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Urocortin and the Brain

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Abstract

Urocortin is a member of the corticotropin-releasing hormone (CRH) family of peptides. In the brain, its potent suppression of food intake is mediated by CRH receptors (CRHR). Urocortin also participates in the regulation of anxiety, learning, memory, body temperature, and shows neuroprotection. This review will summarize the location of urocortin-producing neurons and their projections, the pharmacological evidence of its actions in the CNS, and information acquired from knockout mice. Urocortin interacts with leptin, neuropeptide Y, orexin, and corticotropin in the brain. Also produced by the GI tract, heart, and immune cells, urocortin has blood concentrations ranging from 13 - 152 pg/ml. Blood-borne urocortin stimulates the cerebral endothelial cells composing the blood-brain barrier and crosses the blood-brain barrier by a unique transport system. Overall, urocortin acts on a broad neuronal substrate as a neuromodulator important for basic survival.

Keywords

urocortin; corticotropin-releasing hormone receptor; Edinger-Westphal nucleus; feeding; anxiety; learning; memory; blood-brain barrier

1. Early history of urocortin

Even before accomplishment in 1981 of the long sought goal to isolate corticotropin-releasing hormone (CRH) from mammalian brain (Vale *et al.* 1981), Erspamer and colleagues isolated an amphibian CRH named sauvagine, another in their amazing discoveries of peptides in frog skin (Montecucchi *et al.* 1980; Erspamer *et al.* 1981). About the same time, Lederis et al. found a related CRH compound in fish named urotensin (Lederis *et al.* 1982), now termed urotensin I. Both sauvagine and urotensin are more potent than CRH in stimulating cAMP release from CRHR2-transfected cells.

In 1995, Vale and colleagues reported the cloning of urocortin, a 40 amino acid peptide which has higher affinity for the CRH receptor 2 (CRHR2) than does CRH itself. Based on the highest immunoreactivity to fish urotensin in the midbrain, a urotensin probe was used to screen the rat midbrain cDNA library (Vaughan *et al.* 1995). Urocortin has 63% sequence homology with urotensin, 45% with CRH, and 35% with sauvagine. The first part of its name (uro) reflects its relationship to urotensin and the last part of its name (cortin) reflects its relationship to CRH. Survey of the evolutionary changes of these peptides over species indicates that they occurred early during chordate evolution (Chang and Hsu 2007). The species differences of urocortin seem to be greater than those of CRH. For instance, hamster and rat urocortins differ by two

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amino acids but their CRH sequences show complete homology (Robinson *et al.* 1999). Thus, the distribution and perhaps even function might differ among species.

Potent suppression of food intake is one of the most noticeable biological effects exerted by urocortin. Urocortin is produced both in brain and peripheral organs. Circulating urocortin can also reach the brain by a unique mechanism. The urocortin discussed here is sometimes termed urocortin-1 to distinguish it from the subsequently discovered urocortin-2 (stresscopin-related peptide) and urocortin-3, all of which interact differently with the BBB (Kastin and Akerstrom 2002). This review mainly focuses on our understanding of urocortin and the brain.

2. Cerebral distribution: the Edinger-Wesphal nucleus (EWN), adjacent areas, and other regions

The highest level of cerebral urocortin production occurs in the midbrain, where the most robust immunoreactivity for fish urotensin was identified in the original report (Vaughan *et al.* 1995). Until 2003, urocortin was thought to be located in the EWN. The EWN is the accessory nucleus of the third oculomotor nerve, which supplies pre-ganglionic parasympathetic input by the ciliary nerve resulting in pupil constriction and lens accommodation. The more medial part of the EWN also innervates choroidal neurons of the ciliary ganglion projecting to the choroid, the vascular layer of the eye lying between the retina and the sclera, and regulates its blood flow. Thus, it is conceivable that the projections from urocortin neurons to the eyes might affect circadian rhythms with consequent effects on behavior and food ingestion.

