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

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

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):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
CHAPTER 2

The expression of Corticotropin releasing factor in the developing rat cerebellum:
a light and electron microscopic study

J. D. Swinny¹, D. Kalicharan¹, J. IJkema-Paassen², J.J.L. van der Want¹,
A. Gramsbergen²

¹ Laboratory for Cell Biology and Electron Microscopy, & Graduate School of
  Behavioural and Cognitive Neuroscience, University of Groningen,
  The Netherlands;
² Department of Medical Physiology, University of Groningen, The Netherlands.
ABSTRACT

In the cerebellum, climbing fibers containing corticotropin releasing factor (CRF) are crucial for the induction of long term depression at the Purkinje cell-parallel fiber synapse, a type of synaptic plasticity proposed as the cellular basis of learning. However, CRF expression commences at late embryonic stages, prior to the formation of any functional synapses. Prevailing thought leans towards the supposition of CRF, initially playing a role in establishing the cerebellar circuitry, with a shift in adulthood to that of a neuromodulator. In the present study, we detail the spatial and temporal distributional profile of CRF to further understand the possible roles of this peptide in the developing rat cerebellum. The study showed that the expression of CRF was not evenly distributed rather exhibiting inter- and intra-lobular heterogeneity, being localised in variable amounts in mossy fiber rosettes and climbing fiber terminal profiles from as early as postnatal day 3. In the posterior regions especially lobules IX and X, CRF labelling was more prominent in comparison to the central (V-VIII) and anterior lobules (I-IV). This disparate lobular distribution persisted through to adulthood. No cerebellar neurons were conclusively found to contain CRF. Furthermore, between postnatal days 6 to 9, in the posterior lobules, CRF labelling at the level of the Purkinje cell clearly showed the characteristic climbing fiber terminal arrangement related to the capuchon stage whereas in the anterior lobules, labelling is confined to the perisomatic profiles. These differences might signify that translocation of the climbing fibers from the Purkinje cell soma to its dendrite is not uniform for all climbing fibers with CRF possibly playing a facilitative role in this process. CRF immunoreactivity was also zonally arranged in sagittal bands across the molecular layer, these appearing as early as postnatal day 9 and persisting through to adulthood indicating that not all climbing fibers are immunoreactive. Since not all climbing fibers contain CRF, the process of long term depression and hence learning could possibly not be uniform across the cerebellum but be limited to certain zones. The above study lends credence to the supposition that CRF serves, at least, a dual role in the cerebellum, facilitating postnatal development as well as acting as a neuromodulator once functional synapses have formed.
INTRODUCTION

The processes involved in the development of cerebellar circuitry eventually manifest in a structure pivotal for the fine-tuning of motor coordination (see Welsh et al. 1995; Wickelgren 1998) and higher cognitive functions (Muller et al. 1998). An understanding of these events could provide a convenient paradigm for neuronal learning as well as that of brain behaviour relationships. Attention has recently shifted towards factors purportedly involved in development and plasticity in the cerebellum (Sotelo and Chedotal, 1997; Chen and Tonegawa, 1997), one of these being corticotropin-releasing factor (CRF) (King et al. 1997).

The conventional view of CRF has always been that of an agent involved in the hypothalamus-pituitary-adrenal response to stress (Vale et al. 1981) as well as other central nervous system and immune disorders (De Souza, 1995). Recently, CRF has been found to play an important neuromodulatory role in the adult cerebellum. Miyato et al. (1999) have shown this neuropeptide to be crucial in the induction of long term depression (LTD), a form of synaptic plasticity proposed as the cellular basis of learning in the cerebellum (Ito, 1982). Also, Bishop and King (1992) have shown that in the adult cerebellum, CRF augments synaptic efficacy at the mossy fiber – climbing fiber – Purkinje cell synapses.

