Localization of the type 1 corticotropin releasing factor receptor (CRF-R1) in the embryonic mouse cerebellum

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Received 2 June 2003; revised 6 August 2003; accepted 11 August 2003

Abstract

Corticotropin releasing factor (CRF) is present in the adult, as well as in the embryonic and postnatal rodent cerebellum. Further, the distribution of the type 1 CRF receptor has been described in adult and postnatal animals. The focus of the present study is to determine the distribution and cellular relationships of the type 1 CRF receptor (CRF-R1) during embryonic development of the cerebellum. Between embryonic day (E)11 and E12, CRF-R1 immunoreactive puncta are uniformly distributed in the ventricular zone, the site of origin of Purkinje cells, nuclear neurons, and GABAergic interneurons, as well as the germinal trigone, the birthplace of the precursors of granule cells. Between E13 and 18, the distribution of immunolabeled puncta decreases in both the ventricular zone and the germinal trigone and increases in the intermediate zone, as well as in the dorsal aspect of the cerebellar plate. Between E14 and 18, antibodies that label specific populations of cerebellar neurons were combined with the antibody for the receptor to determine the cellular elements that expressed CRF-R1. At E14, CRF-R1 immunoreactivity is co-localized in neurons immunolabeled with PAX-2, an antibody that is specific for GABAergic interneurons. These neurons continue to express CRF-R1 as they migrate dorsally toward the cerebellar surface. Between E16 and 18, Purkinje cells, immunolabeled with calbindin, near the dorsal surface of the cerebellum express CRF-R1 in their cell bodies and apical processes. CRF has been shown to have a depolarizing effect on adult and postnatal Purkinje cells. Further, CRF has been shown to contribute to excitability of hippocampal neurons during embryonic development by binding to CRF-R1; depolarization induced excitability appears to be critical for cell survival. The location of the type one CRF receptor and the presence of its primary ligand, CRF, in the germinal zones of the cerebellum and in migrating neurons suggest that this receptor/ligand interaction could be important in the regulation of neuronal survival through cellular mechanisms that lead to depolarization of embryonic cerebellar neurons.

Introduction

It is now well established that corticotropin releasing factor (CRF) is present in climbing fibers and mossy fibers in the adult mammalian cerebellum (Cummings & King, 1990; Cummings et al., 1988, 1989; King et al., 1997; Mugnaini et al., 1989; Palkovits et al., 1987; Potter et al., 1991; Powers et al., 1987). CRF modulates Purkinje cell responsiveness to both excitatory and inhibitory amino acids by decreasing an after-hyperpolarizing response (Bishop, 1990; Bishop & King, 1992; Fox & Gruol, 1993). Further, CRF has been shown to be an essential component in the development of long term depression (Miyata et al., 1999).

In the developing cerebellum CRF is present prior to the onset of cell migration, differentiation, and synaptogenesis (Bishop & King, 1999; Overbeck & King, 1999; Yamano & Tohyama, 1994). Although a function for CRF as a neuromodulator in adult circuits is clearly emerging, the role of CRF during embryonic and early postnatal development is not understood. In part, this is due to the fact that a neuromodulatory effect of CRF, similar to that observed in the adult cerebellum, cannot be detected until P9 (Bishop, 2002). Several afferent systems known to contain CRF, such as olivary and vestibular axons, reach the developing cerebellum during embryonic stages of development (Morris et al., 1988; Paradies & Eisenman, 1993). The target of the early arriving vestibular axons is not known, as granule cells, their principle target in the adult, have not yet been born at these early stages of cerebellar development. In addition, the major phase of olivary synaptogenesis with Purkinje cells occurs at late embryonic and early postnatal ages (Chedotal & Sotelo,
This suggests that prior to P9, CRF may function through non-synaptic mechanisms in the developing cerebellum.

However, even if CRF acts through mechanisms that do not involve synaptic contacts, a receptor for the peptide must be present to mediate putative developmental affects on immature cerebellar neurons. To date, it is not known if there are functional receptors for CRF during embryonic stages of cerebellar development. CRF binds to two different receptors designated the type 1 (CRF-R1) and the type 2 (CRF-R2) receptors (Behan et al., 1996; Chalmers et al., 1996; Chen et al., 2000; De Souza, 1995; Grigoriadis et al., 1993; Lovenberg et al., 1995). The focus of the present study is on the embryonic distribution and cellular relationships of CRF-R1 for several reasons. First, previous studies have shown that CRF has a higher affinity for CRF-R1 compared to its affinity for CRF-R2 (Behan et al., 1996; Bittencourt & Sawchenko, 2000; Chalmers et al., 1996; De Souza, 1995). Although CRF binds to CRF-R2, the primary ligand for this type of CRF receptor is urocortin, urocortin II and urocortin III (Gottowik et al., 1997; Lewis et al., 2001; Li et al., 2002; Million et al., 2002; Skelton et al., 2000; Vaughan et al., 1995). Although the distribution of CRF during embryogenesis has been described previously (Bishop & King, 1999), there are no data on the ontogeny of the ligands with the highest affinity for CRF-R2. This precludes meaningful interpretation of the function of this receptor during ontogeny.