More recent studies, however, suggest that urocortin is produced by midbrain sympathetic neurons adjacent to the EWN. Double-labeling immunohistochemistry has been performed on brain samples from human, rats, and pigeons. In human postmortem brains, urocortin staining neurons in the EWN are not choline acetyltransferase (ChAT)-positive, indicating that they are not parasympathetic cholinergic neurons projecting to the ciliary ganglion (Ryabinin *et al.* 2005). In the pigeon, urocortin-positive neurons are also ChAT-negative, located more rostral to the EWN or oculomotor nucleus, and do not overlap with the EWN (Cavani *et al.* 2003). Thus, urocortin-positive neurons in the midbrain seem to be part of the brain circuitry involved in the sympathetic nervous-mediated behavioral response to stress (Koob and Heinrichs 1999; De Fanti and Martinez 2002).

Besides the EWN and its adjacent regions, urocortin in rodents is also produced in other areas of the brainstem, hypothalamus, pituitary, and substantia nigra. The other parts of the brainstem include the lateral superior olive, the facial, hypoglossal and ambiguual motor nuclei. In the hypothalamus, urocortin is mainly present in the supraoptic nucleus and caudal lateral areas (Vaughan *et al.* 1995). The reports of the presence of immunoreactive urocortin in the hypothalamus and anterior pituitary, however, are inconsistent (Oki and Sasano 2004b; Vasconcelos *et al.* 2003; Shi *et al.* 2000). Urocortin fibers have also been found in the spinal cord (Vasconcelos *et al.* 2003; Korosi *et al.* 2007).

There appear to be strain differences. Weitemier et al. failed to detect urocortin immunoreactive cells in the supraoptic nucleus of the hypothalamus, facial nucleus, or substantia nigra of either C57 or DBA mice, in contrast to some rat studies (Weitemier *et al.* 2005). Regardless of species, most studies show that the basal, constitutive expression of urocortin is not high. Nevertheless, urocortin appears to show rapid induction. For example, restraint stress significantly elevates urocortin mRNA in the rat hypothalamus 1 h later (Shi *et al.* 2000). The regulatory changes are indicative of the physiological roles of urocortin, which are discussed in the subsequent sections of this review.

In the human brain, urocortin immunoreactivity and mRNA are found not only in the hypothalamus and pons, but also in the cerebral cortex and cerebellum. The distribution is distinct from that of CRH (Takahashi *et al.* 1998). Vasconcelos, Bittencourt, and their colleagues conducted a thorough study of the capuchin monkey (Vasconcelos *et al.* 2003). It shows that urocortin-immunopositive cell bodies and mRNA are present mainly in the EWN and lamina IX of the spinal cord, with diffuse projection of fibers to many areas. The immunopositive fibers with the highest level of expression are seen in the amygdala, septal region, hypothalamus, EWN, trigeminal complex, vestibular nuclei, spinal cord, and ependyma of the lateral ventricles (Vasconcelos *et al.* 2003). Thus, it seems that primates show a wider distribution of urocortin, in contrast to the predominant location in the brainstem and hypothalamus of rodents.

Besides rodents and primates, there also are some localization studies in other species. In the earthworm, immunohistochemistry showed the distribution of urocortin cell bodies and fibers distinct from those for CRH (Lubics *et al.* 2003). The urocortin staining fibers in the pharyngeal wall of the worm probably originate in the cerebral ganglia, suggesting a regulatory role in feeding behavior.

3. Distribution of urocortin in peripheral organs and its blood concentration

With increasing awareness of the role of the (blood-brain barrier) BBB in regulating the blood-to-brain entry of peripheral peptides, peripheral sites of production of urocortin assume added importance. The gastrointestinal (GI) tract and its associated macrophages constitute a major source of urocortin production in the periphery. The first description of urocortin in the periphery used a heterologous radioimmunoassay involving an antiserum to urotensin, not urocortin, together with a urocortin label and standard to show immunoactivity in the duodenum (Vaughan *et al.* 1995). Urocortin mRNA has been found in rat duodenum, small intestine, and colon, and in situ hybridization showed urocortin-containing cells in both the submucosal and myenteric plexus (Harada *et al.* 1999). Both mRNA and the urocortin peptide have been detected in macrophages in the lamina propria of the human colon as early as 3 months of age, implying an immune stimulus (Muramatsu *et al.* 2000). There was some specificity to this localization in the colon since immunoreactive urocortin was not found in macrophages from other human tissues, including liver, spleen, lung, ovary, or fetal and neonatal colonic cells. Urocortin mRNA expression correlates with the severity of inflammation in patients with ulcerative colitis (Saruta *et al.* 2004), and immunoreactive urocortin is higher in the gastric mucosa of humans with active H. pylori gastritis than in normal controls, again indicating an inflammatory or immunological association (Chatzaki *et al.* 2003).

