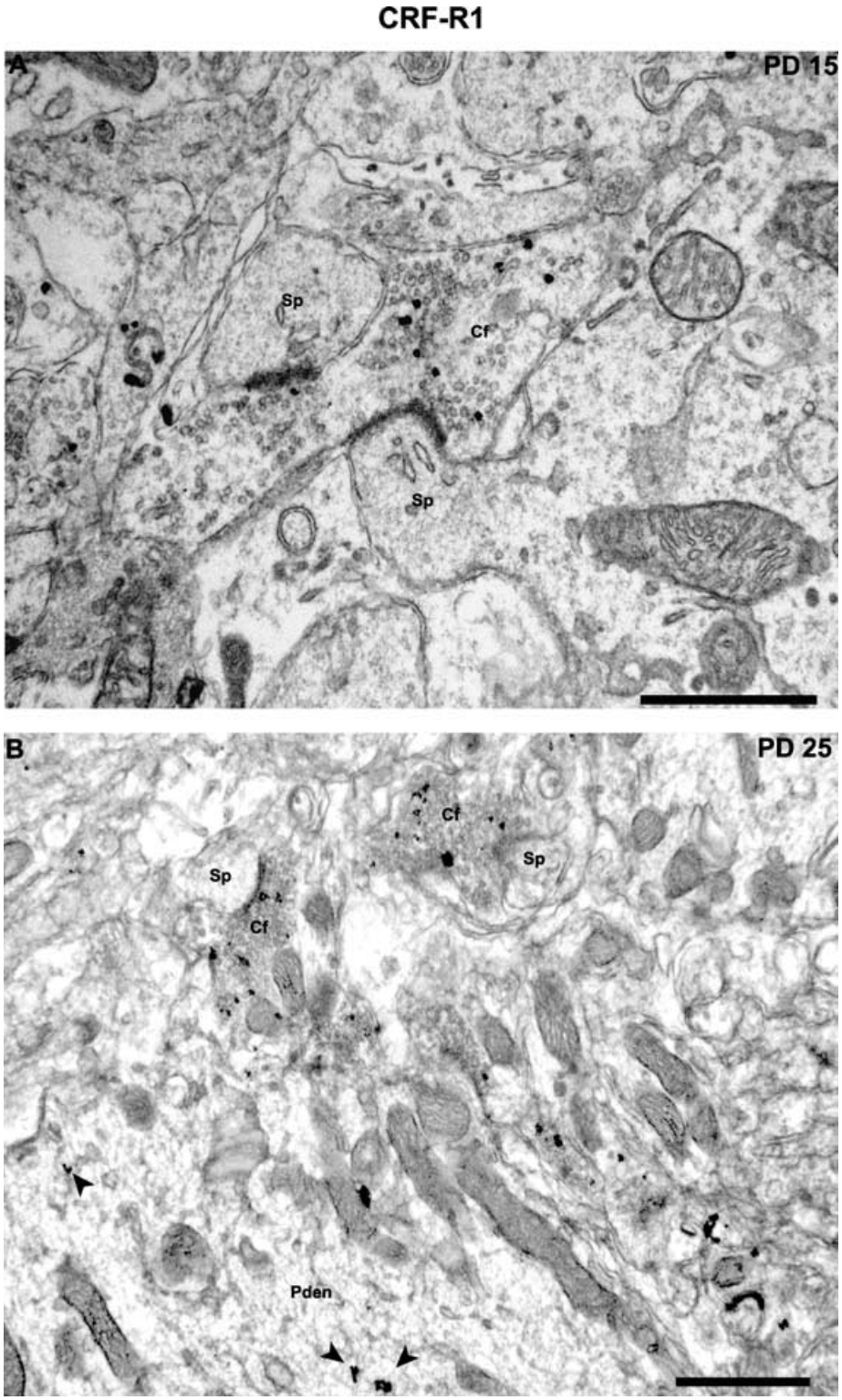



FIG. 5. Electron micrographs of climbing fibres showing dense and selective corticotropin-releasing factor receptor type one (CRF-R1) immunoreactivity. (A) An immunoreactive climbing fibre terminal making synaptic contacts on spines of Purkinje cell dendritic spine at post-natal day (PD) 15. (B) Similar 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.

at this stage. These are presumably climbing fibres, primary Purkinje cell axons or recurrent axonal collaterals (Fig. 3F). CRF-R2-immunoreactive boutons were in greater abundance in comparison to CRF-R1 (see Fig. 2E). Between PD 15 and 25, Purkinje cell immunoreactivity increased, mainly localized on somatic membranes and near the initial axonal segment. Some Purkinje cells exhibited immunoreactivity in the proximal dendritic regions (Fig. 3G–I).