In contrast to its more established role in the adult cerebellum, the function of CRF in the postnatal cerebellum remains enigmatic. Van den Dungen et al. (1988) showed that from postnatal day (PD) 8 in the rat, CRF is found in the two main afferent systems viz. mossy fibers and climbing fibers. However, Bishop and King (1999) have shown that in the mouse, CRF immunoreactivity is present in the cerebellum from birth, long before these afferent systems have formed their adult connections. Up to PD15 there is also extensive immunoreactivity in the external granular layer. This layer consists largely of migrating neurons. The presence of CRF this early could be indicative of it subserving a different role in this time period as opposed to its purported neuromodulatory function in the adult structure. The above hypothesis has recently been enhanced by Ha et al. (2000) who have shown that CRF induces proliferation of cerebellar astrocytes in a dose dependent manner. The influence of CRF on the myriad of developmental processes involved in the establishment of functional circuitry such as axonal target recognition and synapse formation (Oberdick et al., 1998; Sotelo and Chedotal, 1997; Sotelo, 1999) and ongoing plasticity of olivocerebellar axis (Strata and Rossi, 1998) remains to be elucidated.

Labelling studies in the mouse have shown that at early stages, CRF is evenly distributed throughout the cerebellum with a more discrete lobular localisation in the adult structure (Overbeck and King, 1999; Yamano and Tohyama, 1994). This lobular
localisation of CRF appears to be species specific, e.g. in the mouse (Overbeck and King, 1999), most CRF labelled profiles occur in lobules VIII, IX & X and in the rabbit (Errico and Barmack, 1993), in lobules VIII & IX. In the opossum (Cummings et al., 1994), a far more even distribution had been described. By implication of the heterogeneous lobular distribution at adulthood, not all climbing and mossy fibers contain CRF suggestive of a rather discrete role in specified areas of the cerebellum. This raises the question of the role that CRF plays in the development of cerebellar circuitry and whether a specific relationship might exist with its expression and motor development in the early postnatal period. Also, since CRF is important for LTD and that CRF expression develops differently in distinct parts of the cerebellum, is the phenomenon of LTD uniform throughout this structure?

To address these issues, as a first step in the present study, we mapped the topographical distribution of CRF immunoreactivity in the developing rat cerebellum with a view to eventually relating its localisation to cerebellar development and motor behaviour.

**Experimental procedures**

**Immunocytochemistry**

Black-hooded Lister rats were studied at the following postnatal days: 3, 6, 9, 12, 15, 20, 25, 30 and 40. The day of birth was considered PD 0. An average of six animals were used at each age. Ethical 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 anaesthetised with ether and perfused transcardially with first a solution containing 2% PVP, 0.4% NaNO₃ in 0.1 M phosphate buffer (pH 7.4), followed by 4% paraformaldehyde, 0.2% picric acid in 0.1M phosphate buffered saline (PBS) (pH 7.4). The brains were removed and stored in the same paraformaldehyde/picric acid/PBS solution.

For light microscopy, brains were stored overnight in 30% sucrose to cryoprotect the tissue. The tissue was frozen and 20mm thick cryosections were cut in the sagittal plane. Two animals per age group were also sectioned in the transverse plane. Immunoreactivity was visualised by the avidin-biotin-peroxidase complex method, performed as described previously by Yamano and Tohyama, 1994. Briefly, free-floating sections were immersed for two hours in a pre-incubation medium containing 1% normal goat serum, 1% bovine serum albumin, 0.1% glycine, 0.1% lysine, 0.1% cold water fish gelatine and 0.05% triton in 0.1M TRIS buffered saline (TBS) (pH 7.4). The sections were then incubated with rabbit anti-CRF antibodies (Peninsula Laboratories, San Carlos, CA), reactive for human and rat (diluted 1:1000 with pre-
incubation medium) overnight at room temperature. After washing with TBS, the sections were incubated with biotinylated goat anti-rabbit IgG (diluted 1:200 in pre-incubation medium) for two hours at room temperature. After further washing, the sections were incubated with avidin-biotin-complex (Vectastain® ABC kit) for one hour at room temperature. Immunoreactivity was visualised by incubation with 5mg 3,3 diaminobenzidine and 0.03% hydrogen peroxide in 10ml of 0.01 M PBS (pH 7.4) for 5 to 10 minutes at room temperature. The reaction was stopped by washing the sections in TBS. The sections were mounted on gelatine-coated slides, counter stained with cresyl violet for 2 minutes, dehydrated and coverslipped with Permount.