Consistent with the localization of urocortin in GI macrophages and plasma cells (Muramatsu *et al.* 2000; Saruta *et al.* 2004), human lymphocytes produce urocortin, but not CRH (Bamberger *et al.* 1998). Urocortin expression also occurs in the thymus and spleen, and these are altered by injection of lipopolysaccharide (Baigent and Lowry 2000; Kageyama *et al.* 1999).

High levels of urocortin mRNA are also seen in the heart of human and rodents (Baigent and Lowry 2000; Kimura *et al.* 2002; Hashimoto *et al.* 2004; Takahashi *et al.* 2004). In rats, urocortin mRNA is present in adrenals, heart, skeletal muscle, liver, kidney, spleen, and testis, but not in fat (Shi *et al.* 2000), even though adipose tissue is the site of production of many adipokines, including leptin. Beneficial effects of urocortin as a therapeutic agent have been suggested in conditions such as heart failure (Suda *et al.* 2004; Charles *et al.* 2006; Rademaker *et al.* 2005). Urocortin is also expressed in the human fetus, placenta (Petraglia *et al.* 1996; Watanabe *et al.* 1999), skin (Slominski *et al.* 2000), and prostate (Arcuri *et al.* 2002). The

functions of urocortin in the maternal-fetal unit are not known, but urocortin can stimulate the myometrium (Florio *et al.* 2004) and may support fetal adaptation to postnatal life (Florio *et al.* 2005).

Circulating concentrations of urocortin in human plasma vary in different studies, perhaps related to the different assays used. An early report measuring urocortin in human plasma with HPLC verification found concentrations of 16.6 pg/ml for men and 12.8 pg/ml for women, not significantly different from each other or from the levels in pregnant women (Watanabe *et al.* 1999). In term infants, plasma level of urocortin measures 152 pg/ml. At this time, the maternal level of urocortin averages 133 pg/ml (Florio *et al.* 2005). The specificity of the assay was shown by the lack of cross-reactivity with human CRH, urocortin II or III, ACTH, sauvagine, urotensin 1, or thyroglobulin, although neither non-pregnant control values nor verification of intact urocortin by HPLC was reported. In a different study, normal values of 19.5 pmol/l (about 92 pg/ml) in men and 14.2 pmol/l (about 67 pg/ml) in women were reported (Ng *et al.* 2004). In this study, a biotynlated urocortin was used in a competitive immunoluminometric assay. Plasma samples were acidified with 1% trifluoroacetic acid before extraction on C18 columns so that free as well as bound urocortin was probably measured. Using an RIA similar to that used for the sheep studies described next, a New Zealand group found basal plasma urocortin concentrations in healthy men of about 45 pg/ml (about 9.6 pmol/l) (Davis *et al.* 2004).

Urocortin concentrations have also been measured in other animals. The New Zealand group used an HPLC-verified assay to measure plasma urocortin in normal sheep (15.2 pmol/, about 71 pg/ml) and those with heart failure (19.1 pmol/l, about 90 pg/ml) (Charles *et al.* 2006). The positive arteriovenous gradient across the liver, kidney, and hindlimb indicates secretion by these organs. Another study in sheep found basal values of 10.3 pmol/l (about 48 pg/ml) (Rademaker *et al.* 2005). Some of the differences in values among various studies and species may be related to binding proteins. Regardless, the circulating concentrations in sheep and humans are lower than those for leptin.

4. Feeding behavior

4.1. Effects of urocortin on feeding after CNS administration and potential mediators

In the feeding circuitry, urocortin seems to be downstream to melanocortin (MC)-4 receptors in the hypothalamus. Obese MC4R knockout mice do not respond to leptin but urocortin can still exert anorectic effects (Marsh *et al.* 1999).

