CHAPTER 4

Increased density and stabilization of climbing fiber-Purkinje cell synapses in mice overexpressing corticotropin-releasing factor

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In preparation
Abstract

Corticotropin-releasing factor (CRF) plays a crucial role in generating the stress response. During early development CRF also acts as a neuromodulator and triggers neurite outgrowth. Here, we evaluate the effects of CRF on afferent systems of cerebellar Purkinje cells using mice over-expressing CRF, particularly in brain. We hypothesize that CRF not only stimulates dendritic development, but additionally is involved in the process of axon-target development and synapse formation more specifically of climbing fiber afferent input. We demonstrate that over expression of CRF does not affect Purkinje cells in cerebellum, but leads to marked changes in the pattern of distribution of climbing fibers. However, the parallel fiber system, that does not express CRF, is not affected by the overabundance of CRF. Thus, CRF might be strategically distributed and hence involved in the regulation of the formation and stabilization of synapses between climbing fibers and Purkinje cells, selectively with respect to the proximal and middle parts of the dendritic trees of Purkinje cells, and is not involved in the parallel fiber input on the Purkinje cell.
Introduction

Corticotropin-releasing factor (CRF) is involved in the regulation of a wide variety of physiological phenomena including, but not limited to, anxiety, depression, cognitive and feeding disorders (Gold, 1996; Muller et al., 1998; Arborelius et al., 1999; Dautzenberg and Hauger, 2002). CRF is strongly expressed in the CNS, with major sites of expression in the paraventricular nucleus of the hypothalamus, cerebral cortex, cerebellum and the amygdalo–hippocampal complex (Swanson et al., 1983; Bittencourt et al., 1999; Swinny et al., 2002). In the cerebellum, CRF is expressed in the two major afferent systems: mossy fibers and climbing fibers (Palkovitz et al., 1987). Two CRF receptor subtypes have been identified in the cerebellum: CRF-R1 and CRF-R2. Both receptors are coupled to classic G-protein-mediated signal transduction cascades (Chalmers et al., 1996; Bishop et al., 2000). It has been shown that urocortin (UCN), a CRF-like peptide with similar functions, has a greater affinity for CRF-R2 than CRF (Vaughan, et al., 1995; Latchman, 2002), while both peptides have similar affinities for CRF-R1. CRF-R2 exists in two isoforms: full and short length (Lee et al., 2004; Gounko et al., 2006; Tian et al., 2006), which are thought to have different functions in the cerebellum.

The cerebellar expression of CRF starts at early embryonic stages, sooner than any functional connections have been formed (Bishop and King, 1999; Chang et al., 1993). It has been demonstrated that CRF-like peptides are involved in the development of the Purkinje cell (Swinny et al., 2004; Gounko et al., 2005). The presence of CRF is crucial for the induction of long term depression (Miyata et al., 1999) and hence for the functioning of afferent systems. CRF has been shown to increase the sensitivity of cerebellar neurons to excitatory neurotransmitters (Bishop, 1990). Nevertheless, the particular effects of these peptides on the organization of afferent systems of the cerebellum are not well understood.
CRF deficits are implicated in a variety of neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's disease (De Souza, 1995; Behan et al., 1995). In contrast, chronically elevated levels of CRF have been reported in several stress-related and affective disorders in the human, including major depression (Mitchell, 1998; Arborelius et al., 1999). In patients with a major depression, elevated levels of CRF have been detected in the cerebrospinal fluid (Nemeroff et al., 1984), as well as increased numbers of CRF-expressing neurons in the paraventricular nucleus of the hypothalamus (Raadsheer et al., 1994). Molecular links between the overabundance of CRF and the initiation and progression of neurological disorders have not yet been established.

In several earlier studies, we and others extensively studied the effects of the application of CRF in organotypic slice cultures grown ex vivo (Swinny et al., 2004; Chen et al., 2004; Gounko et al., 2005). Although a significant body of data has been collected using slice cultures, they have limitations of relying on relatively short-term acute application, i.e. up to 10 days in vitro. This is largely due to cell viability in slice cultures. In the present study we investigate the functional significance of CRF on the afferent system of Purkinje cells using CRF-overexpressing mice (CRF-OE), in which elevated levels of CRF are present throughout development. The CRF-OE mouse model is not only suitable to study physiological effects of CRF as a neurotrophic factor and neurotransmitter, but may shed more light on the potential role of upregulated CRF in the onset and development of the aforementioned neurological conditions. Our data suggest that over-expressing CRF induces the development of the synapses for climbing fibers, but not for parallel fibers. In addition, CRF appears to be involved in competition between CF and PF terminal input. Therefore, it contributes into the morphological plasticity in the connectivity between afferent inputs of Purkinje cells.
Experimental procedures

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

Perfused cerebella of wild-type and CRF-overexpressing (CRF-OE) eight-week old male mice were used (n=3/group), kind gifts from Dr. T. Kozicz, University of Nijmegen, The Netherlands.