**Evaluation of sections**
For light microscopical evaluation of the labelling pattern, sagittal and transverse sections were studied. At each age, images of sections were projected onto paper using a projection microscope and these were plotted by hand. From these plots, a composite sketch was drawn summarising the labelling pattern for that age group. Selected sections were photographed on a Zeiss microscope and digitally adjusted to enhance the contrast.

For electron microscopy 50mm sections were cut on a vibratome and processed as for light microscopy with immunoreactivity being enhanced according to the gold substituted silver peroxidase method (van den Pol, 1985). Sections were then 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. Ultrathin sections were cut on an LKB Ultratome, counterstained with uranyl acetate and lead citrate and inspected with a Philips CM 100 transmission microscope.

**Controls**
Sections were processed as above with the omission of the primary antibody.

**RESULTS**
The light microscopical analysis of sagittal sections used in this study showed a distinct lobular distribution of CRF which changed with age, hence emphasis will be placed thereon. The nomenclature of Altman and Bayer, (1997) was adopted (fig 1 A).

**Postnatal day 3**
At this stage, only the five major lobes of the cerebellum can be distinguished viz. the anterobasal lobe (lobules I-III), the anterodorsal lobe (lobules IV-V), the central
Figure 1. Schematic representation of mid-sagittal sections through the vermis of the rat cerebellum indicating CRF labelling at different postnatal ages. (A) Lobular nomenclature adopted according to Altman and Bayer (1997). (B) At PD 3 CRF labelling was evenly distributed and appeared in the form of dot-like puncta (arrow) and isolated fiber-like profiles in the white matter (arrowhead). (C) At PD 6 the posterior lobules showed the most labelling in the form of fibers (arrowhead) as well as rosettes (arrow) in the internal granular layer as well as fibers around the Purkinje cell soma. On occasion, isolated fibers penetrated into the molecular layer. The least amount of labelling was to be found in the anterior lobules (especially I and II) and it was located primarily in the region of the Purkinje cell body. The internal granular layer was almost empty. (D) At PD 12 there was an overall increase in the intensity of labelling throughout all lobules with many more CRF positive climbing fibers having penetrated into the molecular layer of lobules IX and X. Also, clearly distinguishable climbing fibers became evident in the anterior lobules. (E and F) PD 15 to 40 showed the final maturation of the labelled profiles. The most intense labelling was found in the apical regions of lobules IX and X closely followed by the central lobules (V, VI, VIII) and finally the anterior lobules (I-IV).
the expression of corticotropin

lobes (lobules VI-VIII), the posterior lobe (lobule IX) and the inferior lobe (lobule X) (fig 1 B). On the whole, CRF labelling was extremely weak, taking the appearance of dot-like puncta and tiny individual fibers (fig 2 A and B). Although weak and difficult to visualise and conclude any precise lobular specificity, these profiles tended to appear in the posterior lobe and inferior lobe and to a lesser extent in the central lobe. The dot-like puncta occurred mainly in the formative internal granular layer with the fiber-like profiles at the level of the Purkinje cells as well as in the external granular layer. It was impossible to distinguish whether these were immature mossy or climbing fibers. Also in evidence were isolated fibers entering the cerebellum presumably from the inferior cerebellar peduncle.

**Postnatal day 6**

At this stage, the adult-like lobular architecture of the cerebellum could be clearly distinguished (fig 1 C). Coupled with the rapid maturation of cerebellar morphology was a striking increase in the intensity of the labelled profiles. The labelling was

---

**Figure 2.** Nomarski interference photomicrographs of CRF labelling at early stages.

(A and B) At PD 3 isolated profiles (solid arrow heads) and puncta (arrows) appeared laterally (A) as well as more medially (B) in the developing lobules. (C) At PD 6 in lobule II, labelling was concentrated at the level of the Purkinje cell soma (open block arrows) with labelling in the internal granular layer extremely weak. (D) In lobule X at this stage, fiber labelling was in the internal granular layer (open arrow head), the Purkinje cell layer as well as the molecular layer (block arrow).
found almost exclusively in the developing main cerebellar afferents viz. mossy fibers and climbing fibers. However, at these early stages, there was also extensive labelling in the external granular layer.

