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Postnatal Developmental Profile of Urocortin 1 and Cocaine- and Amphetamine-Regulated Transcript in the Perioculomotor Region of C57BL/6J Mice

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Abstract

Urocortin 1 (Ucn 1) is an endogenous corticotropin releasing factor (CRF)-related peptide. Ucn 1 is most highly expressed in the perioculomotor urocortin containing neurons (pIIIu), previously known as the non-preganglionic Edinger-Westphal nucleus (npEW). Various studies indicate that these cells are involved in stress adaptation and the regulation of ethanol (EtOH) intake. However, the developmental trajectory of these neurons remained unexamined. Expression of the cocaine- and amphetamine- regulated transcript (CART), which co-localizes with Ucn 1 in the perioculomotor area (pIII) has been examined prenatally, but not postnatally. The goal of the current study was to characterize the ontogenetic profile of Ucn 1 and CART during postnatal development in C57BL/6J (B6) mice. B6 mice were bred, and brains were collected at postnatal days (PND) 1, 4, 8, 12, 16, 24 and 45. Brightfield immunohistochemical staining for Ucn 1 and CART showed that Ucn 1 –immunoreactivity (ir) was absent at PND 1, while CART –ir was already apparent in pIIIu at birth, a finding indicating that although the pIIIu neurons have already migrated to their adult position, Ucn 1 expression is triggered in them at later postnatal stages. Ucn1 –ir gradually increased with age, approaching adult levels at PND 16. This developmental profile was confirmed by double-immunofluorescence, which showed that Ucn 1 was absent in CART –positive cells of pIII at PND 4 and that Ucn 1 and CART are strongly but not completely co-localized in pIII at PND 24. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis confirmed that Ucn 1 mRNA levels are significantly lower at PND 4 and PND 12 than in adult animals. The lack of brain Ucn 1 immunoreactivity at birth and the gradual postnatal increase in Ucn 1 in pIIIu suggests that this peptide plays a greater behavioral role in adulthood than during the early postnatal development of an organism.

Keywords

development; Edinger-Westphal; subgriseal; corticotropin releasing factor; neuropeptide

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1. Introduction

Urocortin 1 (Ucn 1) is a member of the corticotrophin releasing factor (CRF) family of peptides and is known to act with high affinity on both CRF1 and CRF2 receptors (Vaughan et al., 1995). In the brain Ucn 1 is most highly expressed in the perioculomotor urocortin containing neurons (pIIIu) (May et al., 2008; Ryabinin et al., 2008; Spangler et al., 2009), a brain region previously referred to as the non-preganglionic Edinger-Westphal nucleus (npEW) (Bittencourt et al., 1999; Kozicz et al., 1998; Ryabinin et al., 2005; Vaughan et al., 1995; Weitemier et al., 2005). To a lesser degree, Ucn 1 is also expressed in the lateral superior olive (Bittencourt et al., 1999; Weitemier et al., 2005). The distribution of this peptide has been examined in various animal models in both the central nervous system and the periphery (Boorse and Denver, 2006; Cunha et al., 2007; Kozicz et al., 1998; Kozicz and Arimura, 2002; Kozicz et al., 2002; Lim et al., 2006; May et al., 2008), but the developmental trajectory of Ucn 1 immunoreactivity (ir) has been limited in the literature.

Ucn 1 expression has been reported in fetal ovine pituitary and human colon (Holloway et al., 2002; Muramatsu et al., 2000). In the nervous tissue, many studies have found that exogenous application of Ucn 1 has a neuroprotective and neurotrophic role, suggesting that Ucn 1 could play a role during development (Abuirmeileh et al., 2007; Brar et al., 2000; Calle et al., 2005; Choi et al., 2006; Facci et al., 2003; Gounko et al., 2005; Swinny et al., 2004b). However, this interpretation is complicated by the fact that exogenous application of Ucn 1 does not distinguish between the endogenous actions of Ucn 1 and other CRF-like peptides. It has been previously reported that CRF and CRF receptors show brain region-specific profiles of expression, appear in the brain already prenatally, but are not found in pIIIu at any age (Avishai-Eliner et al., 1996; Baram and Lerner, 1991; Bittencourt et al., 1999; Bugnon et al., 1982; Chalmers et al., 1995; Eghbal-Ahmadi et al., 1998; Grino et al., 1989; Korosi and Baram, 2008; Potter et al., 1992). To evaluate the potential role of Ucn 1 in development, it would be beneficial to study the ontogenic profile of Ucn 1 expression. To this date only one study attempted to characterize this profile (Swinny et al., 2004a). This study performed in the rat, reported presence of Ucn 1 –positive fibers in cerebellum and Ucn 1 –positive cells in inferior olive at postnatal day (PND) 3, an increase of Ucn1 –positive fibers in the cerebellum at PND 8 and PND 15 and presence of Ucn 1 –positive cells at PND 15. These findings suggested a substantial change in brain Ucn 1 expression during postnatal development warranting future more detailed investigations.