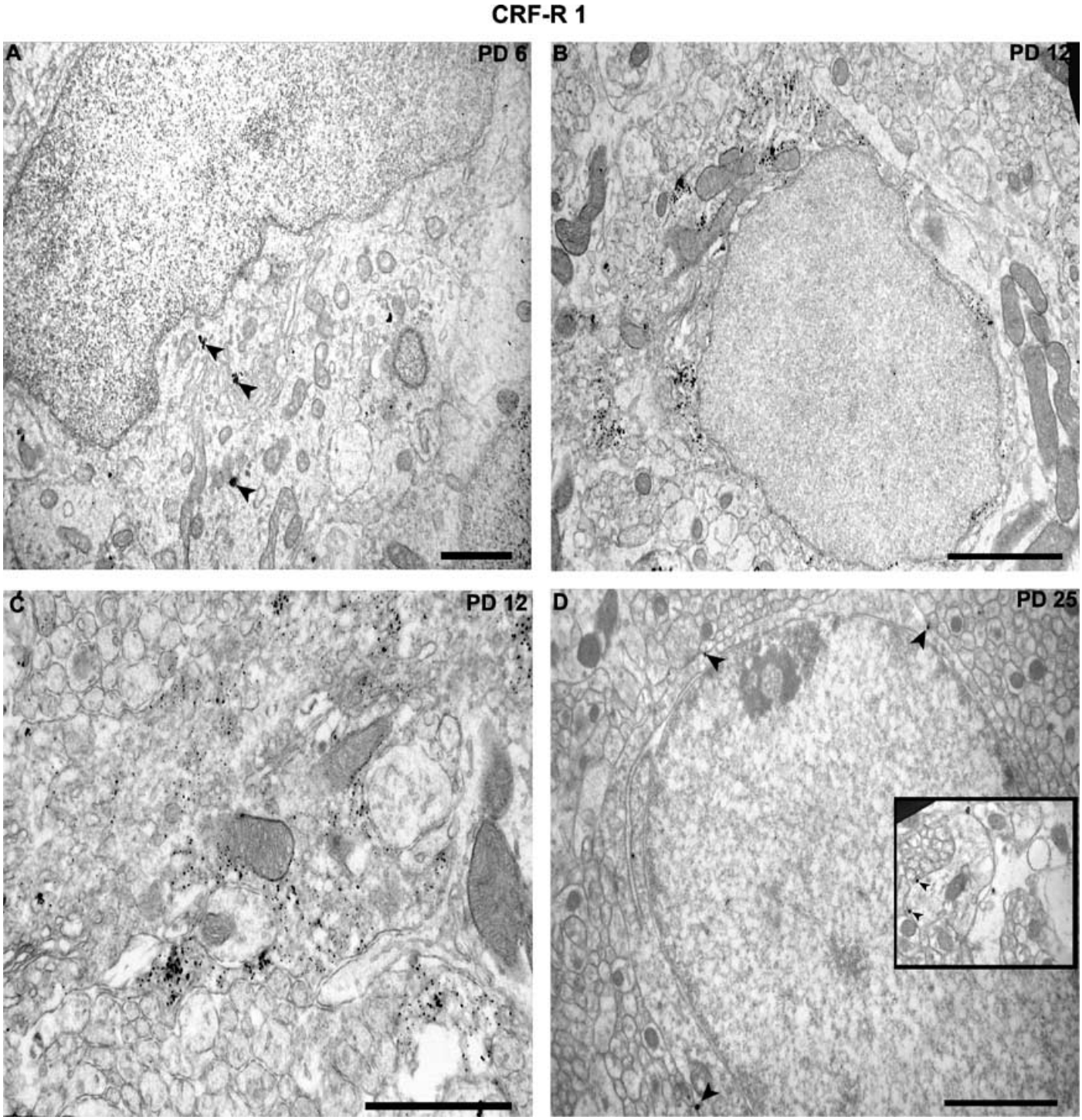



FIG. 6. Electron micrographs of corticotropin-releasing factor receptor type one (CRF-R1) immunoreactivity within interneurons in the molecular layer. (A) An overview of a stellate cell at post-natal day (PD) 3 identified by its pale nucleus (in comparison to basket cells) showing reaction product within 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) 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 (A–C) are of pre-embedding immunocytochemistry and (D) of post-embedding immunocytochemistry. Scale bars: (A and C), 1 μm; (B and D), 2 μm.

### *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 localized either within the trans Golgi network, in small vesicles or in multivesicular bodies (Fig. 4A 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 PD 6–11 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. 4C). 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 post-embedding techniques, during the entire period of ontogeny, CRF-R1 immunoreactivity in the Purkinje cell was never found on or close to synaptic membrane specializations (Fig. 4D).

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. 4A). 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. Post-embedding labelling showed that the reaction product was localized in the cytoplasm and was not related to membranes (data not shown) (Fig. 5A and B).

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-R1 immunoreactivity. The reaction product was mainly localized in somata and dendrites and not in terminals. The pattern of somatic and dendritic expression persisted into adulthood, with post-embedding labelling showing reaction product often membrane bound in the somata and dendrites (Fig. 6).