Lobules X and IXa,b displayed fairly dense labelling. However, it was not evenly distributed throughout the lobule, predominating in the lateral regions and especially at the lobular apex (fig 2 D). The most abundant labelling occurred in the internal granular layer in the form of mossy fiber rosettes as well as axons of climbing fibers. It was difficult to notice any discernable difference in the intensity of labelling between mossy fibers and climbing fibers. There was little or no labelling in the folia. Between PD 6 and 9, some labelled fibers, presumably climbing fibers at the level of the Purkinje cells were between the capuchon stage and that of pericellular nesting.

The distributional pattern was similar in lobules V, VI, VII and VIII and IXc, but with much less intensity as well as frequency in terms of labelled profiles. Here, the labelling was mainly in the internal granular layer. Mossy fiber rosettes as well as climbing fibers could on occasion be clearly distinguished. The climbing fibers

Figure 3. Photomicrographs of CRF labelling at PD 12. (A) shows that in lobule II, labelling was in the form of puncta primarily at the Purkinje cell layer and that it was not evenly distributed throughout the lobule appearing weak in the folial regions. (B) Shows that isolated fibers had penetrated into the molecular layer (arrows). (C) In contrast, lobule X showed a far more even distribution of labelled profiles. These being in the internal granular layer, Purkinje cell layer and molecular layer. (D) Shows that certain climbing fiber profiles (arrow head) were clearly at a different stage of development compared with those in lobule II, having penetrated higher into the molecular layer.
sporadically showed pericellular nests on the somata of Purkinje cells. The appearance of the labelling in the entire anterobasal lobe (lobules I, II and III) as well as the remaining lobule of the anterodorsal lobe (IV) was in stark contrast to the rest of the cerebellum taking on the form of numerous densely labelled puncta or pericellular nests surrounding the Purkinje cell soma without any axonal processes being labelled.

Figure 4. Photomicrographs comparing CRF labelling in lobules II and X at PD 15 to 40. (A) In lobule II at PD 15, the pattern of labelling resembled that of the other lobules, however, labelled profiles were still far less numerous than in lobule X at the corresponding stage. (B) An enlargement shows that labelling was concentrated at the apex of lobule IIa. (C) Shows that lobule X was far more extensively labelled, however, there also existed regions of weaker labelling especially in the folium (arrow heads). (D) Shows the full extent of labelled profiles especially in the apex. (E) Shows the maturity of climbing fibers in lobule X and (F) shows mossy fiber rosettes (arrows) as well as fibers in the internal granular layer at PD 24.
(fig 2C). The internal granular layer was almost completely absent of any mossy or climbing fibers. This pattern of labelling began to taper off at PD 12 when fiber-like profiles appeared.

Electron microscopic data showed that labelled profiles, presumably climbing fibers, had at this stage made synapses on the soma of Purkinje cells (fig 5 C) as well as on the proximal dendrites (fig 5 D). It was difficult to notice any discernable differences between synapses made in the various lobules.

**Postnatal days 9 and 12**

At these stages, there was an overall increase in the intensity of the labelled profiles (fig 1 D). In those lobules with an already intense labelling (X and IX), there was a more even distribution throughout these lobules (fig 3 C). One also observed single translocations of climbing fiber profiles from the soma of Purkinje cell to their dendrites in the molecular layer (fig 3D). Evidence for the apparent increased number of synapses formed in the molecular layer was apparent at the electron microscopic level (fig 6 B).