Many reports have shown that urocortin is more potent than CRH in suppressing appetite. Koob and colleagues injected urocortin intracerebroventricularly (icv) in doses as low as 10 ng and found decreased food intake in food-deprived as well as free-feeding rats (Spina *et al.* 1996). The major cellular sites of expression of urocortin in the rat do not contain CRH mRNA, although the distribution of urocortin fibers correlates well with the distribution of the CRHR2, but not CRHR1, receptor (Spina *et al.* 1996). This indicates that urocortin serve functions different from those of CRH, and that CRHR2 plays a major role in mediating the effects of urocortin. Nevertheless, CRH knockout mice show increased urocortin expression in the EWN (Weninger *et al.* 2000). Another early study showed that the anorexic effect of urocortin was decreased by pretreatment with antisense oligonucleotides to CRHR2 mRNA (Smagin *et al.* 1998). Since urocortin is more potent than CRH in reducing food intake, and since urocortin binds more avidly to CRHR2 than does CRH, it is generally concluded that CRHR2 plays the major role in the satiety effects of urocortin.

In any study of a substance that decreases feeding, it is necessary to eliminate the possibility of a generalized toxic effect explaining the hypophagia. The conditioned taste avoidance

paradigm is the standard for this purpose. Benoit et al. showed that the reduced food intake after urocortin was unaccompanied by the conditioned taste aversion observed after a dose of CRH producing a comparable reduction of food intake (Benoit *et al.* 2000).

There are several neuronal substrates mediating the effects of urocortin. The first and foremost are the hypothalamic nuclei. Injected into the paraventricular nucleus (PVN) of the rat hypothalamus, urocortin decreased NPY-, nocturnal-, and deprivation-induced feeding in doses ranging from 1-100 pmol (about 5-500 ng/ml) (Wang *et al.* 2001a). It did not increase brain c-Fos, and lower doses were not associated with conditioned taste aversion. These results indicate the PVN as a major site for the anorectic actions of urocortin. However, Ohata et al. injected 2.5 μg urocortin into various hypothalamic nuclei of the rat, including the PVN, and only found inhibition of food intake when injected into the ventromedial nucleus (Ohata *et al.* 2000). The arcuate nucleus and the nucleus of the solitary tract may also be involved (Daniels *et al.* 2004; Sinnayah *et al.* 2003).

Apart from the hypothalamus, the dorsal raphe nucleus is also responsive to urocortin. Injected into the dorsal raphe of mice, urocortin reduced overnight food ingestion more than the vehicle, CRH, or antisauvagine-30 (Weitemier and Ryabinin 2006). This also resulted in reduced fluid consumption, but not ethanol preference. Nonetheless, the interactions of urocortin and serotonin in the dorsal raphe nucleus are more likely part of the anxiety circuitry, along with the basolateral amygdala. Acute bilateral injection of urocortin into the basolateral amygdala reduced total interaction time in the social interaction test and increased c-Fos expression in the serotonergic neurons, indicating its anxiogenic role (Spiga *et al.* 2006b). While it is clear that the dorsal raphe nucleus is a neuroanatamical substrate for feeding modulation, it seems difficult to separate the anxiogenic effects and feeding reduction, as will be further discussed in the next section of this review.

Metabolic activity reflects the sympathetic outflow which may participate in the satiety effect of urocortin. In rats, icv injection of urocortin (1 μg) causes an increase in whole body oxygen consumption, accompanied by an increase in body temperature. This effect can be abolished by ganglionic blockade, suggesting that the effects of urocortin are at least partially mediated by central activation of sympathetic outflow and increase of energy expenditure (De Fanti and Martinez 2002). Acting in accordance with the reduction of food intake, urocortin thus serves as a catabolic signal. However, there are also studies showing decreased oxygen consumption in lean and ob/ob mice by urocortin treatment (Asakawa *et al.* 2001), or lack of changes as seen in marsupials, discussed below (Hope *et al.* 2000). Species differences and variations in experimental conditions may explain the different findings.

The motility of the GI tract can be affected by central urocortin. Urocortin injected icv in mice decreased the gastric emptying of a solid meal (Nagata *et al.* 2005). This raised the possibility of a contributory role of the stomach in the observed reduction in food intake and body weight. However, neither urocortin antiserum nor a general CRHR antagonist affected fed or fasted gastroduodenal motility (Kihara *et al.* 2001). The effects of urocortin on gastrointestinal motor function are reviewed elsewhere (Martinez *et al.* 2004).

