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THE CORTICOTROPIN-RELEASING FACTOR (CRF)-SYSTEM AND MONOAMINERGIC AFFERENTS IN THE CENTRAL AMYGDALA: INVESTIGATIONS IN DIFFERENT MOUSE STRAINS AND COMPARISON WITH THE RAT

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Abstract—Corticotropin-releasing-factor (CRF) containing systems and monoaminergic afferents of the central amygdaloid nucleus (Ce) are crucial players in central nervous stress responses. For functional analyses of specific roles of these systems, numerous mouse models have been generated which lack or overexpress individual signal transduction components. Since data concerning system morphologies in murine brain are rarely available, mouse studies are usually designed and interpreted based on previous findings in rats, although interspecies differences are frequent. In the present study, *in situ* hybridization for CRF mRNA and correlative immunocytochemistry for CRF and monoaminergic afferents revealed numerous CRF mRNA-reactive neurons in the lateral Ce subnucleus (CeL) codistributed with dense dopaminergic fiber plexus in mice as has been demonstrated in rats. However, while in rats the lateral capsular Ce (CeLc) displays only scarce CRF immunoreactive (CRF-ir) innervation, particularly dense CRF-ir fiber plexus were observed in the CeLc in mice, with differences in labeling densities between different strains. CRF-ir terminal fibers overlap with the moderate serotonergic innervation of this subnucleus in mice. Additionally, CRF mRNA-reactive neurons were found immediately dorsal to the amygdala in the region of the interstitial nucleus of the posterior limb of the anterior commissure/amygdalostriatal transition area in both species. In mice, this region displayed dense CRF-ir fiber plexus, with variations between the strains. The results indicate that in mice and rats dopaminergic afferents represent the primary monoaminergic input to the CRF neurons in the CeL. In mice only, CRF-ir afferents provide dense innervation of CeLc neurons. Since the CeLc lacks dopaminergic input in both species but possesses moderate serotonergic afferents, CRF/serotonin interactions may occur selectively in mouse CeLc. The observed interspecies and interstrain differences in CRF input and CRF/monoaminergic interactions may influence the interpretation of findings concerning Ce functions in stress and fear in mouse models.

Key words: stress, fear, species differences, *in situ* hybridization, immunohistochemistry.

The central nucleus of the amygdala (Ce) is a key component of neural circuits mediating stress- and fear-induced vegetative, behavioral and affective changes (see Eliava et al., 2003 for review). It represents one of the major output nuclei of the amygdaloid complex. Its projections to basal forebrain, hypothalamic and brainstem nuclei evoke autonomic, endocrine, and somatomotor responses to sensory stimuli, which are related to the amygdala from various brain areas, modified in extensive intraamygdaloid connectional systems, and finally transmitted to the Ce (e.g. Pitkänen et al., 1997; Jolkkonen and Pitkänen, 1998). Morphological studies in various species from rodents to human have shown that the Ce is composed of several distinct subnuclei (e.g. McDonald, 1982; Cassell et al., 1986; Price et al., 1987; DeOlmos, 1990; Alheid et al., 1995; Urban and Yilmazer-Hanke, 1999). These subnuclei differ considerably in their cyto- and chemoarchitecture, and in their connectivity. In the rat, neurons producing various peptides and peptidergic terminal fiber plexus are selectively localized to medial, intermediate, lateral, or lateral capsular subnuclei (e.g. Cassell et al., 1986; Cassell and Gray, 1989). Unique extra- and intraamygdaloid connectional characteristics of the subnuclei have been amply documented in numerous tracing studies (see Jolkkonen and Pitkänen, 1998, for review). Significant subnuclear differences are particularly obvious concerning the monoaminergic (adrenergic, noradrenergic, dopaminergic and serotonergic) innervation (Fallon and Ciofi, 1992; Asan, 1993, 1995, 1997b, 1998).