#### *CRF-R2*

Within the first post-natal week, pre- and post-embedding labelling showed that Purkinje cell immunoreactivity was purely cytoplasmic, particularly in the region of the rough endoplasmic reticulum. Membrane-bound reaction product was not evident at this early stage (Fig. 7A). At PD 12, the reaction product was evident in somata, while climbing fibre terminals making synaptic contact on somatic spines were also immunoreactive (Fig. 7B). Using post-embedding labelling, PD 15 was shown to be the first stage at which reaction product was localized bound to plasma membranes (Fig. 7C). 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 peri-synaptic. Membrane-bound reaction product was also associated with vacuolization, presumably due to endocytotic receptor internalization (Fig. 7D–F).

In the molecular layer, CRF-R2 immunoreactivity was present in Purkinje cell dendritic spines contacted by parallel fibres. Labelling in spines was only expressed from PD 9 through to PD 15. During this period, parallel fibre terminals were always free of immunoreactivity (Fig. 8A and B). Labelling in parallel fibre terminals was evident at later stages. In the pre-synaptic terminal, immunogold particles were visualized close to the synaptic membrane specialization (Fig. 8C–E).

CRF-R2 immunoreactivity was expressed in climbing fibres from early stages (see Fig. 7B) through to adulthood. It could be documented, using pre- and post-embedding labelling, that the reaction product was often associated with vesicles and was rarely bound to pre-synaptic membranes (Fig. 9).

## Discussion

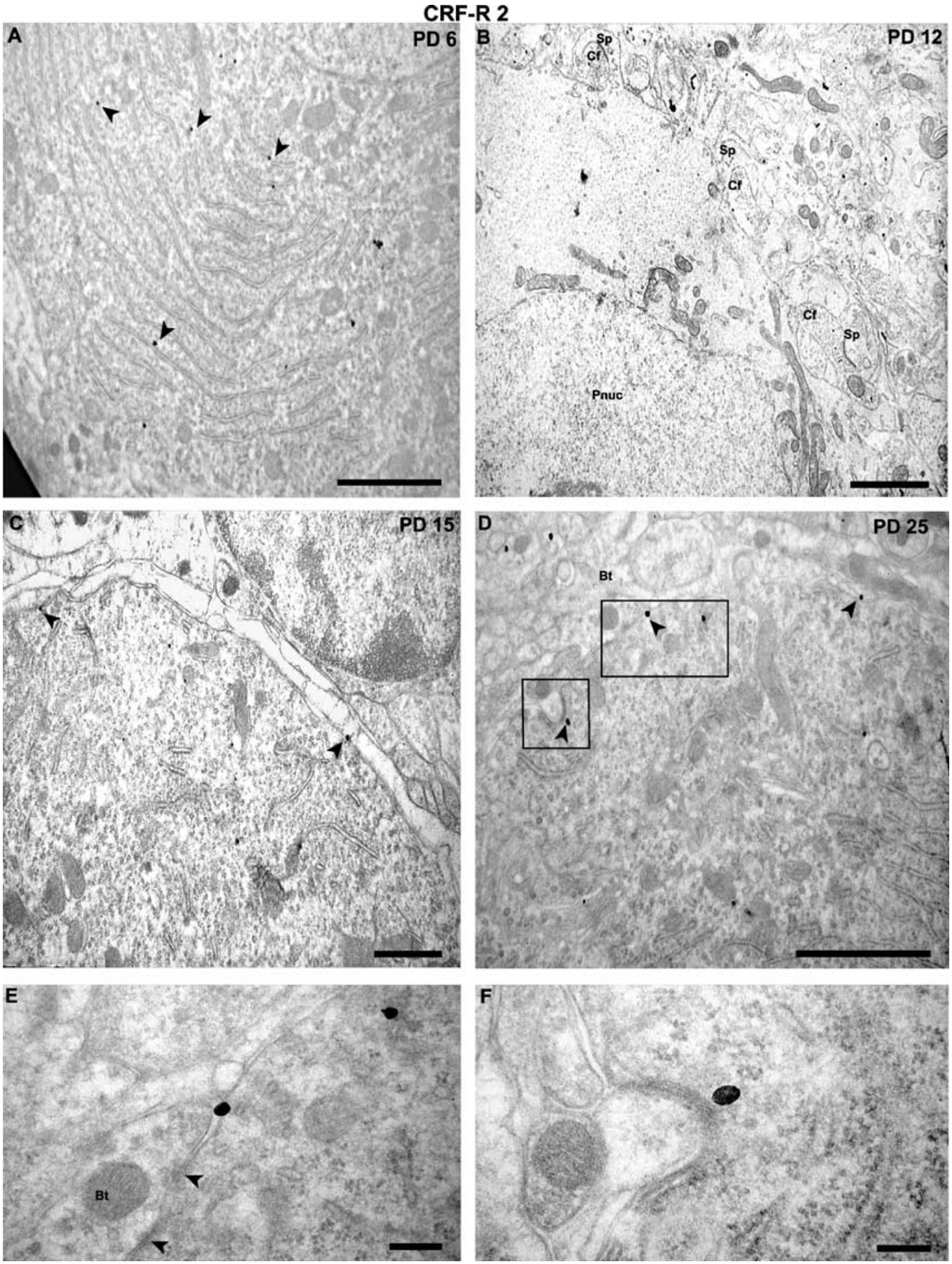
The current study is the first to provide a detailed account of the localization of CRF-R1 and CRF-R2 at different developmental stages at the subcellular level. We have largely concentrated on the developing Purkinje cells as they develop their dendritic tree and afferent input during the first three post-natal weeks (Sotelo, 1978) and are also considered the organizing 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 localized pre-synaptically or post-synaptically and secondly, do cerebellar afferents express CRF receptors? Such data are crucial for an integrative analysis of light microscopic observations on CRF and urocortin localization, 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 localization 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 the 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 localization of receptors, especially G-protein-coupled receptors which are expected to be predominantly membrane bound. We have used post-embedding immunocytochemistry using immunogold particles as this is regarded as the method to provide the most accurate localization (Lopez-Bendito *et al.*, 2001). However, the trade-off, compared with the peroxidase method, is a decreased intensity on labelling and this is evident in this study.

