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The localization of brain sites of anxiogenic-like effects of urocortin-2

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ABSTRACT

The influence of intracerebroventricullary-administered urocortin-2, a selective corticotropin-releasing factor receptor 2 (CRF_2) agonist, on rat anxiety-like behaviour, the expression of c-Fos and CRF, and plasma corticosterone levels was examined in the present study. When applied to animals exposed to the conditioned fear-induced context, urocortin-2 enhanced a conditioned freezing fear response. Urocortin-2 also significantly decreased rat exploratory activity in the open field test. Exogenous urocortin-2 increased the conditioned fear-induced expression of c-Fos in the central amygdala (CeA), and parvocellular neurons of the paraventricular hypothalamic nucleus (pPVN), and revealed the effect of conditioned fear in the medial amygdala (MeA). In the fear-conditioned animals, immunocytochemistry showed an increase in the density of CRF-related immunoreactive complexes in the lateral septum (LS), 35 min after urocortin-2 administration and 10 min after the conditioned fear test, compared with saline-pretreated fear-conditioned animals. These data suggest a role of urocortin-2 in the behavioural and immunocytochemical responses to stress, in which it strengthens the measures of anxiety-like responses.

1. Introduction

The poor correlation between sites of expression of corticotropin-releasing factor (CRF) and CRF_2 receptors as well as the relatively low affinity of CRF for the CRF_2 receptor suggested the presence of another ligand, the existence of which was evidenced in the cloning of urocortins (Perrin and Vale, 1999). The CRF family of peptides now consists of four members, including CRF, urocortin-1/urotensin-I, urocortin-2, and urocortin-3. The characterisation of CRF and related peptides as well as the cloning of CRF_1 and CRF_2 receptors, which display distinct affinity for CRF ligands, combined with the development of selective CRF receptor antagonists and agonists, have demonstrated the importance of CRF_1 and CRF_2 receptors in the stress-related endocrine (activation of hypothalamic–pituitary-adrenal axis), behavioural (anxiety/depression, altered feeding), autonomic (activation of sympathetic nervous system), and immune responses (Dautzenberg and Hauger, 2002; Maruyama et al., 2007). While urocortin-1 and CRF both display a similar high affinity for the CRF_1 receptor, the affinity of urocortin-2 and urocortin-3 for the CRF_2 receptor is more than 10-fold higher than that of rat/human CRF (Skelton et al., 2000).

CRF family peptides are expressed at different densities throughout the central nervous system and in peripheral tissues (Oki and Sasano, 2004). CRF_2 receptor mRNA displays comparable expression in rat and mouse brain, distinct from, and more restricted than, that of the CRF_1 receptor. Intense expression of CRF_2 receptor mRNA is observed in structures of the olfactory system, corticomedial parts of the amygdala, fields CA1-CA4 of the hippocampus, the ventromedial hypothalamus, the lateral septal nucleus, the ventromedial hypothalamic nucleus, the choroid plexus and several brain stem nuclei (Chalmers et al., 1995; Rybnikova et al., 2003).

Comparative pharmacology of CRF receptor agonists suggested that CRF and urocortins mimic, and CRF receptor antagonists consistently reduce, functional consequences of stressor exposure, such as enhanced anxiety (Smagin et al., 2001). Earlier findings indicated a role for urocortins and CRF_2 receptors in mediation of anxiety and panic responses within the basolateral amygdala and lateral septum of rat brain (Sajdyk et al., 1999; Pelleymounter et al., 2002). For example, the selective CRF_2 receptor agonist urocortin-2 (injected intracerebroventricullary; i.c.v.) increased the acoustic-startle response, albeit with less efficacy than the non-selective CRF receptor agonist h/r-CRF (Risbrough et al., 2003). Ovine CRF infusion significantly decreased novel object touches and climbs, and increased latency to first novel object contact. Urocortin-2 had a similar, but less dramatic, effect, decreasing only climbing behaviour (Clark et al., 2007). These and other results suggest that one of the modes through which the CRF system promotes anxiety is the recruitment of stress-like states after stimulation of CRF_2 receptors within the brain.

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The immediate response mode driven by corticotropin-releasing hormone was suggested to organise via CRF_1 receptors the behavioural, sympathetic and hypothalamic–pituitary-adrenal (HPA) responses to a stressor. In the delayed response mode, the urocortins, acting through CRF_2 receptors, seem prominent (de Kloet, 2003). Overall, some experimental data indicate that CRF and urocortins may coopertate in the effects of stress on the body and brain responses to environmental stimuli.

However, urocortin-2 also increased open arm exploration 4 h after injection (Valdez et al., 2002). Human urocortin-2 (hUcn2) induced mild motor suppressive effects and delayed anxiolytic-like effects (Valdez et al., 2002). Urocortin-deficient mice have normal stress responses but show heightened anxiety-like behaviours in the elevated plus maze and open-field tests (Vetter et al., 2002). Urocortin-3 (a CRF_2 receptor agonist, i.c.v.) did not alter rat social interaction, but it decreased anxiety-like behaviour in the defensive withdrawal test at a 2-nmol dose (Zhao et al., 2007). Collectively, these set of data suggest a role for central CRF_2 receptors in the inhibition of behavioural responses to stressors.