In all species studied to date, the Ce is a major site of extrahypothalamic expression of corticotropin releasing factor (CRF). This "anxiogenic" peptide plays an important role in orchestrating central stress responses, both via the hypothalamo–hypophysial–adrenal axis and via extrahypothalamic circuits, and has been implicated in stress-related and

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Abbreviations: AP, alkaline phosphate; AStr, amygdalostriatal transition area; BL, basolateral amygdaloid nucleus; Ce, central amygdaloid nucleus; CeL, lateral central amygdaloid nucleus; CeLc, lateral capsular central amygdaloid nucleus; CeM, medial central amygdaloid nucleus; CRF, corticotropin releasing factor; DIG, digoxigenin; IAL, interaural level; IHC, immunohistochemistry; IPAC, interstitial nucleus of the posterior limb of the anterior commissure; -ir, immunoreactive; ISH, *in situ* hybridization; M, medial amygdaloid nucleus; NGS, normal goat serum; NT, neurotensin; PBS, phosphate-buffered saline; pPB, pontine parabrachial nucleus; RT, room temperature; SER, serotonin; SERT, serotonin transporter; SSC, standard saline citrate; Str, striatum; TBS, Tris-buffered saline; TH, tyrosine hydroxylase.

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affective disorders in humans (see Mitchell, 1998; Arborelius et al., 1999). Numerous biochemical, pharmacological and behavioral investigations have documented a causative role of the Ce CRF-system in stress- and anxiety-induced reactions in laboratory rats. Stress increases CRF mRNA and protein levels in the Ce, whereas suppression of CRF transmission in this nucleus reduces stress- and anxiety-related vegetative reactions and behavior (e.g. Rassnick et al., 1993; Swiergiel et al., 1993; Heinrichs et al., 1995, 1997, 1998; Merlo Pich et al., 1995; Gray and Bingaman, 1996; Rodriguez de Fonseca et al., 1997; Hsu et al., 1998; Merali et al., 1998; Basso et al., 1999; Koob, 1999a,b, 2000; Makino et al., 1999; Richter and Weiss, 1999; Richter et al., 1999; Sarnyai et al., 2001; Weiss et al., 2001; Bakshi et al., 2002). Additionally, constitutively increased CRF levels and/or numbers of CRF neurons in the Ce have been associated with a predisposition for anxiety-related behavior in specific rat strains or rat lines (Altemus et al., 1994; Merali et al., 2001; Yilmazer-Hanke et al., 2002). Indirect evidence indicates that monoaminergic afferents influence the activity of Ce CRF neurons (Raber et al., 1995; Smialowska et al., 1999; Day et al., 2002; Commons et al., 2003). A recent morphological study points to a particularly prominent dopaminergic influence, documenting intense perisomatic dopaminergic innervation of CRF neurons in the rat Ce (Eliava et al., 2003).

Mouse strains differing in their emotionality (e.g. Conti et al., 1994; Stiedl et al., 1999; Dirks et al., 2001; Plappert and Pilz, 2002; Ohl et al., 2003) and genetically altered mice present a powerful tool to study the role of specific neuromodulator systems for behavior. Numerous mouse models have been generated which lack or overexpress certain components of the CRF and/or monoaminergic signal transduction cascades and display behavioral and other abnormalities (e.g. Heinrichs et al., 1997; Contarino et al., 1999; Bale et al., 2000; Glickstein and Schmauss, 2001; Takahashi, 2001; Contarino and Gold, 2002; Dirks et al., 2002, 2003; Müller and Keck, 2002; van Gaalen et al., 2003; Groenink et al., 2003; Holmes et al., 2003a,b; Lesch et al., 2003; Bale and Vale, 2004). Additionally, CRF-containing neural and neuroendocrine systems appear to play a role for stress-induced dysfunctions in other mouse models (e.g. Okuyama et al., 1999; Sakic et al., 1999; Matsuoka et al., 2003; Nomura et al., 2003). Recent studies in these mouse models have demonstrated interactions between monoaminergic and CRF systems in anxiety-related behavior (Dirks et al., 2003; Groenink et al., 2003). Alterations of CRF levels in the Ce have been associated with anxiety-like behavior in mutant mice (Bale et al., 2000) and in stressor-like effects of cytokine-treatment in mice (Hayley et al., 2001).

