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Ontogeny of cocaine- and amphetamine-regulated transcript (CART) peptide and calbindin immunoreactivity in granule cells of the dentate gyrus in the rat

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Abstract

Cocaine- and amphetamine-regulated transcript (CART) peptide was first discovered in the rat striatum following cocaine and amphetamine administration. However, even without psychostimulant treatment, many neuronal groups of the central nervous system, including granule cells of the dentate gyrus, express CART peptide. Earlier studies, based on the prenatal expression of CART peptide in the mesencephalon, suggest that it exerts neurotrophic effects. In the present study, ontogenetic expression of CART peptide in dentate gyrus granule cells was studied using immunohistochemistry in rats from 5 days to 3 months old. Expression was correlated with the expression of another neurochemical marker of granule cells, the calcium binding protein, calbindin.

Calbindin was already present in granule cells at postnatal day 5 (P5), whereas CART peptide was first observed at P12. The first CART peptide- and calbindin-immunoreactive cells were localized to the lateral end of the dorsal blade, to the outer part of granule cell layer adjacent to the molecular layer, which agrees with the localization of the first-generated granule cells in the dentate gyrus. The first calbindin-immnunoreactive mossy fibers were seen at P9 in the stratum lucidum of CA3, and the entire projection path of mossy fibers expressed calbindin at P18. Mossy fibers were CART peptide-immunopositive at P12, and they were visible in the most distal part of CA3, in CA3a next to CA2. This localization fits with the known spatial organization of mossy fiber axon terminals. An adult-like expression of CART peptide and calbindin in the hippocampal formation was detectable at P30.

The late postnatal appearance of CART peptide in dentate granule cells, and their axonal terminals, indicates that CART peptide may play a neurotrophic role in late developmental events, such as synaptogenesis. However, this does not exclude the possibility of a neuromodulatory role for this peptide.

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Granule cells of the dentate gyrus are important elements of the basic neuronal circuitry of the hippocampal formation, playing a crucial role in directing information from the entorhinal cortex to Ammon's horn. Their functional importance is highlighted by experiments showing that elimination of granule cells, either following neonatal irradiation with X-rays or adrenalectomy in adulthood, results in impaired spatial navigation of rodents (Sloviter et al., 1989, 1993; Conrad and Roy, 1993; Czurkó et al., 1997; Czéh et al., 2001).

In addition to glutamate, granule cells and their axons, the mossy fibers, express a large variety of neurochemical markers, such as the opioid-peptides enkephalin (Gall et al., 1981; McGinty et al., 1982) and dynorphin (McGinty et al., 1982), and the calcium-binding protein calbindin D-28k (CB) (Baimbridge and Miller, 1982; Sloviter, 1989; Celio, 1990). More recently, cocaine- and amphetamine-regulated transcript (CART) peptide has been localized to granule cells and mossy fibers in the hippocampal formation of the rat (Koylu et al., 1998; Seress et al., 2004). The opioid peptides, dynorphin and enkephalin, are known to play a role in hippocampal long-term potentiation (LTP), although they appear to exert opposite effects on the excitability of their target cells, the CA3 pyramidal neurons (Morris and Johnston, 1995). Studies on transgenic animals with reduced CB expression revealed that

Abbreviations: CART, cocaine- and amphetamine-regulated transcript; CB, calbindin; LTP, long-term potentiation; P, postnatal day; str., stratum

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LTP was impaired in hippocampal CA1 pyramidal cells indicating that CB is necessary for LTP and synaptic plasticity in all areas of the hippocampal formation (Molinari et al., 1996; Jouvenceau et al., 1999).

CART peptide, which mRNA was originally found in the striatum of rats following administration of cocaine and amphetamine (Douglass et al., 1995), is present in various neuronal populations and axonal bundles of the central nervous system without any psychostimulant treatment (Koylu et al., 1997, 1998; Seress et al., 2004; Ábrahám et al., 2005). Its localization in dense core vesicles of synaptic terminals suggests that CART acts as a neuromodulator or co-transmitter (Smith et al., 1997). Certainly, the main functions of CART peptide in feeding, reward and reinforcement, as well as in stress responses, are proposed to be achieved through neuromodulation (for review, see Dominguez et al., 2004). Recently it was reported that exogenously applied CART peptide facilitated NMDA-receptor mediated nociceptive neurotransmission (Hsun Lin et al., 2005). Moreover, based on the prenatal expression of CART peptide in brainstem and hypothalamus, and like other central nervous system peptides, such as CRF, tachykinins, VIP, PACAP or galanin, CART peptide may play a significant role in histogenesis and may exert neurotrophic functions (Lindholm et al., 1998; Hill et al., 1999; Ha et al., 2000; O'Meara et al., 2000; Brischoux et al., 2002; Fisher et al., 2003; Wierup et al., 2004; Risold et al., 2006). Nevertheless, this is at odds with reports that CART knock-out mice are born healthy and viable, with neither macroscopic nor microscopic morphological brain abnormalities (Asnicar et al., 2001).