### *Corticotropin-releasing factor receptors in cerebellar afferents*

From PD 3–4, CRF-R1 and CRF-R2 are expressed in climbing fibres and mossy fibre rosettes while only parallel fibres express CRF-R2. The 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, pre-synaptically. The pre-synaptic localization of these receptors is in accordance with physiological data from other brain regions (Lawrence *et al.*, 2002; Lewis *et al.*, 2002). The reason for and consequences of this pre-synaptic interaction are unclear. However, pre-synaptic localization of receptors, acting as autoreceptors (Merighi, 2002), is not uncommon for neuropeptides. For example, both CGRP and its receptors are localized 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


CRF-R 2
A
PD 6
B
PD 12
Sp
Cf
Sp
Cf
Cf
Sp
Pnuc
C
PD 15
D
PD 25
Bt
E
Bt
F

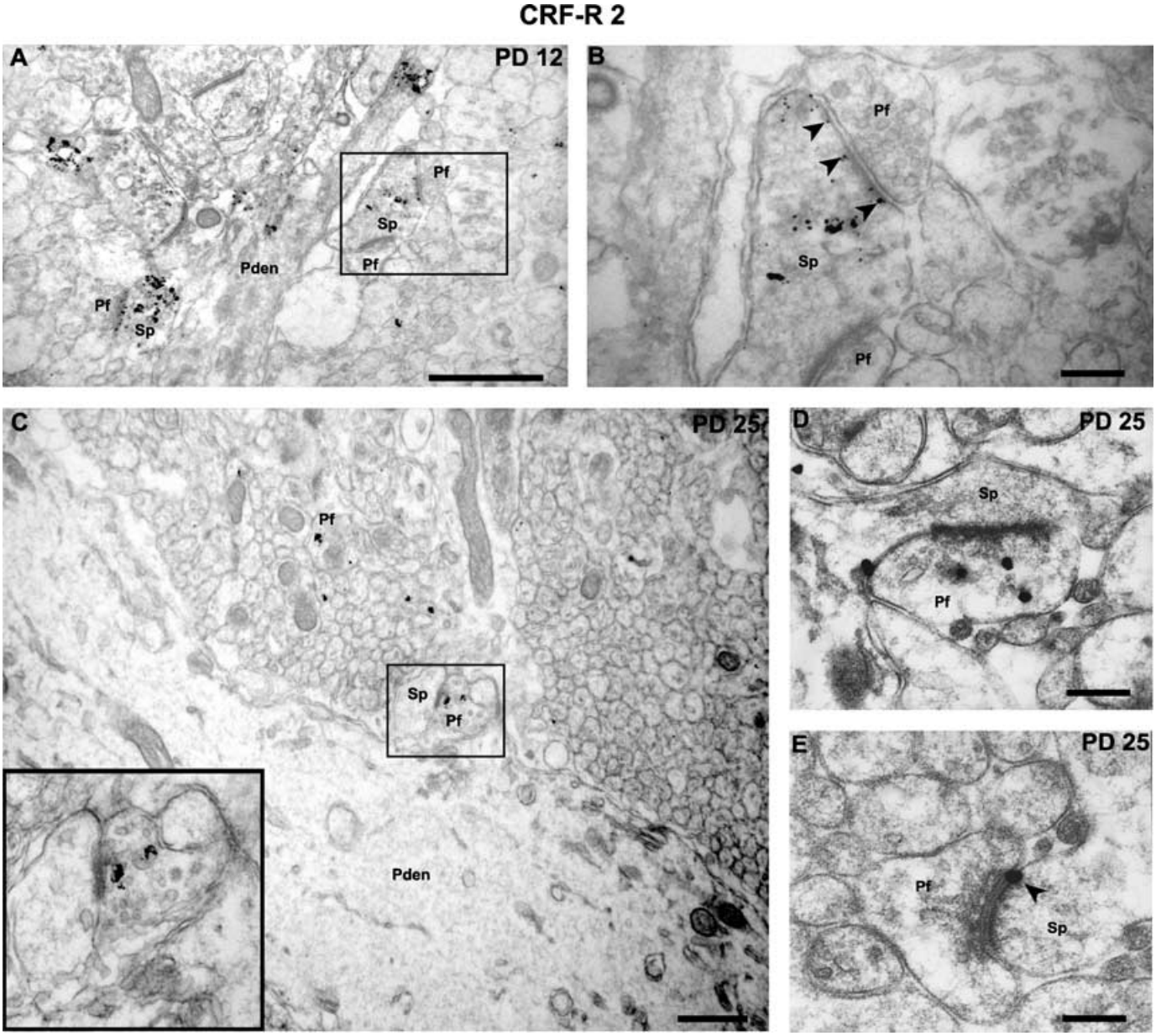



FIG. 8. Electron micrographs of corticotropin-releasing factor receptor type two (CRF-R2) at Purkinje cell–parallel fibre contacts at post-natal day (PD) 12 and 25. (A) An overview showing parallel fibres making synaptic contact with immunoreactive dendritic spines at PD 12. Note the reaction product also contained within the dendritic branch. (B) A magnified view of the boxed area in (A), showing scattered reaction product within the spine and in the region of the post-synaptic density (arrowheads). (C) An overview of parallel fibre–Purkinje cell contacts at PD 25 showing immunoreactivity selectively localized 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 they were either in the lumen of the parallel fibre terminal or (E) membrane bound in the perisynaptic region (arrowhead). (A–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 and C), 1 μm; (B, D and E), 200 nm.

the neuropeptide and/or other co-stored modulators (Malcangio & Bowery, 1999; Khakh & Henderson, 2000). The pre-synaptic localization 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 to the

FIG. 7. Electron micrographs of corticotropin-releasing factor receptor type two (CRF-R2) immunoreactivity in Purkinje cells at different post-natal stages. (A) An overview of the soma of a Purkinje cell at post-natal day (PD) 6 showing immunogold particles closely associated with rough endoplasmic reticulum (arrowheads). (B) 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) A region of a soma of a Purkinje cell at PD 15. Immunogold particles are cytoplasmically localized and membrane bound (arrowheads). (D) A Purkinje cell soma at PD 25 with membrane-bound immunogold particles (arrowheads). (E) 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) A magnified view of the square boxed area in (D) showing an immunogold particle that is closely attached to the pre-synaptic membrane specialization. Bt, basket cell terminal; Cf, climbing fibre; Pnuc, Purkinje cell nucleus; Sp, Purkinje cell somatic spine. Scale bars: (A–D), 2 μm; (E and F), 200 nm.