4.2. Effects of peripheral administration of urocortin on feeding behavior

Peripheral injection of minute doses of urocortin dose-dependently (0.003-3 nmol/mouse) produces higher and more prolonged inhibitory effects on food intake than does CRH, CCK-8, or leptin (Asakawa *et al.* 1999). One hour after intraperitoneal (ip) administration, as little as 14 ng of urocortin significantly reduced cumulative food intake in lean mice fasted for 16 hours. Only the highest dose (3 nmol/mouse, equivalent to about 14 μg/mouse) reduced water intake, a dose 1000-times higher than that reducing food intake. Peripheral administration of urocortin also decreased food intake and body weight in ob/ob mice. Another group found that 1-10 μg/

kg ip dose-dependently reduces the 2-hour cumulative food intake in mice (Wang *et al.* 2001c).

The ability of urocortin to reduce food intake is accompanied by a reduced motivation to eat. This was shown by ip administration of a small dose of urocortin (5-10 μg/kg) to food-deprived male rats in an operant bar press task (Kinney *et al.* 2000). In marsupials, ip administration of 10 μg/kg urocortin decreased food intake, being about 50-times more potent that CRH, an effect not blocked by a CRHR1 antagonist. This was not accompanied by a change in whole body oxygen consumption (VO_2) or plasma cortisol (Hope *et al.* 2000). Overall, the effects of peripheral urocortin on feeding behavior appear to be primarily mediated by direct effects in the CNS, with a possible contributory influence from the GI tract.

5. Effects of urocortin on anxiety, the stress response, and learning behavior

Anxiogenic effects of urocortin have been suggested by pharmacological studies. Urocortin icv reduces exploratory behavior in the elevated plus maze test in mice (Moreau *et al.* 1997) and rats (Jones *et al.* 1998). Intracerebral injection of urocortin into the amygdala elicits anxiety-like behavior in the social interaction test (Spiga *et al.* 2006a; Gehlert *et al.* 2005), and it impairs maternal defense behavior, presumably by increasing anxiety (D'Anna *et al.* 2005). CRHR1 seems to mediate the effects of urocortin on anxiety, in contrast to CRHR2 which mainly mediates its effects on food intake. But since the distribution of urocortin coincides with that of CRHR2 rather than CRHR1, the question arises whether the results from central injections of urocortin into regions with predominant CRHR1 receptor expression show essential physiological roles.

It is established, nonetheless, that urocortin participates in the stress response, shown by its induced expression. Even in the EWN, restraint stress for 3 h causes a 3-fold increase of urocortin mRNA. This upregulation can be blocked by chronic glucocorticoid treatment in wildtype mice. In CRH knockout mice, there is a higher level of urocortin expression than in the wildtype mice, and glucocorticoid treatment does not affect it. The findings appear to suggest a role of EWN-originated urocortin in the regulation of the autonomic nervous system during stress (Weninger *et al.* 2000).

Anxiolytic effects of urocortin, however, have been suggested by studies with urocortin knockout and CRHR2 knockout mice. Mice lacking urocortin have increased, not decreased, anxiety-like behavior in the elevated plus maze and open field (Vetter *et al.* 2002). In a more recent study by a different group, urocortin knockout mice also have impaired adaptation to repeated restraint and a decreased response to cold (Zalutskaya *et al.* 2007). CRHR2 knockout mice are hypersensitive to stress and show increased anxiety-like behavior, again supporting an anxiolytic role of urocortin (Bale *et al.* 2000).

The discrepancy between pharmacological studies and the knockout studies may be explained by non-selective activation of several receptors systems in the brain by centrally injected urocortin. Moreover, urocortin can increase grooming and exploratory behavior (de Groote *et al.* 2005). As a general oversimplification, activation of CRHR1 may increase anxiety-type behavior while activation of CRHR2 decreases it. Since urocortin acts on both receptors, it is reasonable to expect that different variables may influence the balance of actions between these or even other receptors.

Urocortin facilitates the acquisition, consolidation, and retrieval of a passive avoidance (inhibitory) response in mice, suggesting improved learning and memory (Telegdy *et al.* 2005). Similar performance-enhancing effects of urocortin occur in rats involving consolidation of passive avoidance learning and acquisition of navigation in the Morris water maze, reflecting spatial learning and memory. The effects are biphasic, task-dependent, and

cannot be explained by the putative anxiogenic properties of urocortin. Acquisition of spatial navigation was only improved on the initial day of testing, in contrast to drugs which reduce arousal, and this is consistent with the conclusion that urocortin facilitates performance of novel rather than familiar stimuli (Zorrilla *et al.* 2002). Some of these results could be caused by increased arousal but not by hyperactivity. Electroencephalographic effects, differing, from those observed with CRH, indicate that urocortin functions as a mild CNS stimulant to enhance arousal (Slawecki *et al.* 1999).