Thus, the intent of the present study is to determine the distribution and cellular relationships of the type 1 CRF receptor and to relate these data to the distribution of its primary ligand, CRF, during embryonic development. The present observations on the ontogeny of the receptor also will be correlated with developmental events occurring at different stages of embryonic ontogeny including proliferation and migration of neurons from the two major germinial sites, namely the ventricular zone and the germinal trigone.

Methods

All procedures conform to the standards for use of laboratory animals established by the Institute of Laboratory Animal Resources, U.S. National Academy of Sciences. All efforts were made to minimize both the suffering and number of animals used in this study. A protocol for these studies insuring humane use of animals has been reviewed and approved by an Institutional Laboratory Animal Care and Use Committee.

Timed-pregnant female mice were anesthetized with Avertin (0.2 ml/10 gm body weight). Embryonic day (E) 0 was defined as the first day a vaginal plug was observed following mating. A laparotomy and caesarian section was performed to remove pups from the uterus at embryonic days (E) 11, 12, 13, 14, 15, 16, and 18. Animals between E11 and 15 were decapitated and their heads placed in 4% paraformaldehyde for 24–30 hours. Animals between E14–17 were chilled on ice and perfused through the heart with 4% paraformaldehyde. The heads were then placed in the same fixative for 24 hours. The heads of both immersion and perfusion fixed animals were then transferred to Sorensen’s phosphate buffer that contained 20% sucrose for an additional 24 hours, until they sank. They were then frozen on crushed dry ice and stored at −70 °C until they were cut on a cryostat at 20 μm in the sagittal or transverse plane. At least 3 animals from each age were used in this study. Sections were thaw mounted onto glass slides and processed for immunohistochemistry.

IMMUNOHISTOCHEMICAL PROCEDURES

Peroxidase anti-peroxidase procedures

Alternate slides were placed in an antibody generated in a goat against CRF-R1 obtained from Santa Cruz Biotechnology, Santa Cruz, CA. The antibody was diluted 1:3000 in phosphate buffered saline (PBS) that contained 0.3% Triton X-100 (PBT). The sections were then rinsed in PBS and sequentially placed in goat IgG (1:500 in PBT) and goat peroxidase anti-peroxidase (1:500 in PBT) for 1 hour each at room temperature with constant agitation. Following a final rinse in PBS the sections were processed using the glucose oxidase procedure (Shu et al., 1988) to visualize the distribution of the receptors.

Double immunolabeling procedures

A double-labeling paradigm was used to identify cells that express the receptor protein. In this paradigm, cell specific antibodies, generated in species other than goat, were combined with the receptor antibodies in a single solution and applied to the same sections.

An antibody generated in mouse against calbindin, used at concentrations of 1:500, was used to identify Purkinje cells. Two different antibodies, PAX-2 generated in mouse, and gamma amino butyric acid (GABA), generated in rabbit, were used to identify basket, stellate, and Golgi cells. Both were diluted 1:500 in PBT. The calbindin and GABA antibodies were obtained from Sigma. The PAX-2 antibody was obtained from Zymed. Slides were incubated in a “cocktail” containing a primary antibody for CRF-R1 and a primary antibody for one of the cell specific markers for 30–48 hours at 4 °C with constant agitation. Slides were then rinsed in PBS and placed in a solution that contained a mixture of two different secondary antibodies. CY-3 (goat IgG; red fluorescence) was used to label the receptor sites and CY-2 (mouse IgG; green fluorescence) to label specific cells. Both CY-2 and CY-3 were obtained from Jackson ImmunoResearch, West Grove, PA.

CRF RECEPTOR ANTIBODY

CRF-R1 is an affinity-purified polyclonal antibody raised against amino acid sequence 396–415 mapping at the carboxy terminus (intracellular domain) of the CRF-R1 precursor of human origin which is identical to the corresponding mouse and rat sequences. The region used to generate the antibody (siptprosfhisikqvsw) has 85% homology with the CRF type 2 receptor, containing 3 substitutions (siptpspdfhisikqtw). Thus, this particular antibody has the potential to react with both types of CRF receptors. However, Chen et al. (2000) further characterized the same CRF-R1 antibody used in this study by Western blotting and determined there was no cross-reactivity with CRF-R2 when used at a dilution of 1:500 as...