Immunocytochemistry

Immunoreactivity was visualised by the avidin-biotin-peroxidase complex (ABC). All further steps were performed in phosphate buffer (PB, 100 mM, pH 7.3). The sections (30 μm thick) were permeabilized with 0.3% Triton X-100, and nonspecific binding blocked with 1% bovine serum albumin (Sigma, USA). Primary monoclonal antibody mouse anti-calbindin D-28K for Purkinje cells (1:5000; Swant, Switzerland) and rabbit anti-CRF (1:1000; Peninsula Laboratories, USA) were used at 4°C overnight. After washing with PB, the slices were incubated with a secondary antibody ABC - biotinylated rabbit anti-mouse IgG, 1:200 or goat anti-rabbit (DakoCytomation, Denmark) for 2 h at room temperature and after wash 1 h for ABC (Vectastain Elite, VectorLabs, USA). Immunoreactivity was visualised by incubation with 5 mg 3,3′-diaminobenzidine HCl (DAB) and 0.03% hydrogen peroxide in 10 ml of PB for 10 min at room temperature. The reaction was stopped by washing the sections in cold PB.

For double-labeling immunofluorescence, the sections were treated at 37°C for 10 min with pepsin (DakoCytomation, Denmark) in 0.2N HCl. The sections were permeabilized and
nonspecific binding blocked with 0.3% Triton X-100, 1% bovine serum albumin (BSA) (Sigma, USA). The primary antibodies used were guinea pig polyclonal VGluT1 (1:500; gift from Prof. M. Watanabe, Hokkaido University School of Medicine, Japan) and mouse monoclonal VGluT2 (1:500; Chemicon, the Netherlands) at 4°C overnight. Primary antibodies were detected with Cy3 donkey anti-guinea pig (1:500; Jackson, the Netherlands) and Alexa 633-conjugated donkey anti-mouse (1:500; Molecular Probes, USA) antibodies. The slices were washed again and mounted in Dako mounting medium (DakoCytomation, Denmark). To identify localization of proteins confocal laser scanning microscopy (Leica DMRX with confocal TCS NT unit; Germany) was used.

**Western blot**

Murine cerebella (WT and CRF-OE) were homogenized in lysis buffer solution containing 50 mM Tris–HCl buffer, pH 7.4, 1.0% Triton X-100, 150 mM NaCl, 5 mM EDTA and protease inhibitor cocktail. Lysates were cleared from tissue debris by centrifugation at 1000g for 5 min. Protein concentrations were determined by a bicinchoninic acid protein assay, and equal amounts of total protein were separated on 10% SDS-PAGE gels (15% for CRF) and electrotransferred onto polyvinylidene fluoride membrane (Millipore, Amsterdam, The Netherlands). After routine blocking with 5% BSA in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 2 h, the membranes were incubated with rabbit anti-CRF (1:500), mouse monoclonal anti-calbindin D-28K (1:5000), guinea pig polyclonal VGluT1 (1:1000) or mouse monoclonal anti-VGluT2 (1:1000) in TBS-T at 4°C overnight. Mouse anti-GAPDH antibody (Ambion, Huntingdon, USA) was used at 1:8000 as loading control. The immunoreaction was detected using HRP-labeled secondary antibodies (Sigma, St Louis, MO, USA or Amersham Biosciences, Little Chalfont, UK) and standard standard enhanced chemiluminescence.
Statistical analysis

The data about the number of Purkinje cells in cerebellum were expressed by their mean values ± standard error. Statistical differences were measured by the Student's t-test. Care was taken to avoid double counting of Purkinje cells in the same section.

Results

First, we used an immunolight microscopic and Western blot analysis with an antibody against CRF to confirm that CRF is overexpressed in CRF-OE cerebellum. At the light microscopic level, CRF immunoreactivity was present around cell bodies of Purkinje cells (Fig. 1 A, B), in cerebellar interneurons and climbing fibers (Fig. 1 C, D; dark arrows). The labeling intensity was

