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DIFFERENTIAL RESPONSES OF CORTICOTROPIN-RELEASING FACTOR AND UROCORTIN 1 TO ACUTE PAIN STRESS IN THE RAT BRAIN

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Abstract—It has been hypothesized that corticotropin-releasing factor (CRF) and its related neuropeptide urocortin 1 (Ucn1) play different roles in the initiation and adaptive phases of the stress response, which implies different temporal dynamics of these neuropeptides in response to stressors. We have tested the hypothesis that acute pain stress (APS) differentially changes the dynamics of CRF expression in the paraventricular nucleus of the hypothalamus (PVN), oval subdivision of the bed nucleus of the stria terminalis (BSTov) and central amygdala (CeA), and the dynamics of Ucn1 expression in the midbrain non-preganglionic Edinger–Westphal nucleus (npEW). Thirty minutes after APS, induced by a formalin injection into the left hind paw, PVN, BSTov, CeA and npEW all showed a peak in cFos mRNA expression that was followed by a robust increase in cFos protein-immunoreactivity, indicating a rapid increase in (immediate early) gene expression in all four brain nuclei. CRF-dynamics, however, were affected by APS in a brain nucleus-specific way: in the PVN, CRF-immunoreactivity was minimal at 60 min after APS and concomitant with a marked increase in plasma corticosterone, whereas in the BSTov not CRF peptide but CRF mRNA peaked at 60 min, and in the CeA a surge of CRF peptide occurred as late as 240 min. The npEW differed from the other centers, as Ucn1 mRNA and Ucn1 peptide peaked at 120 min. These results support our hypothesis that each of the four brain centers responds to APS with CRF/Ucn1 dynamics that are specific as to nature and timing. In particular, we propose that CRF in the PVN plays a major role in the initiation phase, whereas Ucn1 in the npEW may act in the later, termination phase of the adaptation response to APS.

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Abbreviations: AP, alkaline phosphatase; APS, acute pain stress; BSTov, oval subdivision of the bed nucleus of the stria terminalis; CeA, central amygdale; CRF, corticotropin-releasing factor; DIG, digoxigenin; EDTA, ethylenediaminetetraacetic acid; HPA-axis, hypothalamo-pituitary-adrenal axis; IgG, immunoglobulin G; NBT/BCIP, nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate; NDS, normal donkey serum; npEW, non-preganglionic Edinger–Westphal nucleus; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PVN, paraventricular nucleus; SEM, standard error of the mean; SSC, standard saline citrate buffer; SSD, specific signal density; Ucn1, urocortin 1.

Key words: non-preganglionic Edinger–Westphal nucleus, hypothalamic paraventricular nucleus, central amygdala, bed nucleus of the stria terminalis, HPA-axis, stress response.

The sensation of pain is an early warning to alert the organism for the presence of actual or potentially damaging stimuli (Woolf and Salter, 2000). Acute pain triggers the individual to avoid the harmful situation (Millan, 1999) and initiates, via the release of corticotropin-releasing factor (CRF) from the hypothalamic paraventricular nucleus (PVN), the activation of the hypothalamo-pituitary-adrenal (HPA)-axis (Johnston et al., 1985; Imaki et al., 1992; Kozicz, 2001; Viau and Sawchenko, 2002). Besides the PVN, many other brain centers play a role in autonomic, endocrine and behavioral responses to pain. In the present study we focus on those stress-associated brain centers that use, like the PVN, CRF as their (main) neurotransmitter, viz. the oval subdivision of the bed nucleus of the stria terminalis (BSTov) and the central amygdala (CeA). Moreover, we pay attention to the midbrain non-preganglionic Edinger–Westphal nucleus (npEW), which contains the brain's largest amount of the CRF-family member, urocortin 1 (Ucn1). Both CRF and Ucn1 have been strongly implicated in pain and stress responses (Kozicz, 2001, 2007; Bale and Vale, 2004). Upon activation of the PVN, CRF induces in pituitary corticotropes the synthesis of pro-opiomelanocortin, which is processed to adrenocorticotropic hormone that subsequently stimulates the adrenal cortex to secrete corticosteroids. These give feed-back to the brain and stimulate neuronal networks to evoke some aspects of adaptation behavior, other aspects being elicited by centrally acting CRF (Makara et al., 1969; Taylor et al., 1998; Greisen et al., 1999; de Kloet, 2000; de Kloet et al., 2005). The PVN contains several subpopulations of neurons that respond to a variety of physical and psychological stressors including pain, as is evident from stress-induced expression of the immediate early gene cFos and the CRF gene (Imaki et al., 1992, 1995; Viau and Sawchenko, 2002; Palkovits, 2008). The PVN receives strong input from centers in the limbic system (Herman et al., 2005) including the CRF-producing CeA and BSTov. The CeA plays a key role in HPA-axis regulation and stress-related behaviors (Davis and Shi, 1999), especially in response to stressors that threat to disturb homeostasis (Xu et al., 1999). The CeA activates the PVN indirectly, especially via the BSTov (Jankord and Herman, 2008; Ulrich-Lai and Herman, 2009). The CeA and BSTov are major sites of extra-hypothalamic expression of CRF and CRF