Some of the variability in behavioral tests can be explained by the inverted U-shaped dose-response curve first shown for the central actions of a peptide in animals in 1971 and in human beings in 1978 (Kastin *et al.* 1984). Time of administration of urocortin during the behavioral task, pretraining in the specific task, as well as the type and difficulty of the task itself are additional sources of variation.

6. Other CNS effects of urocortin

Urocortin appears to be pyrogenic. Two groups of investigators have shown that icv injection of about 1 μg urocortin increases body temperature (De Fanti and Martinez 2002; Telegdy *et al.* 2006). This can be blocked by a prostaglandin inhibitor.

Perhaps one of the most promising effects of urocortin that may lead to therapeutic development is neuroprotection. In cultured rat hippocampal neurons, picomolar amounts of urocortin protect the cells against oxidative and excitotoxic insults (Pedersen *et al.* 2002). Urocortin was 10-fold more potent than CRH in these cells. In another study of cultured rat cerebellar, hippocampal, and cortical neurons, the addition of urocortin and other CRH-related peptides prevented PI3K- or β-amyloid 1-42-induced apoptotic death, effects reversed by a CRHR1 antagonist (Facci *et al.* 2003).

7. Interactions of urocortin with other peptides/polypeptides

7.1. Leptin

To determine whether the urocortin gene on human chromosome 2 is a candidate for susceptibility to obesity, single nucleotide polymorphism analysis and association studies have been performed in French Caucasians. There was no strong association of the urocortin gene and obesity-related phenotypes (Delplanque *et al.* 2002). This contrasts with the studies on leptin, as the same locus shows a linkage with leptin levels in an African American population (Comuzzie *et al.* 1997; Rotimi *et al.* 1999). However, it cannot be ruled out that urocortin indirectly affects the genetic trait in the development of obesity.

Kotz and colleagues performed a series of studies investigating the interactions of urocortin and leptin on feeding behavior. After injection of 200 pmol (about 1 μg) of urocortin into the PVN of rats, the plasma concentration of leptin was increased (Kotz *et al.* 2002). Nevertheless, the leptin released may not be the mediator for the anorexigenic effects of urocortin, since the effects of urocortin typically occur sooner. Leptin is considered a long-term satiety signal, exerting more chronic effects through induction of gene transcription via Janus kinase (JAK)-2 and signal transducer and activator of transcription (Stat). Moreover, as the authors point out, urocortin-initiated leptin signal would not explain the lack of effect of urocortin on the uncoupling protein (UCP)-2 in brown adipose tissue or the urocortin-induced decrease in muscle UCP-3 (Kotz *et al.* 2002). Release of leptin from adipocytes generally takes at least 30 min as compared with the rapid release of insulin from pancreatic islets. Thus, the satiety effect of urocortin is direct and can be further enhanced by its induction of leptin.

The synergistic effect of leptin and urocortin on suppression of food intake is shown by co-treatment of rats with doses of leptin and urocortin that are not effective when given alone. In rat serum, urocortin is bound to a large protein, and addition of leptin displaces the urocortin so that it associates non-covalently with what appears to be leptin. Leptin also shows facilitatory effects on urocortin transport across the BBB, as will be further discussed below (Kastin *et al.* 2002). We also have new evidence from cellular studies that urocortin facilitates the signaling of leptin (Pan et al., unpublished observations). Therefore, urocortin and leptin interact with each other in a cooperative manner to reduce food intake.

7.2. Neuropeptide Y (NPY)

NPY stimulates food intake, an action opposite to that of urocortin. In the rat EWN, most of the urocortin-positive neurons also express the Y5 receptor for NPY, and receive input from NPY-positive terminals. When NPY is injected into the lateral ventricle, there is a strong activation of urocortin neurons shown by c-Fox immunoreactivity. NPY treatment also causes a rapid (within 2 h) induction of urocortin mRNA in these neurons (Gaszner *et al.* 2007). Thus, NPY has stimulatory effects on urocortin-positive neurons in this region, mediated by Y5 (and, to a lesser extent, Y1) receptors. In this sense, it appears, that urocortin provides a negative feedback to counteract the stimulatory effect of NPY on feeding. However, urocortin injection into the PVN inhibits NPY-stimulated feeding (Wang *et al.* 2001b), indicating that the interactions are not reciprocal.