---

**Figure 5.** (A) Low-power photomicrograph and (B) magnified region of CRF labelling in the flocculus and paraflocculus regions. (C) Photomicrograph of a transverse section at PD 18 showing the tendency for labelled profiles to be arranged in sagittal bands. (D) Photomicrograph of a control section showing no labelling.
The anterior and central lobules still lagged far behind the posterior and inferior lobules in terms of extent and intensity of labelled profiles. From PD 12 onwards, the punctate profiles in lobules I-III were on the wane giving rise to isolated mossy fiber and climbing fiber profiles (fig 3 A and B). The climbing fibers appeared to be at an earlier stage of development than those in lobules IX and X having not yet translocated from the Purkinje cell soma to its dendrites. The mossy fibers as well appeared far less numerous and labelled less intense than those in lobules IX and X.

PD 9 was the first stage to observe a zonal arrangement of labelled profiles in the transverse plane. This occurred at the molecular layer as well as in the internal granular layer. Fig 5 C shows this arrangement more clearly at PD 18. The above distributional pattern of labelling is followed through to adulthood.

**Figure 6.** Electron micrographs of CRF labelled profiles PD 6. (A) Survey of CRF labelling in lobule II and (B) in lobule X. Calibration bars = 0.5µm. Labelling was found primarily around the Purkinje cell somata at the level of the nucleus (Pc nuc). In lobule X, CRF containing profiles made synaptic contacts which resembled those in lobule II. These varicose profiles contained spherical vesicles and occasional mitochondria. (C) These synaptic contacts were at the level of the Purkinje cell soma (peri-somatic) (arrow heads) or (D) on the stubby dendrites of the Purkinje cell (arrows). Calibration bars = 0.25µm.
Chapter 2

Postnatal day 15

At this stage the distributional pattern was characterised by a rapid advancement in the number and intensity of labelled profiles in those areas (anterior and central lobules) that up till now were slower to develop (fig 1 D). The intensity as well as the extent was greatly increased but still did not approach that of lobules IX and X. In lobules IX and X, the appearance of synapses formed on the Purkinje cell soma was infrequent with most climbing fibers traversing the soma (fig 7 A) to form synapses in the molecular layer.

![Figure 7](image1.png)

**Figure 7.** Electron micrographs of CRF labelled profiles at PD 12 in lobule X. (A) Survey at the level of the Purkinje cell soma. Calibration bar = 2µm. (B) Enlargement showing a CRF profile making a triple synapse with a Purkinje cell spine (arrow heads). Calibration bar = 0.25µm.

![Figure 8](image2.png)

**Figure 8.** Electron micrographs of CRF labelled profiles at PD 15 & 24. (A) At PD 15, a labelled CRF fiber traversing the soma of a Purkinje cell (arrow heads). At this stage, somatic synapses have not been encountered. The soma contains a large substantive cisternae characteristic for a Purkinje cell. (B) Shows at PD 24, a labelled plexus (arrows) appositioned between fibers and the Purkinje cell soma. No synapses were found at the level. Calibration bars = 1.5µm.
the expression of corticotropin

Only lobules II b, III and VI c still exhibited labelling of a weak intensity and an uneven distribution. However, even in these lobules, there was an overall increase as well as more labelled profiles that were at the level of the Purkinje cell layer or had penetrated into the molecular layer. The remaining lobules of the anterior (I, IIa, IV) and central lobes (V, VI a,b, VII, VIII) all showed dense though still uneven labelling in both the internal granular layer and molecular layer.

Postnatal days 24-40
At adult stages, we observed the greatest degree of labelling, occurring in the posterior and inferior lobules, although lobule IX c showed slightly less but still more than anterior and central lobules (fig 1 F; fig 4 A and B). Even in adulthood, labelling was not even within these lobules, predominating in the apex and appearing weak in the folial and lateral regions of the lobules (fig 4 C and D). Also, labelling was more abundant in the vermal lobes (in comparison to the hemispheric regions), appearing also in the flocculus and paraflocculus (fig 5 A and B). At the electron microscopic level, these regions had profiles, which contained either less labelled product or were completely empty. The labelling was contained in climbing fibers (fig 4 E) and mossy fiber rosettes (fig 4D and 8) as well as plexus at the level of the Purkinje cell soma (fig 7 B).