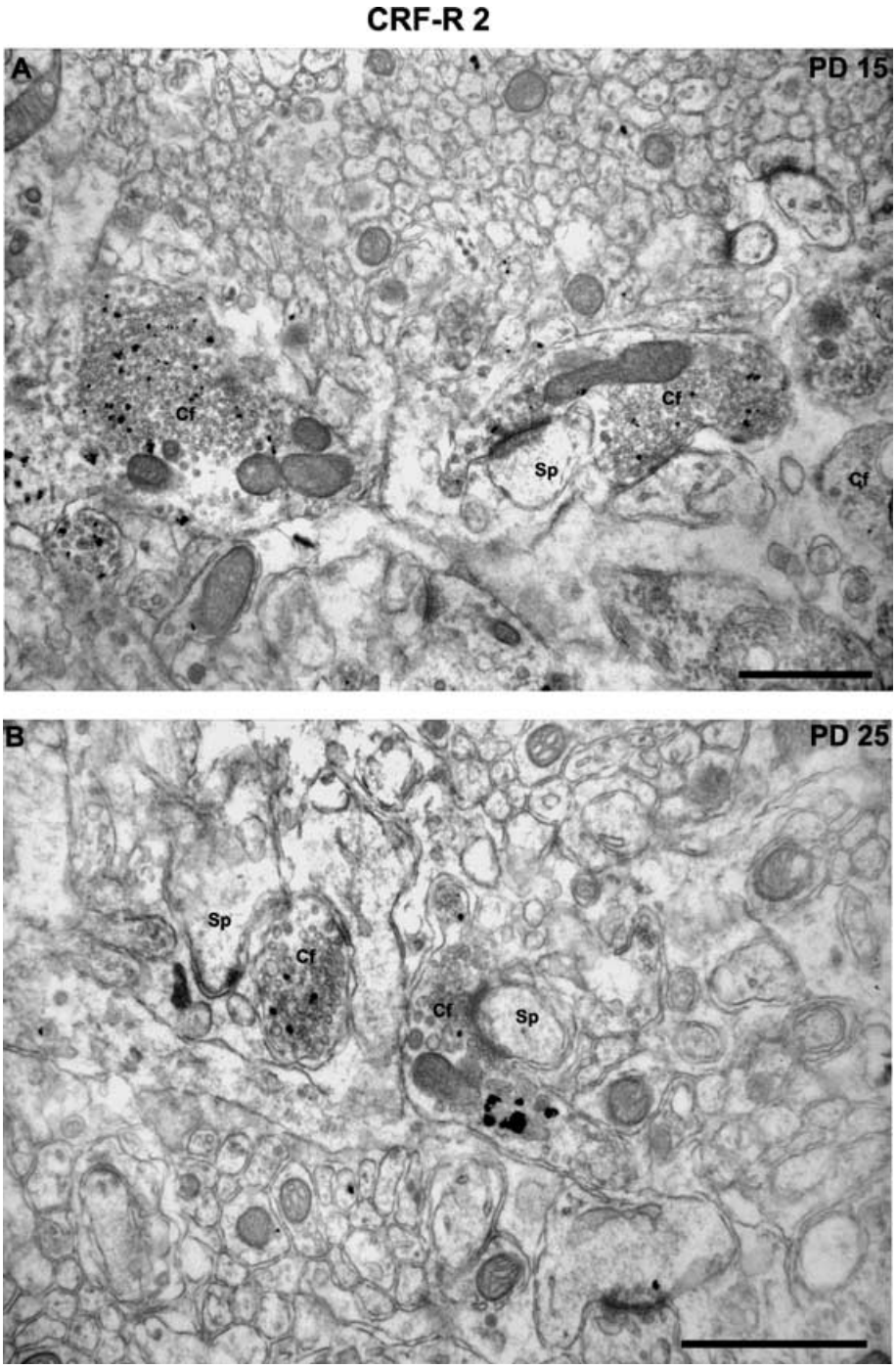



FIG. 9. Electron micrographs of corticotropin-releasing factor receptor type two (CRF-R2) immunoreactivity in climbing fibre profiles. (A) Two characteristic climbing fibre profiles making synaptic contacts on spines of Purkinje cells at post-natal day (PD) 15. (B) Similar immunoreactive profiles at PD 25. Cf, climbing fibre; Sp, Purkinje cell dendritic spine. Scale bars: 1 μm.

post-synaptic membrane (Merighi, 2002) CRF-R1 or CRF-R2 was never visualized on post-synaptic sites of climbing fibre contacts, namely the proximal stubby spines. CRF-R1 was consistently visualized 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 as both CRF receptors (Miyata *et al.*, 1999) and metabotropic glutamate receptors (Ichise *et al.*, 2000) are essential for LTD. The metabotropic glutamate receptors are also necessary for the elimination of supernumerary climbing fibres (Ichise *et al.*, 2000), processes deemed crucial in the correct wiring and functioning of the cerebellar circuitry.

### *Corticotropin-releasing factor receptors in Purkinje neurons*

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 & Bayer, 1997), and later into the primary dendrites and dendritic branches. Also, CRF-R1 immunoreactivity was not evidently bound to membranes; however, it always occurred in the cytoplasm of somata or dendrites. These observations are rather unexpected as CRF-R1 belongs to the family of G-protein-coupled receptors which are usually membrane bound. However, this cytoplasmic localization 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 internalization 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 9–15 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 & 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 in 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 post-synaptically 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 cell dendrites. Dendritic development is governed by intrinsic patterns (Threadgill *et al.*, 1997) and extrinsic cues (Whitford *et al.