Interpretation of the results of physiological, pharmacological and behavioral investigations in terms of the function and/or functional interactions of different modulator systems requires detailed knowledge of the morphology of involved neural systems. To date, appropriate data from murine brain are few, and results of studies in mice are most often interpreted based on findings in rats, although interspecies differences are frequent. Thus, differences in distribution and densities of CRF receptors (Radulovic et al., 1998; van Pett et al., 2000), and in the effects of psychological stress on noradrenaline, dopamine and serotonin (Konstandi et al., 2000), have been documented between the mouse and rat amygdala. Therefore, to provide a basis for analyses of various mouse models, the present study was designed to document in detail the subnuclear localization of CRF-producing neurons, CRF-immunoreactive (CRF-ir) terminals and monoaminergic afferents in the Ce in different mouse strains and to compare the data with results from the Wistar rat. Additionally, possible interrelations between the monoaminergic innervation and CRF-containing neurons and fiber systems of the mouse Ce were studied.

EXPERIMENTAL PROCEDURES

Tissue preparation

All animals were purchased from Charles River (Sulzfeld, Germany; Wistar rats; C57BL/6J mice) or bred in our animal facilities (Würzburg: Wistar rats, C57BL/6J, 129Sv mice; Magdeburg: inbred BA//c, BALB/cJ, C3H/HeJ, C57BL/6J, CPB-K, DBA/2J, NMRI mice; for details see Yilmazer-Hanke et al., 2003). All experiments were done conforming to the guidelines on the ethical use of animals according to the German Law for the Protection of Animals, and were designed to minimize the number of animals used and their suffering. For *in situ* hybridization (ISH) or combined ISH/immunohistochemistry (IHC), adult Wistar rats (n=4) and adult C57BL/6J mice (n=8; Charles River) were decapitated in ether anesthesia. The brains were dissected and cut into frontal slices of 3–5-mm thickness. The slices were immediately frozen in liquid-nitrogen-cooled isopentane and stored at −80 °C. For IHC, adult Wistar rats (n=4) and adult mice of different strains were used: BA//c (n=4), BALB/cJ (n=4), C3H/HeJ (n=3), C57BL/6J (n=13), CPB-K (n=3), DBA/2J (n=5), NMRI (n=3), and 129Sv (n=2). The animals were deeply anesthetized with ether or sodium pentobarbital (180 mg/kg i.p.; Merial GmbH, Hallbergmoss, Germany) and, after a short prerinse, perfusion fixed for 10 min using a 4% formaldehyde fixative in 0.1 M phosphate buffer pH 7.4 freshly prepared from paraformaldehyde. After perfusion, the brains were dissected and tissue blocks containing the amygdala were postfixed for 2–3 h in fixative at room temperature (RT). Tissue blocks were then washed in 0.01 M phosphate buffered saline (PBS; pH 7.4), successively infiltrated with 10 and 20% sucrose in PBS, frozen in liquid-nitrogen-cooled isopentane and stored at −80 °C. Serial 40 μm frontal cryostat or vibratome sections were prepared. One series of sections from the mouse brains was immediately mounted and Nissl stained for cytoarchitectonic studies.

IHC

Sections were preincubated in 5% normal goat serum (NGS; Sigma, Deisenhofen, Germany) diluted in 0.5% Triton X-100 (Sigma) in PBS for 1.5 h and were then immediately transferred to the primary antibody solution consisting of PBS with 0.5% and 1% NGS (incubation buffer). Antibodies used were polyclonal rabbit-anti-tyrosine hydroxylase (TH; Calbiochem, Schwalbach, Germany; dilution 1:500), polyclonal rabbit-anti-serotonin (SER; Chemicon, Hofheim, Germany; 1:45,000), polyclonal rabbit-anti-SER transporter (SERT; Oncogene, Bad Soden, Germany; 1:1000), polyclonal rabbit-anti-neurotensin (NT; Amersham, Braunschweig, Germany; 1:500), and polyclonal rabbit-antiserum generated against rat/human CRF (Peninsula via Bachem, Weil, Germany; dilution 1:1000–1:15,000). CRF-immunoreactions were done on serial sections from all mouse strains (for comparative analysis of density of CRF-ir fibers 1:15,000) and from rats, TH-, SER- and SERT-immunoreactions on serial