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receptors (Merchenthaler et al., 1982; Van Pett et al., 2000) and their CRF mRNA and CRF contents are upregulated by stressful and painful stimuli (Ulrich-Lai et al., 2006; Kim et al., 2010).

The Ucn1-producing npEW changes its activity in response to a variety of acute and chronic stressors (Weninger et al., 2000; Kozicz, 2001; Gaszner et al., 2004). While the PVN has a fast response to acute stimuli, with cFos peaking within an hour (Cullinan et al., 1995; Viau and Sawchenko, 2002), the npEW responds very slowly to acute (pain) stress, with cFos and Ucn1 contents peaking only 4 h after stress initiation (Weninger et al., 2000; Kozicz, 2001). In line with this notion we hypothesize that CRF in the PVN, acting via CRF receptor 1 (CRFR1), plays a major role in the stress adaptation response initiation, whereas Ucn1 in the npEW would rather be involved in the later, termination phase of the adaptation response, acting on CRFR2 (Coste et al., 2000; de Kloet et al., 2005). In the present study we have tested this hypothesis by comparing responses to an acute pain stress paradigm (APS) by the PVN, BSTov, CeA and npEW.

A frequently used method to induce APS in rat is the injection of formalin into the hind paw (Dubuisson and Dennis, 1977; Vissers et al., 2004; Mravec et al., 2007). This stressor causes moderate, continuous pain and evokes a complex behavioral response consisting of licking, biting and shaking of the affected limb, for a period of about 1 h after injection (Tjølsen et al., 1992; Abbott et al., 1995). Using this paradigm, we have studied the temporal dynamics of pain stress-related changes in (immediate early) gene expression (cFos mRNA and cFos protein as markers) and in mRNA and peptide contents of CRF (in the PVN, CeA and BSTov) and of Ucn1 (in the npEW), using quantitative *in situ* hybridization and immunohistochemistry, respectively.

EXPERIMENTAL PROCEDURES

Animals and stress paradigm

Twenty-five male albino Wistar-R Amsterdam rats, 12–14 weeks old, bred in our Animal Facility of the Department of Anatomy, Pécs, were paired-housed in standard plastic cages (40×25×20 cm^3) in a temperature- and humidity-controlled environment. They were kept on a 12 h light/12 h dark cycle (lights on at 6:00 AM, light intensity 200 lx) and were allowed access *ad libitum* to rodent chow and tap water throughout the experiment. Rats were acclimatized to these conditions for 1 week before starting the experiments. Fifty microlitres 4% paraformaldehyde in pyrogen-free saline (PFA; Sigma Chemical, St. Louis, MO, USA) was injected subcutaneously into the left hind paw of an APS animal (n=5 per group). Immediately after injection, rats were put back in their home cages, and sacrificed 30, 60, 120 or 240 min later by anesthetization and decapitation, as described below. Control animals (n=5) were treated in the same way, but had not been injected. All efforts were made to minimize the number of animals used and their suffering, and all procedures were conducted in accordance with the Declaration of Helsinki and the animal use guidelines based on the law of 1998, XXVIII, for animal care and use in Hungary, and approved by the Medical Faculty Advisory Committee for Animal Resources of Pécs University. Chemicals were obtained from Merck (Darmstadt, Germany) unless stated otherwise.