*, 2002). Corticotropin-releasing factor, by coupling to CRF-R1, has been shown to mediate neurite outgrowth in catecholaminergic immortalized neurons (Cibelli *et al.*, 2001). Further investigations are needed to determine whether CRF, or particularly urocortin, is implicated in the early intrinsic pattern of Purkinje cell dendritic development. As 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 pre-synaptic expression of CRF receptors, together with colocalized 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 localization within different regions of the Purkinje cell dendritic tree alludes to their participation in the intrinsic programme regulating the outgrowth and maturation of Purkinje cell dendrites. Functional studies are underway to test the above hypotheses.

## Acknowledgements

The assistance of F. Dijk, G. Kors and H. de Weerd in tissue processing for electron microscopy is gratefully acknowledged. The expert photographic assistance of B. Hellinga and D. Huizinga is also greatly appreciated.

## Abbreviations

CRF, Corticotropin-releasing factor; CRF-R1, CRF receptor type one; CRF-R2, CRF receptor type two; PBS, phosphate-buffered saline; PD, post-natal day.

## References

Aguilera, G., Harwood, J.P., Wilson, J.X., Morell, J., Brown, J.H. & Catt, K.J. (1983) Mechanisms of action of corticotropin-releasing factor and other regulators of corticotropin release in rat pituitary cells. *J. Biol. Chem.*, **258**, 8039–8045.

Altman, J. & Bayer, S.A. (1997) Development of the cerebellar system. In *Relation to its Evolution, Structure and Function*. CRC Press, Location, xxx–xxx.

Bilezikjian, L.M. & Vale, W.W. (1983) Stimulation of adenosine 3′,5′-monophosphate production by growth hormone-releasing factor and its inhibition by somatostatin in anterior pituitary cells in vitro. *Endocrinology*, **113**, 1726–1731.

Bishop, G.A. (1990) Neuromodulatory effects of corticotropin releasing factor on cerebellar Purkinje cells: an in vivo study in the cat. *Neuroscience*, **39**, 251–257.