In the anterior lobules, labelling also was inhomogeneous predominating in apical regions (fig 4 A and B). However, these lobules showed greater areas of weak or no labelling.

Figure 9. Electron micrographs of CRF labelling in mossy fiber profiles. (A) At PD 12, mossy fibers containing CRF were numerous. These contained numerous spherical vesicles, mitochondria, and made multiple synapses (arrow heads). (B) By PD 24, mossy fiber rosettes had increased in size. Calibration bars = 0.25 µm.
DISCUSSION

CRF distribution

The present study shows that in the rat cerebellum, the distribution of CRF labelling is highly dependent upon the age as well as the region under view. The posterior aspects of the cerebellum (especially lobules IX and X) showed intense labelling in the two main afferent systems i.e. mossy fibers and climbing fibers from as early as PD 6 with this pattern persisting into adulthood with a striking increase in intensity and frequency of labelled profiles at later stages. However, in anterior lobules between the stages of PD 6-12, labelling appeared weak and predominantly around the Purkinje cell with a subsequent change later to more intense labelling in the mossy fibers and climbing fibers. These findings add to those of Palkovits et al. (1987) and van den Dungen et al. (1988) who failed to elucidate any lobular distribution of CRF. Another important finding was that not all climbing fibers were CRF-positive with variations occurring within as well as between lobules. The same was noticeable for the mossy fiber system.

The posterior lobules IXa & b and X which displayed the most intense labelling and moreover, which appeared earliest, essentially receive mossy fiber input from the vestibular system as well as climbing fibers from the dorsal cap of the inferior olive (Voogd and Glickstein, 1998). The early and overwhelming presence of CRF in the vestibulo-cerebellar system, the phylogenetically oldest part of the cerebellum, could possibly signify a crucial role for this peptide in the maturation and subsequent functioning of this system, more so than in any other function involving the cerebellum. In contrast to other lobules, climbing fibers and mossy fiber rosettes appeared here to be equally CRF-positive making it difficult to discern any dominance between the two systems. Although heavily labelled, even within these lobules, labelled profiles predominated within certain areas especially the apex (as opposed to the folial and lateral regions). This rather discrete localisation of CRF in only certain regions of the cerebellum is perhaps suggestive of the role of this neuropeptide being confined to these specific areas.

Other areas of the cerebellum lagged behind in terms of the temporal as well as the quantitative expression of CRF. In lobules V, VI a & b as well as VIII, the CRF distribution developed slightly slower than IX and X and never reached their level of intensity. In the anterior lobules, the labelling first appeared predominantly around the Purkinje cell soma in the form of punctate profiles. From PD 12 onwards, the labelled profiles took on the appearance of climbing fibers and mossy fibers, not dissimilar from those in other lobules, although far less frequent and intense.
Importantly, up to this stage, the internal granular layer appeared almost devoid of any labelled profiles in these anterior lobules. Somatotopical maps indicate a large degree of overlap in terms of afferent input to these areas from precerebellar nuclei, indicating perhaps a more generic role for CRF in these areas. The fact that CRF occurs predominantly in the vermis is suggestive of it being involved more in spinocerebellar function such as the control of axial muscles and posture. The fact that CRF was never evenly distributed throughout the lobule but located either apically or laterally might be in keeping with the functional compartmentation of the cerebellum (Voogd and Glickstein, 1998). Since the source of CRF is from the precerebellar nuclei, the heterogeneous distribution of CRF within the cerebellum indicated in this study could be a marker for the differential functional development of these nuclei. It raises the question whether this disparate distribution is suggestive of this neuropeptide serving different functions in different parts of the cerebellum. Alternatively, it could merely be an indication of the extent to which these precerebellar nuclei are represented in the cerebellar cortex.