7.3. Orexin A

Orexins (hypocretin) also stimulate feeding. Orexin-A and -B are expressed by a specific population of neurons in the lateral hypothalamic area. These neurons project to numerous brain regions, particularly the monoaminergic and cholinergic nuclei of the hypothalamus, midbrain, and pons. The orexinergic system regulates feeding and sleep-wakefulness (Sakurai 2002). The orexin-positive neurons are mainly located in the lateral hypothalamus, electrical stimulation of which induces anorexia and death (Bernardis and Bellinger 1996). While orexin A (hypocretin 1) injection into the lateral hypothalamus stimulates feeding, urocortin injection into the lateral septum significantly inhibits the effect of orexin A. This feeding suppression is not associated with conditioned taste aversion (Wang and Kotz 2002). Thus, urocortin can exert inhibitory input to the orexigenic system in the lateral hypothalamus.

7.4. Corticotropin (ACTH)

Although urocortin can release ACTH from cultured anterior pituitary cells, evidence including the use of anti-urocortin serum indicates that endogenous urocortin may not be involved in the regulation of ACTH secretion from the pituitary gland in vivo (Ozawa *et al.* 1997; Oki and Sasano 2004a). Urocortin also can stimulate ACTH release from cultured human placental cells, regulate placental vessel resistance to blood flow, and stimulate uterine myometrial strip contractility, suggesting a possible role in pregnancy and parturition (Florio *et al.* 2004).

8. Blood-brain barrier to urocortin

8.1. Animal studies

Urocortin is a 4.7 kD peptide that is relatively stable in the circulation. However, its basal permeability across the BBB is low compared with many other ingestive peptides and larger proteins. The blood-to-brain influx rate of the radioactively labeled tracer by itself is no faster than the vascular marker albumin (Kastin *et al.* 2000).

Co-administration of 5 μg/mouse of leptin, but not CRH, however, enables blood-borne urocortin to enter the brain at a faster rate (Kastin *et al.* 2000). The converse does not occur: urocortin does not affect leptin transport. More importantly, the entry of a tracer amount of

activated urocortin is saturable, being inhibited by excess urocortin. This shows the selective responsiveness of the transport system. Entry of urocortin into the hypothalamus, a part of the brain fully protected by the BBB (Kastin and Pan 2006; Peruzzo *et al.* 2000), is higher than into brain cortex (Kastin *et al.* 2000). Leptin-activated urocortin transport also can be inhibited by co-administration of an antibody against the extracellular domain of the leptin receptor (Kastin *et al.* 2002) and by the selective CRHR2β antagonist antisauvagine-30 (Pan *et al.* 2004). Overall, the facilitatory effect of leptin on urocortin permeation suggests that the phenomenon of leptin resistance in obese subjects can be partially overcome by urocortin.

HPLC showed that urocortin crosses the BBB in intact form, and capillary depletion showed that it enters the parenchyma of the brain rather than being trapped in the microvessels composing the BBB. Neither leptin nor urocortin affect the entry of albumin, indicating no leakage or increased permeability of the BBB. Capillary zone electrophoresis has suggested an association of urocortin with leptin in blood (Kastin *et al.* 2002). Unlike CRH (Martins *et al.* 1996; Martins *et al.* 1997), there is no efflux transport system out of the brain for urocortin. Thus, co-administration of leptin activates the saturable, unidirectional, transport of urocortin across the BBB.

Preatreatment of mice with glucose increases urocortin permeation across the BBB. If urocortin plays a physiological role in inhibiting food intake, it would be expected that high blood glucose would increase the entry of urocortin into the brain while low glucose would decrease transport. But when glucose is injected at the same time as urocortin, no change in the rate of entry is observed. Presumably, simultaneous administration does not allow enough time for feedback. However, pretreatment with glucose 0.5, 1, and 2 h before injection of urocortin, while blood glucose levels are significantly elevated, does increase the transport of urocortin across the BBB (Kastin and Akerstrom 2001).