Bishop, G.A., Seelandt, C.M. & King, J.S. (2000) Cellular localization of corticotropin releasing factor receptors in the adult mouse cerebellum. *Neuroscience*, **101**, 1083–1092.

Bloch, B., Dumartin, B. & Bernard, V. (1999) In vivo regulation of intraneuronal trafficking of G protein-coupled receptors for neurotransmitters. *Trends Pharmacol. Sci.*, **20**, 315–319.

Born, W., Muff, R. & Fischer, J.A. (2002) Functional interaction of G protein-coupled receptors of the adrenomedullin peptide family with accessory receptor-activity-modifying proteins (RAMP). *Microsc. Res. Techn.*, **57**, 14–22.

Chang, C.P., Pearse, R.V. 2nd, O'Connell, S. & Rosenfeld, M.G. (1993) Identification of a seven transmembrane helix receptor for corticotropin-releasing factor and sauvagine in mammalian brain. *Neuron*, **11**, 1187–1195.

Chen, R., Lewis, K.A., Perrin, M.H. & Vale, W.W. (1993) Expression cloning of a human corticotropin-releasing-factor receptor. *Proc. Natl Acad. Sci. U.S.A.*, **90**, 8967–8971.

Chen, Y., Brunson, K.L., Muller, M.B., Cariaga, W. & Baram, T.Z. (2000) Immunocytochemical distribution of corticotropin-releasing hormone receptor type-1 (CRF (1))-like immunoreactivity in the mouse brain: light microscopy analysis using an antibody directed against the C-terminus. *J. Comp. Neurol.*, **May 8**, 420 (3), 305–323.

Cibelli, G., Corsi, P., Diana, G., Vitiello, F. & Thiel, G. (2001) Corticotropin-releasing factor triggers neurite outgrowth of a catecholaminergic immortalised neuron via cAMP and MAP kinase signalling pathways. *Eur. J. Neurosci.*, **13**, 1339–1348.

Giguere, V. & Labrie, F. (1983) Additive effects of epinephrine and corticotropin-releasing factor (CRF) on adrenocorticotropin release in rat anterior pituitary cells. *Biochem. Biophys. Res. Commun.*, **110**, 456–462.

Grigoriadis, D.E. & De Souza, E.B. (1989) Heterogeneity between brain and pituitary corticotropin-releasing factor receptors is due to differential glycosylation. *Endocrinology*, **25**, 1877–1888.

Hatten, M.E. (1999) Central nervous system neuronal migration. *Annu. Rev. Neurosci.*, **22**, 511–539.

Ichise, T., Kano, M., Hashimoto, K., Yanagihara, D., Nakao, K., Shigemoto, R., Katsuki, M. & Aiba, A. (2000) mGluR1 in cerebellar Purkinje cells essential for long-term depression, synapse elimination, and motor coordination. *Science*, **288**, 1832–1835.

Khakh, B.S. & Henderson, G. (2000) Modulation of fast synaptic transmission by presynaptic ligand-gated cation channels. *J. Auton. Nerv. Syst.*, **81**, 110–121.

Lawrence, A.J., Krstew, E.V., Dautzenberg, F.M. & Ruhmann, A. (2002) The highly selective CRF(2) receptor antagonist K41498 binds to presynaptic CRF(2) receptors in rat brain. *Br. J. Pharmacol.*, **136**, 896–904.

Lewis, K., Li, C., Perrin, M.H., Blount, A., Kunitake, K., Donaldson, C., Vaughan, J., Reyes, T.M., Gulyas, J., Fischer, W., Bilezikjian, L., Rivier, J.,

Sawchenko, P.E. & Vale, W.W. (2001) Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc. Natl Acad. Sci. U.S.A.*, **98**, 7570–7575.

Lewis, M.W., Hermann, G.E., Rogers, R.C. & Travagli, R.A. (2002) In vitro and in vivo analysis of the effects of corticotropin releasing factor on rat dorsal vagal complex. *J. Physiol. (Lond.)*, **543**, 135–146.

Lopez-Bendito, G., Shigemoto, R., Lujan, R. & Juiz, J.M. (2001) Developmental changes in the localisation of the mGluR1alpha subtype of metabotropic glutamate receptors in Purkinje cells. *Neuroscience*, **105**, 413–429.

Lovenberg, T.W., Liaw, C.W., Grigoriadis, D.E., Clevenger, W., Chalmers, D.T., De Souza, E.B. & Oltersdorf, T. (1995) Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. *Proc. Natl Acad. Sci. U.S.A.*, **92**, 836–840.

Malcangio, M. & Bowery, N.G. (1999) Peptide autoreceptors: does an autoreceptor for substance P exist? *Trends Pharmacol. Sci.*, **20**, 405–407.

Merighi, A. (2002) Costorage and coexistence of neuropeptides in the mammalian CNS. *Prog. Neurobiol.*, **66**, 161–190.

Miyata, M., Okada, D., Hashimoto, K., Kano, M. & Ito, M. (1999) Corticotropin-releasing factor plays a permissive role in cerebellar long-term depression. *Neuron*, **22**, 763–775.