![Figure 1](image-url)
significantly higher in CRF-OE (Fig. 1 B, D) cerebellum as compared to WT (Fig. 1 A, C). Fig. 1E further confirms the strong upregulation of CRF in CRF-OE cerebellum by Western blot analysis. Next, we wanted to know whether the overexpression of CRF leads to changes in Purkinje cell number and/or density. To this end, we performed Western blot analysis of D28K calbindin, a calcium-binding protein specific for Purkinje cells (Kadowaki et al., 1993; Hatten and Heintz, 1995). Our analysis did not reveal any differences in calbindin levels in control vs. CRF-OE cerebella (Fig. 3, upper panels). Equal protein loading was validated by Western blot with GAPDH antibody (Fig. 3, lower panels). This data may either indicate that the number of Purkinje cells as well as calbindin level per cell remained unchanged, or that the number of cells did change, but so did the calbindin level per cell. In order to discriminate between these two possibilities, cerebella of CRF-OE and WT mice were stained for calbindin, and the number of Purkinje cells per mm cortex was counted. We counted all Purkinje cells in 10 sagittal sections (per animal), made across the whole cerebellum (n=2 for WT and n=2 for CRF-OE mice). We did not find any statistically significant differences in the linear density of Purkinje cell for WT

![Figure 2](image-url). The linear density of Purkinje cells per mm cortex in WT and CRF-OE. The graph shows absence of differences in linear density of Purkinje cells for WT and CRF-OE cerebella (mean ± SE = 49.4 ± 0.9 cells/mm for WT and 49.7 ± 1.5 cells/mm or CRF-OE; P=0.854). See text for more details.
and CRF-OE cerebella (mean ± SE = 49.4 ± 0.9 cells/mm for WT and 49.7 ± 1.5 cells/mm or CRF-OE; \( P=0.854 \)) (Fig. 2). Taken together, our analysis of the linear density of Purkinje cells and Western blot data suggest that neither the linear density of Purkinje cells nor the overall number of cells were influenced by CRF overexpression (Fig. 2).

**Figure 3.** Western blot analysis of WT and CRF-OE cerebella showing increase in expression selectively for VGluT2. Top panel - Western blot of control and CRF-OE cerebella with anti-calbindin antibody, second panel - anti-VGluT1 (for parallel fibers), third panel – anti VGluT2 (marker for climbing fibers) and anti-GAPDH loading control (bottom panel). See text for more details.

CRF and related factors have recently been demonstrated to be involved in the Purkinje cell morphogenesis and in development and stabilization of Purkinje cell connections with other neurons (Swinny et al., 2004; Chen et al., 2004; Gounko et al., 2005). We therefore performed a series of experiments in order to identify whether a chronic overabundance of CRF will lead to abnormalities in the Purkinje afferents. In the adult cerebellum, vesicular glutamate transporter type 1 (VGluT1) is predominantly expressed in the parallel fiber system, but not in climbing fibers. Vesicular glutamate transporter type 2 (VGluT2) is expressed in climbing fibers. We used antibodies against the two vesicular glutamate transporters (VGluTs) to evaluate the density and patterns of climbing and parallel fiber terminals in the vicinity of Purkinje cells. At the protein level, both antibodies recognized protein bands at approx. 65 kDa in the cerebellum of the adult mouse (Fig. 3, second and third panels). For VGluT1, levels were not significantly different from
CRF-OE cerebella (Fig. 3, second panel). In contrast, VGluT2 level was found to be strongly upregulated in CRF-OE mouse cerebella (Fig. 3, third panel). Blurred VGluT2 band reflect its glycosylation in the biosynthetic pathway and this is in line with published data (Miyazaki et al., 2003).

**Figure 4.** (for colorinformation: see page 154) Double-labeling immunofluorescence experiment demonstrating the pattern of distribution of afferent system of Purkinje cells in WT (A-C) and CRF-OE (D-F) cerebella. The number of VGluT2-positive synapses increased in CRF-OE cerebellum. A, D - VGluT1 labeling, B, E – VGluT2 labeling and C, F - merged images. Size bars, 40 μm.

Using the double-immunofluorescence staining we analyzed the pattern of distribution of both transporters in cerebella of CRF-OE and WT mice. In both cases, VGluT1 and VGluT2 immunoreactivities were present in the molecular and Purkinje cell layers of the cerebellar cortex (Fig. 4). The pattern of VGluT1 was identical in WT (Fig. 4 A, C) and CRF-OE (Fig. 4 D, F) cerebella. The immunoreactive puncta were present around the somata of Purkinje cells and in the lower part of the molecular layer. Both proximal and distal parts of PC dendritic trees were labeled, and the highest density of staining was present in the neuropile regions (Fig. 4 A, C, D, F). In contrast, the pattern of VGluT2 immunoreactivity was different in WT and CRF-OE
cerebella (Fig. 4). This is in agreement with our Western blot data (Fig. 2). In both control and CRF-OE cerebella immunolabeling was concentrated in the lower and middle part of the molecular layer (Fig. 4 B, C, E, F), along the unstained silhouettes of dendritic shafts of Purkinje cells. However, both the intensity of labeling of individual synapses and the number of VGluT2-positive synapses were dramatically increased in CRF-OE cerebellum compared to WT (Fig. 4 B, C, E, F). In addition, we observed small, strongly immunopositive puncta around the somata of Purkinje cells in CRF-OE cerebella, but not in wild type (Fig. 4 B, C, E, F). Our data, thus, indicate that over-expressing of CRF leads to an increase of VGluT2, but not of VGluT1 level in the cerebellum, and induces the increasing translocation of VGluT2 into a new subset of synapses contacting somata of Purkinje cells.