Pretreatment of mice with insulin also positively modulates urocortin influx across the BBB. Opposite to the effect of glucose, insulin (which decreases blood glucose levels) would be expected to decrease the entry of an anorexigenic peptide such as urocortin if such effects were physiological. Although simultaneous iv injection of insulin and urocortin does not affect urocortin entry into the brain, administration of insulin 1 or 2 h before the urocortin, resulting in hypoglycemia, decreases the blood-to-brain entry of urocortin as expected (Kastin and Akerstrom 2001). Thus, urocortin transport into brain in response to hyperglycemia or hypoglycemia reacts appropriately for a satiety peptide.

The proinflammatory cytokine and adipokine tumor necrosis factor α (TNF) also potentiates the permeation of urocortin across the BBB. Like leptin, the TNF-activated transport system for urocortin is inhibited by antisauvagine-30, the CRHR2β antagonist, and it tends ($p < 0.06$) to be reduced by a leptin receptor antibody. Both of the TNF receptors are involved in this system since antibodies to either p55 or p75 abolish TNF-induced activation of urocortin influx (Pan *et al.* 2004). Thus, three anorexigenic peptides/polypeptides interact at the BBB. Instead of inhibiting each other's entry into the brain, however, two of them (leptin and TNF) facilitate the entry of the third (urocortin).

8.2. Findings from cultured cells

Endocytosis and exocytosis assays in cells overexpressing either CRHR1 or CRHR2 show that both receptors can mediate the transport of urocortin across the BBB in intact form. Since cerebral microvessels from mouse brain express both receptor subtypes, receptor-mediated transport of urocortin can be executed by either CRHR1 or CRHR2 (Tu *et al.* 2007a). Once endocytosed, the intracellular urocortin can remain intact for an hour, an observation rare in studies of clathrin or caveolae-mediated pathways.

In immortalized rat brain microvessel endothelial (RBE4) cells, leptin increases the endocytosis of urocortin in a time- and dose-dependent manner (Tu *et al.* 2007b). Fluorescently labeled urocortin shows vesicular trafficking through early endosomes and can be sorted to the Golgi complex by 20 min. This leptin-stimulated internalization is saturable and not accompanied by changes in the γ-glutamyl transpeptidase or nitric oxide synthase activity of the endothelial cells.

The facilitatory effect of leptin on urocortin cellular internalization is consistent with the findings from mouse studies mentioned earlier. Leptin does not increase the characteristic signaling of urocortin by intracellular cAMP production; rather, urocortin facilitates leptin signaling by Stat3 signaling. Both Stat1 and Stat3 activities, induced by leptin signaling through the ObRb receptor, can be enhanced by the presence of CRHR1 or CRHR2 and co-treatment with urocortin (Pan et al., unpublished observations). Thus, the actions of urocortin on leptin signaling are not reciprocal. This unidirectional cooperativity between urocortin and leptin may not only help overcome leptin resistance, but it may also have general implications for signaling crosstalk between different classes of receptors.

9. Conclusions

Urocortin is present both in the brain and peripheral organs, and the permeation of urocortin across the BBB can be affected by factors related to nutrition and hormonal status. Within the brain, the projections of urocortin-positive neurons are even more widespread than the neuronal cell bodies. It seems that the production of urocortin in the brain is most prevalent along the midline structures of the mesencephalon and diencephalon, and the projections hardly reach the cerebral cortex. Perhaps the permeation of urocortin across the BBB partially overcomes the lack of projections of urocortin-positive fibers to the cortical regions. Despite much work on the effects of urocortin on feeding behavior, anxiety, learning, and memory, there are many unsolved questions about the CNS pathways and cellular interactions of urocortin with other peptides.

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Abbreviations

ACTH
corticotropin

BBB
blood-brain barrier

ChAT
choline acetyltransferase

CNS
central nervous system

CRH
corticotropin-releasing hormone

CRHR
corticotropin-releasing hormone receptor

EWN
Edinger-Wesphal nucleus

GI
gastrointestinal

ICV
intracerebroventricularly

JAK
Janus kinase

MC
melanocortin

NPY
neuropeptide Y

PVN
: paraventricular nucleus

Stat
: signal transducer and activator of transcription

TNF
: tumor necrosis factor

UCP
: uncoupling protein