**CRF during maturation**

The present data show that CRF is present in the rat cerebellum much earlier than PD 8 as previously reported by van den Dungen et al. (1988). They also reported the disappearance of the pericellular nest stage of labelling around the Purkinje cells from PD 16 to 18 onwards, which is in contrast to our study where we found that depending on the region underview, this type of labelling had disappeared as early as PD 6 in the posterior lobules and around PD 12 in the anterior lobules. However, our study is in concordance with their findings that not all climbing fibers contained CRF and not all CRF positive fibers were equally immunoreactive. Our findings also tend to contrast somewhat with those of Overbeck and King, (1999) who found a far more uniform lobular distribution of CRF positive profiles up till PD 10, after which a differential lobular distribution became more apparent. Our data suggest that this differential lobular distribution occurs much sooner, as early as PD 6. Since this study was performed in a mouse, there appears to be a clear species difference in terms of the ontogeny of CRF-containing profiles.

Bishop and King, (1999) have shown that CRF is expressed in the embryonic murine cerebellum. Given the overlap in its distribution patterns in the two species, it is most likely that it is also expressed in the embryonic rodent cerebellum, though further studies need to corroborate this supposition.

CRF is known to augment the activity of neurotransmitters at the Purkinje cell synapse (Bishop and King, 1992). However, these studies were restricted to adult material. It
could therefore be inferred that this neuropeptide has a vastly different role in postnatal or developing tissue since functional synapses have not yet formed. King and Overbeck, (1999) have proposed that CRF plays a developmental role in the cerebellum with a subsequent shift to that of a neuromodulatory function once the functional circuitry has been established. This hypothesis has been enhanced by the recent publication of Ha et al. (2000) showing that CRF caused proliferation of cerebellar astrocytes in vitro. However, the establishment of the cerebellar circuitry remains subject to a myriad of processes (Strata et al., 1997; Vogel, 1998). We have shown that CRF from a very early age is not ubiquitous, with some afferents to certain regions containing the neuropeptide (posterior regions) and others not (anterior regions). This is in keeping with the findings of Chedotal et al. (1997) that suggest the anterior and posterior regions of the cerebellum are under separate controlling developmental factors despite the consequential rather homogenous morphology. Chedotal and Sotelo, (1993) have also shown that a sub-population of climbing fibers portray a rather unique developmental profile, synapsing rather precociously at PD 0 on the distal dendrites of Purkinje cells and later retracting to form pericellular nests at around PD 6. They termed this early stage of climbing fiber synaptogenesis, the “creeper stage”. These afferents projected exclusively to vermal lobule X. The early expression of CRF in climbing fibers could be a useful marker to study climbing fiber synaptogenesis in other regions of the cerebellum.

For CRF to play a pivotal role in the maturation of the climbing fiber and mossy fiber systems, differences in labelled and non-labelled profiles would be expected. However, ultrastructural analysis failed to elucidate any discernable morphological differences in the maturation processes between CRF containing profiles and those lacking it. Whatever differences were apparent were between afferents containing CRF in different parts of the cerebellum and we have concentrated on the two extremes to highlight the contrasting conditions i.e. lobule II and lobule X. From an early stage (PD 6) there were differences in the labelling picture between CRF containing climbing fibers in lobules II and X. It raises the question whether the disparity in the extent of labelling in these two regions is due to differences in the maturity of the climbing fiber and mossy fiber rosettes in the two regions, differential maturation of the respective precerebellar nuclei or merely the time-onset on CRF production in these nuclei. CRF labelling appeared different in lobule II when compared with that of lobule X. In lobule II, CRF was concentrated primarily in puncta around the soma of the Purkinje cell without any labelling of axonal processes whereas in lobule X, label was clearly visible in the axon as well as in terminals synapsing on the Purkinje cell soma. Ultrastructural analysis clearly showed climbing fibers having made
synapses on the soma of Purkinje cells as well as their proximal dendrites. The translocation of climbing fiber synapses from the Purkinje cell soma to their proximal dendrites appeared to occur at disparate rates in the different lobules suggestive of the maturation of this afferent system occurring at different rates in different parts of the cerebellum. The temporal differences in the expression of CRF mRNA in the precerebellar nuclei probably impacts on the development of those axons containing it. How CRF fits into the chemo-affinity hypothesis espoused by Alcantara et al. (2000) and Vogel, (1998) remains to be elucidated. Comparative electrophysiological recordings in different regions could go some way to confirm whether the early expression of CRF in posterior lobules represents the precocious establishment of functional neuronal networks or merely an anatomic maturation.