Discussion

Purkinje cells receive afferents from two excitatory systems. Climbing fibers originate from cells localized in the contralateral inferior olive, while parallel fibers originate from granule cells of cerebella cortex (Palay and Chan-Palay, 1974). Activities of these two afferent systems induce long term depression and motor learning at the cellular level (Ito, 1989; Linden and Connor, 1995). The input from climbing fibers to the cerebellum is crucial for normal function and organization of cerebellar circuitry. However, the factors controlling the development of this specifically organized pathway, are not fully clarified. In the normal cerebellum, CRF is concentrated in climbing fibers (Palkovits et al., 1987) and thus could be involved in the developmental control of the specificity of axonal projections. The participation of CRF in the regulation of the developmental processes of differentiation and synaptogenesis is likely to occur through binding to their specific receptors and the activation of corresponding signaling cascades. It has been shown that upon the binding of CRF to its receptor CRF-R1, cAMP level is increased.
within neuronal cells (Labrie et al., 1982; Cibelli et al., 2001). The increase in cAMP can lead to further intracellular events such as IP$_3$-dependent calcium release, protein phosphorylation by cAMP-regulated kinases and changes in gene expression, all of which are important in the developmental processes of neuronal maturation and neurite differentiation (Yuste et al., 2000; Wong and Ghosh, 2002).

Here, using Western blot analysis and immunofluorescence we found that overexpression of CRF leads to the upregulation of VGluT2 in the cerebellum. Since it has been previously established that VGluT2 is localized in climbing fibers rather than in parallel fibers (Hioki et al., 2003; Miyzaki et al., 2003), we conclude that CRF is involved in the regulation of the climbing fiber development and stabilization of their contacts with Purkinje cells, i.e. matching with postsynaptic sites. Importantly, our Western blot data as well as immunofluorescence analysis indicate that the level of VGluT1, expressed in parallel, but not in climbing fibers, remained unaffected by chronic overexpression of CRF. Thus, CRF appears to specifically govern synapse formation and maintenance exclusively of climbing fiber-Purkinje cell synapses, but not for parallel fiber-Purkinje cell contacts. This corresponds to previous observations, that in vivo (organotypic slice cultures of rat cerebellum), CRF application of slices leads to significantly less glutamate receptor delta 2 - positive synapses in CRF-treated slices (Gounko et al., 2005). This indicates that CRF positively effects selection and sustenance of climbing fiber – Purkinje cell synapses and negatively the formation of parallel fiber – Purkinje cell synapses.

Recently, it has been demonstrated that an increase of CRF level alters the function of targeted Purkinje cells (Jeong et al., 2006). Jeong et al., (2006) have shown that in the ataxic pongo mouse, neurons of the inferior olivary nucleus and their climbing fibers were more intensely stained for CRF than controls. Furthermore, tyrosine hydroxylase was located in Purkinje cells whereas no tyrosine hydroxylase expression was found in controls. They demonstrated that the
immunostaining for CRF and tyrosine hydroxylase in pogo/pogo cerebellum revealed that the
distribution of tyrosine hydroxylase-immunoreactive Purkinje cells corresponded to terminal
fields of CRF-immunoreactive climbing fibers (Jeong et al., 2006). In relation to our data this
implies that increased levels of CRF leads to the formation of more preterminal boutons in the
vicinity of the Purkinje cell. As earlier shown, deprivation of climbing fiber – Purkinje cell
synapses leads to the development of the contacts of parallel fibers with Purkinje cells at the
same locations (Morando et al., 2001). Probably, the presence of CRF in synapses protects these
postsynaptic sites from elimination and thus save functional and stable contacts of climbing
fibers to Purkinje cells.

In conclusion, we demonstrated that the overexpression of the CRF leads to marked changes in
the pattern of distribution of climbing fibers due to increasing of synapses in the cerebellum.
These changes appear to be leading specifically to the strengthening of synapses and regulation
of synaptogenesis between climbing fibers and Purkinje cells.

References

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