Whereas the CRF_1 receptor appears to contribute to anxiety associated with stress, the role of CRF_2 receptors and their selective endogenous ligands remains unclear and may depend on drug dose, brain location, or testing environment. In the present study, we examined the influence of i.c.v.-administered urocortin-2, a selective CRF_2 receptor agonist, on rat anxiety-like behaviour, the expression of c-Fos and CRF, and plasma corticosterone levels. We have obtained a wide range of information on the localization of central processes targeted by urocortin-2. We have concentrated particularly on the frontal cortex and limbic nuclei to verify and extend previous findings that emphasised the role of these areas in mediating the central effects of stress and CRF-related peptides. For the immunocytochemical analysis, we selected brain regions that participate in the processing of emotional input to the brain and also constitute an important part of the Papez circuit – one of the major pathways of the limbic system involved in the cortical control of emotion, i.e., the prefrontal cortex, hypothalamus, amygdala and hippocampus (cf. Gray, 1983). The aim of this study was to test the hypothesis about the involvement of the neuropeptide urocortin-2, a specific ligand for the CRF_2 receptor, in modulating activity of cortical and limbic areas responsible for anxiety-like behaviours.

2. Materials and methods

2.1. Animals

A cohort of 105 animals was used in the study. Adult male Wistar rats (200 ± 20 g), were bought from a licensed breeder (Polish Academy of Sciences Medical Research Center, 5 Pawinskiego Str., Warsaw, Poland). Animals were housed five per polycarbonate cage (38 × 59 × 20 cm, *w/l/h*) in standard laboratory conditions, under 12 h: 12 h light: dark cycle (lights on at 7 a.m.), in a constant temperature (21 ± 2 °C) and 70% humidity. The rats were given free access to food and water.

The experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609 EEC). The Local Committee for Animal Care and Use at Warsaw Medical University approved all experimental procedures using animal subjects.

2.2. Experimental protocol

These experiments assessed the effects of mouse urocortin-2 (a selective CRF_2 receptor agonist, Ki > 100 nM for CRF_1 receptors, and Ki = 0.7 nM for $CRF_{2\alpha}$ receptors) on rat behaviour in the open field and contextual fear-conditioning tests, plasma corticosterone level, and expression of c-Fos and CRF in brain structures (Reyes et al., 2001). After four days acclimatisation to the vivarium, animals were anaesthetised by intraperitoneal injection of ketamine (100 mg/kg) and fixed in a stereotaxic apparatus (Stoelting & Co., USA). The guide cannulae (0.7 mm o.d., 0.5 mm i.d., 10.0 mm long) were implanted unilaterally into the right lateral ventricle, according to the coordinates from the atlas of rat brain (Paxinos and Watson, 1998): 0.8 mm posterior to bregma, 1.4 mm lateral to bregma and 3.2 mm ventral to dura. The guide cannulae were fixed to the skull with jewellery screws and dental acrylic cement. Seven days later, rats were subjected to behavioural testing. To reduce the stress of injection, all animals were handled prior to the experiment. During the seven days of handling, animals were removed from their home cages and held by an experimenter for 1 min in the same way as during drug administration. On the experimental day (the open field test), animals were divided into the following groups: C – rats pretreated with Ringer's solution (n = 8) or $U_{1.2}$ – rats given urocortin-2 at a dose of 1.2 nmol/rat (n = 8). On the first fear-conditioning test day, animals were divided into the following groups: C_{Sh} – rats pretreated with Ringer's solution, and conditioned to the aversive context (n = 18); $U_{0.24+Sh}$ – rats given urocortin-2 at a dose of 0.24 nmol/rat, and conditioned to the aversive context (n = 15); and $U_{1.2+Sh}$ – rats given urocortin-2 at a dose of 1.2 nmol/rat, and conditioned to the aversive context (n = 12). These groups were all tested in the conditioned fear test. Additionally, three other control groups were examined in a separate experiment in which the animals were not conditioned to the aversive context, but were placed in the conditioning boxes only: C – rats pretreated with Ringer's solution, placed in the conditioning boxes, not conditioned to the aversive context (n = 18); $U_{0.24}$ – rats given urocortin-2 at the dose of 0.24 nmol/rat, placed in the conditioning boxes, not conditioned to the aversive context (n = 14); $U_{1.2}$ – rats given urocortin-2 at the dose of 1.2 nmol/rat, placed in the conditioning boxes, not conditioned to the aversive context (n = 12). The animals received peptide or Ringer's solution 15 min before the conditioned fear test. The rats from C, $U_{0.24}$ and $U_{1.2}$ groups were placed for 10 min in a training box and left undisturbed, without footshocks (a measurement of spontaneous and drug-induced immobility). Ten minutes after the exposure to the aversive context (35 min after drug administration), the animals were decapitated in a different room and trunk blood samples were taken from 50% of the animals: C (n = 9), $U_{0.24}$ (n = 7), $U_{1.2}$(n = 6), C_{Sh} (n = 9), $U_{0.24+Sh}$ (n = 8), $U_{1.2+Sh}$ (n = 6). Additionally, brains were removed, frozen in dry ice-cooled cyclopenthane, and stored at −70 °C for CRF immunocytochemistry. The remaining animals (50%) were decapitated 90 min after the experiment (115 min after drug administration), and their brains were removed, frozen and stored at −70 °C for c-Fos immunocytochemistry.

2.3. Drug treatment

Rats were injected intracerebroventricullary (i.c.v.) with mouse urocortin-2 (Sigma-Aldrich, Poland) at the doses of 0.24 nmol/rat (1 μg/rat) and 1.2 nmol/rat (5 μg/rat). The doses were selected according to previous studies and pilot experiments (Valdez et al., 2002; de Groote et al., 2005; Henry et al., 2006; Staub et al., 2006). The peptide was dissolved in Ringer's solution (mM): sodium chloride – 147, potassium chloride – 4, calcium chloride – 2.4, pH 6, and administered in a volume of 5 μl. For vehicle injections, Ringer's solution alone was used in the same volume.

Intra-cerebral injections of urocortin-2 were given using Hamilton microsyringes connected via tubing with metal needles and a pump (CMA/1000, Sweden), 1.0 mm below of the tip of guide cannula. Solutions were administered over 120 s. The injection