Neurogenesis of granule cells in rat starts on embryonic day 17 (Bayer, 1980). With only 15% of granule cells generated prenatally (Altman and Bayer, 1975), an intensive proliferation of these cells occurs during the first postnatal weeks. Thus, an adult-like morphology of granule cells of dentate gyrus cannot be expected by the end of the third postnatal week.

Dynorphin and enkephalin appear relatively late in granule cells and in their axons. The first dynorphin-immunoreactive mossy fibers can be detected on P6 suggesting that dynorphin appears in morphologically immature fibers soon after they reach their target cells (Gall, 1984). In contrast, granule cells and mossy fibers first exhibit enkephalin immunoreactivity after P10, when mossy fiber bouton elaboration is already well advanced and physiologically competent mossy fiber synapses with CA3 pyramidal cells are already established (Gall et al., 1984). The situation with respect to the appearance of CB during granule cell development is less clear. While some report the first CB-positive granule cells in the dentate gyrus as early as E21 (Enderlin et al., 1987), others do not find them until P3 when CB expression correlates with the onset of synaptogenesis (Rami et al., 1987). Studies on CB-deficient animals support a neuromodulatory role for CB (Molinari et al., 1996; Jouvenceau et al., 1999). This suggests that granule cells of the dentate gyrus may express CB only following neuronal differentiation. Information is not available about the CART peptide expression in granule cells during ontogeny.

In the present study, we have analyzed the expression of CART peptide in granule cells and mossy fibers of the dentate gyrus during the postnatal development of the rat. In addition, the ontogeny of CB in granule cells is revisited because of the controversy in previously published experiments.

1. Experimental procedures

Young (postnatal days: 5, 7, 9, 12, 18, 30, 40, $n = 3$ for each) and adult (3 months old, $n = 3$) male Long-Evans rats were used in this study. The day of birth was considered as day 0. Litters were culled to a maximum of eight pups at the day of birth. From any one litter, a maximum two animals were taken for each age group, ensuring that animals of a given age originated from at least two different litters. For the critical early period (P5–12) littermates were therefore, be used at different ages. Altogether 26 animals from 4 litters were included in this study. Animal housing and care, including anesthesia regime and fixation, were approved by the local ethical committee and were performed in accordance with institutional guidelines for animal experiments and with governmental laws for animal protection, as well as with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Animals were terminally anaesthetized with an overdose of a mixture of ketamine and xylazine then transcardially perfused with phosphate buffer (PB 0.1 M, pH 7.4) followed by 4% paraformaldehyde containing 15% saturated picric acid in PB. After removal from the skull, brains were postfixed, in the same fixative used for the perfusion, for 4 h at room temperature.

1.1. CART peptide immunohistochemistry

Following fixation, brains were cut using a vibratome (Technical Products Incorporation, St Louis, MO) at 60 μm and free-floating sections were collected and processed for immunocytochemistry. After washing in Tris-buffer (TB, pH 7.6), the sections were pretreated with a solution of 1% hydrogen peroxide for 30 min to block endogenous peroxidase activity, then they were preincubated in normal horse serum (1% in TB, Vector Laboratories, Burlingame, CA) containing 0.4% Triton X-100 (Sigma) for 1 h. This step was followed by incubation with the primary monoclonal mouse anti-CART antibody prepared against purified recombinant CART (41–89) (Ca6-1 F4D4, a generous gift from Dr. J.T. Clausen, Novo Nordisc A/S, Bagverd, Denmark) diluted in TB (1:50,000), for three days at 4 °C with continuous shaking. Binding sites were visualized with biotinylated secondary antibody (4 h at room temperature) and the avidin–biotin–peroxidase detection system (Universal Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA). The chromogen was 3,3′-diaminobenzidine (DAB), and the immunoreaction was carried out under visual control, monitoring progress via a light microscope and stopped by the removal of the DAB followed by washes in buffer. After immunocytochemistry, the sections were mounted on slides, air-dried, dehydrated, cleared with xylene and covered with DePeX (Fluka, Switzerland). The specificity of anti-CART antiserum has already been reported (Thim et al., 1998, 1999). Furthermore, if the primary antibody was omitted no immunostaining was observed.

1.2. CB immunohistochemistry

1.2.1. CB immunohistochemistry on free-floating sections

Alternate vibratome sections (60 μm) were used for CART peptide and CB immunohistochemistry. After washing in TB (pH 7.6), the free-floating sections were pretreated with 1% hydrogen peroxide for 30 min. As for the CART peptide immunohistochemistry, this step was followed by a preincubation in normal horse serum (10% in TB, Vector Laboratories, Burlingame, CA) containing 0.4% Triton X-100 (Sigma) for 1 h, then by incubation with the primary monoclonal mouse anti-CB antibody (Swant, Bellinzona, Switzerland), diluted in TB (1:5000), for three days at 4 °C with continuous shaking. Binding sites were detected with biotinylated secondary antibody (4 h at room temperature) and with the aid the avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA). DAB was used as chromogen and the intensity of the immunoreaction was monitored in a light microscope. Mounting, drying and covering of the sections were the same as for CART peptide

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