Palay, S. & Chan-Palay, V. (1974) *Cerebellar Cortex: Cytology and Organisation*. Springer, New York.

Palkovits, M., Leranth, C., Gorcs, T. & Young, W.S. (1987) Corticotropin-releasing factor in the olivocerebellar tract of rats: demonstration by light- and electron-microscopic immunohistochemistry and in situ hybridization histochemistry. *Proc. Natl Acad. Sci. U.S.A.*, **84**, 3911–3915.

Perrin, M., Donaldson, C., Chen, R., Blount, A., Berggren, T., Bilezikjian, L., Sawchenko, P. & Vale, W. (1995) Identification of a second corticotropin-releasing factor receptor gene and characterization of a cDNA expressed in heart. *Proc. Natl Acad. Sci. U.S.A.*, **92**, 2969–2973.

Petralia, R.S., Zhao, H.M., Wang, Y.X. & Wenthold, R.J. (1998) Variations in the tangential distribution of postsynaptic glutamate receptors in Purkinje cell parallel and climbing fiber synapses during development. *Neuropharmacology*, **37**, 1321–1334.

van den Pol, A.N. & Gorcs, T. (1986) Synaptic relationships between neurons containing vasopressin, gastrin-releasing peptide, vasoactive intestinal polypeptide, and glutamate decarboxylase immunoreactivity in the suprachiasmatic nucleus: dual ultrastructural immunocytochemistry with gold-substituted silver peroxidase. *J. Comp. Neurol.*, **252**, 507–521.

Radulovic, J., Sydow, S. & Spiess, J. (1998) Characterization of native corticotropin-releasing factor receptor type 1 (CRFR1) in the rat and mouse central nervous system. *J. Neurosci. Res.*, **54**, 507–521.

Reyes, T.M., Lewis, K., Perrin, M.H., Kunitake, K.S., Vaughan, J., Arias, C.A., Hogenesch, J.B., Gulyas, J., Rivier, J., Vale, W.W. & Sawchenko, P.E. (2001) Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc. Natl Acad. Sci. U.S.A.*, **98**, 2843–2848.

Satake, S., Saitow, F., Yamada, J. & Konishi, S. (2000) Synaptic activation of AMPA receptors inhibits GABA release from cerebellar interneurons. *Nature Neurosci.*, **3**, 551–558.

Shi, F., Nomden, A., Oberle, V., Engberts, J.B. & Hoekstra, D. (2001) Efficient cationic lipid-mediated delivery of antisense oligonucleotides into eukaryotic cells: down-regulation of the corticotropin-releasing factor receptor. *Nucl. Acids Res.*, **29**, 2079–2087.

Sotelo, C. (1978) Purkinje cell ontogeny: formation and maintenance of spines. *Prog. Brain. Res.*, **48**, 149–170.

Swinny, J., Kalicharan, D., Gramsbergen, A. & van der Want, J. (2002) The localisation of urocortin in the adult rat cerebellum: a light and electron microscopic study. *Neuroscience*, **14**, 891–903.

Threadgill, R., Bobb, K. & Ghosh, A. (1997) Regulation of dendritic growth and remodeling by Rho, Rac, and Cdc42. *Neuron*, **19**, 625–634.

Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl Acad. Sci. U.S.A.*, **76**, 4350–4354.

Vale, W., Spiess, J., Rivier, C. & Rivier, J. (1981) Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science*, **213**, 1394–1397.

Van Pett, K., Viau, V., Bittencourt, J.C., Chan, R.K., Li, H.Y., Arias, C., Prins, G.S., Perrin, M., Vale, W. & Sawchenko, P.E. (2000) Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J. Comp. Neurol.*, **428**, 191–212.

Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M.H., Lewis, K., Sutton, S., Chan, R., Turnbull, A.V., Lovejoy, D., Rivier, C., Rivier, J., Sawchenko, P.E. & Vale, W. (1995) Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature*, **378**, 287–292.

Vita, N., Laurent, P., Lefort, S., Chalon, P., Lelias, J.M., Kaghad, M., Le Fur, G., Caput, D. & Ferrara, P. (1993) Primary structure and functional expression of mouse pituitary and human brain corticotrophin releasing factor receptors. *FEBS. Lett.*, **335**, 1–5.

Voogd, J. & Glickstein, M. (1998) The anatomy of the cerebellum. *Trends Neurosci.*, **21**, 370–375.

Wang, H.L., Tsai, L.Y. & Lee, E.H. (2000) Corticotropin-releasing factor produces a protein synthesis-dependent long-lasting potentiation in dentate gyrus neurons. *J. Neurophysiol.*, **83**, 343–349.

Whitford, K.L., Dijkhuizen, P., Polleux, F. & Ghosh, A. (2002) Molecular control of cortical dendrite development. *Annu. Rev. Neurosci.*, **25**, 127–149.

Yi, H., Leunissen, J., Shi, G., Gutekunst, C. & Hersch, S. (2001) A novel procedure for pre-embedding double immunogold-silver labelling at the ultrastructural level. *J. Histochem. Cytochem.*, **49**, 279–284.