Tissue fixation and sectioning

Rats were perfused transcardially under deep anesthesia with pentobarbital (Nembutal, Sanofi-Synthélabo, Budapest, Hungary; 100 mg/kg body weight) with 50 ml 0.1 M sodium phosphate-buffered saline (PBS; pH 7.4), followed by 250 ml of 4% ice-cold PFA in PBS, for 20 min. Then they were rapidly decapitated, and their brains postfixed in fresh 4% PFA, transferred into 30% sucrose in PBS, and when completely submerged, frozen on dry ice. Twenty-five micrometres thick coronal slices of the forebrain, midbrain and of the periventricular zone of the hypothalamus were cut on a freezing microtome (Microm, Walldorf, Germany), and stored in sterile antifreeze solution (0.05 M PBS, 30% ethylene glycol, 20% glycerol) at −20 °C.

Corticosterone radioimmunoassay

From each rat, a 3 ml blood sample was taken from the left ventricle prior to insertion of the perfusion needle, and collected into a pre-cooled vial with 150 μl 7.5% ethylenediaminetetraacetic acid (EDTA), and centrifuged at 3000 g for 10 min. Plasma aliquots were assayed for corticosterone radioimmunoassay as described previously (Gaszner et al., 2004), using [^{3}H] corticosterone (12,000 cpm; 90–120 Ci/mmol, NET-399; Perkin-Elmer, Boston, MA, USA) and CS-RCS-57 antiserum (Jozsa et al., 2005). The inter- and intra-assay coefficients of variation were 9.2 and 6.4%, respectively.

Antiserum characterization

Goat polyclonal antiserum against Ucn1 IgG (sc-1825; 1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA) generated against a peptide mapping at the C-terminus of rat Ucn1, has high specificity, as reported previously (Bachtell et al., 2003a,b). Rabbit polyclonal cFos antiserum (sc-52; 1:4000; Santa Cruz Biotechnology) had been raised against the epitope corresponding to residues 3–16 of human cFos. The high specificities of these antisera have been previously confirmed by their preabsorption with the synthetic peptides to which they have been raised, which abolished staining in all cases (Gaszner et al., 2004, 2009). Rabbit polyclonal CRF antiserum (1:2000; kind gift from Dr. W.W. Vale, The Salk Institute, La Jolla, CA, USA) had been raised against rat CRF and its high specificity was described earlier on the basis of preabsorption with the homologous synthetic peptide (Rivier et al., 1983; Sawchenko et al., 1984). In our studies, no staining was observed after omission of first antisera or their preabsorption with respective antigens.

In situ hybridization

CRF mRNA, Ucn1 mRNA and cFos mRNA were detected using antisense cRNA probes transcribed from their respective linearized cDNA (GenBank accession codes AY128673, U33935 and X06769.1, respectively; probes kindly provided by Dr. W.W. Vale). Sense cRNA probes served as controls (no hybridization signal was seen). Probes were labeled with digoxigenin (DIG)-11-UTP (Roche Molecular Biochemicals, Basel, Switzerland). Hybridizations were carried out at 20 °C unless stated otherwise. Sections were rinsed in PBS for 4×15 min, and fixed in 4% ice-cold paraformaldehyde at 4 °C for 30 min. Subsequently, they were rinsed 4×7 min in PBS followed by pre-incubation for 10 min at 37 °C in proteinase K medium containing 0.1 M Tris–HCl, 0.05 M EDTA and 10 μg/ml proteinase K (Invitrogen, Carlsbad, CA, USA). After rinsing in autoclaved MQ-water, acetylation was performed with 0.25% acetic acid anhydride in 0.1 M tri-ethanolamine buffer (pH 8.0) for 10 min followed by a rinse in 2× concentrated standard saline citrate buffer (2×SSC; pH 7.0) for 5 min. Hybridization mixture (50% deionized formamide, 0.3 M NaCl, 0.001 M EDTA, 1× Denhardt's solution, 10% dextran sulfate) together with