**CRF as a neuro-modulator**

In vitro studies by Bishop and King, (1992) have shown that CRF augments the activity of neurotransmitters at the synapses of climbing fibers and mossy fibers on Purkinje cells. Our study shows that not all climbing fibers contain CRF. This raises the question of how important CRF and the CRF-receptor system is to the functioning of climbing fibers (Bloedel and Bracha, 1998) and whether the dynamics of this afferent system differs between lobules as well as the impact of CRF positive and negative climbing fiber on motor co-ordination (Ebner, 1998; Welsh et al., 1995). It would also be prudent to correlate the distribution of CRF with that of its receptor. Currently, it is known that two CRF receptors are expressed in the cerebellum (Lovenberg et al. 1995; Chang et al 1993; Potter et al. 1994). Bishop et al. (2000) have shown that in the adult mouse, receptor type one is present on the somas and primary dendrites of Purkinje cells of all the lobules of the cerebellar cortex as well as in radial glia of certain vermal lobules. Receptor type two is also distributed throughout all lobules being localised to the basal pole of the Purkinje cell, certain basket and stellate cells as well as granule cells. However, Van Pett et al. (2000) have shown that there is a degree of disparity in terms of CRF receptor mRNA expression between the rat and mouse cerebellum. Notwithstanding, this universal distribution of the receptors could indicate that the reach of CRF in the cerebellum is far and wide though its impact on cerebellar function is discretely regulated to perhaps certain time points in certain regions. Importantly, studies of knock-out mice for the various CRF receptors (Kishimoto et al., 2000; Bale et al., 2000) show that these animals have various motor deficits.
CRF is principally involved in the body’s stress response via the HPA axis. The fact that CRF, as shown by this and other studies, is extensively expressed in the cerebellum from a very early age through to adulthood implies a pivotal role of CRF in cerebellar processes. However, a robust theory incorporating CRF activity with that of current thinking on cerebellar function such as the Marr-Albus-Ito theory remains elusive, resulting in a degree of conjecture regarding the primary role of CRF in this structure. Miyata et al. (1999), using adult cerebellar slices have shown that CRF contained in climbing fibers is crucial for the induction of LTD between the parallel fiber-Purkinje cell synapse. However, a host of other factors have been found to be important for cerebellar LTD (Daniel et al., 1998; Ichise et al., 2000) and Hansel and Linden, (2000) have shown that LTD is possible at climbing fiber – Purkinje cell synapses as well. In both studies, there is no mention of the region of the cerebellum where the recordings were done raising the question whether these phenomena are equally inducible in every cerebellar lobule or does learning within the cerebellum take place via other mechanisms in CRF-poor regions. Kenyon, (1997) has also postulated that another site of long term memory storage could also be the synapses of parallel fibers onto stellate/basket cell interneurons. Since we have found CRF labelling in climbing fibers to exhibit a temporal and spatial heterogeneity, the questions arise as to how soon LTD can manifest itself and whether LTD is limited to certain regions of the cerebellum. Also, is LTD the predominant mechanism underlying memory storage in the cerebellum? It could be suggestive of learning and memory being fairly disparate processes in different regions of the cerebellum.

Conclusions

This study shows that there is a prominent CRF cerebellar expression throughout the lifetime of the rat indicative of a pivotal role in the functioning of this structure. The study also shows that CRF is not evenly distributed throughout the cerebellum but predominates in the posterior aspects, especially vermal lobules IX and X suggesting that the role of CRF could be more dominant in these regions. Further studies in progress to manipulate the expression of this neuropeptide will shed some light on its function in the cerebellum.

REFERENCES


Chapter 2