The developmental expression profile of cocaine- and amphetamine- regulated transcript (CART), a peptide that strongly but not completely co-localizes with Ucn 1 in the perioculomotor area (pIII) (Kozicz, 2003; Lazar et al., 2004; Lima et al., 2008; Xu et al., 2009), has been examined prenatally (Brischoux et al., 2002). It was reported that CART is expressed during early embryonic development, and the finding that these cells migrate through the ventral tegmentum area and settle in the pIII, has led to the argument that CART may be one of the earliest neuropeptides with a neuromodulatory role that is expressed in the brain (Brischoux et al., 2002; Risold et al., 2006). Although, this prenatal developmental profile was reported, the postnatal developmental expression profile of CART has only been examined in other brain regions (Abraham et al., 2007).

Various studies indicate that cells in the pIIIu are sensitive to stress (Cunha et al., 2007; Gaszner et al., 2004; Kozicz, 2007), ethanol (EtOH) administration and self-administration (Bachtell et al., 1999; Chang et al., 1995; Ryabinin et al., 1997; Sharpe et al., 2005; Topple et al., 1998), and administration of other drugs of abuse (Bachtell et al., 2002a; Spangler et al., 2009). A greater number of Ucn 1 –positive cells in pIIIu can be found in many ethanol-preferring strains of mice and rats, including C57BL/6J (B6) mice, versus alcohol avoiding strains (Bachtell et al., 2002b; Bachtell et al., 2003; Fonareva et al., 2009; Ryabinin and

Weitemier, 2006; Turek et al., 2005) and both Ucn 1 and CART play an important role in the actions of alcohol and other addictive drugs in adult animals (Bachtell et al., 2004; Dandekar et al., 2008; Jaworski et al., 2008; Ryabinin et al., 2008; Salinas et al., 2006; Turek and Ryabinin, 2005; Weitemier and Ryabinin, 2005). However, since the postnatal development of these peptides has not been extensively studied, it remains unknown whether these peptides may play a role in in responses to stress and addictive drugs during infant and juvenile stages of ontogeny.

The goal of the current study is to characterize the postnatal developmental expression profile of Ucn 1 with a focus on pIIIu and compare it to CART with the use of immunohistochemistry and quantitative reverse transcription polymerase chain reaction (qRT-PCR).

2. Results

Brightfield immunohistochemistry revealed that Ucn 1 –positive cells in the B6 mice were completely absent at PND 1, and only one of three mice showed Ucn 1 –ir in the pIIIu at PND 4. Furthermore, no additional Ucn 1 –ir was observed in the slices stained laterally from the midline at PND 1 or PND 4. At PND 8, Ucn 1 –ir was present in pIIIu of all mice examined and had increased 3.2 fold from levels at PND 4. Another 2.4 fold increase was observed at PND 12, with mature cell counts leveling off at PND 16, as similar values were observed in the late juvenile/early adolescent mice at PND 24. Representative sagittal sections of the pIIIu and Ucn 1 –positive cells can be seen in Figures 1 and 2.

CART –positive cells were strongly labeled in pIII starting at PND 1. The number of detected CART –labeled cells doubled during the first postnatal week and then slightly declined. At PND 24, CART –ir was 1.4 fold of PND 1 counts (Figures 1 and 3).

Double fluorescence immunohistochemistry of Ucn 1 and CART performed in additional mice on coronal sections at PND 4 and PND 24 showed positive CART labeling at PND 4, while Ucn 1 was completely absent from the pIIIu. At PND 24, fluorescent labeling of both Ucn 1 and CART was present. Double labeling confirmed that Ucn 1 and CART are largely co-localized in pIII, but a small number of cells showed either only Ucn 1 or only CART staining (Figure 4).

Quantitative real-time polymerase chain reaction (qRT-PCR) performed on dissections of pIIIu from PND 4, PND 12 and PND 45 confirmed the increase in Ucn 1 expression during postnatal development (Figure 5). Specifically, the relative level of Ucn 1 mRNA at PND 4 was twice lower than on PND 12 and 12.5 times lower than at PND 45, an effect reflected in a significant ANOVA ($F(2,21) = 10.3$, $p=0.0008$). Post-hoc analysis with Fisher's Protected Least Significant Difference (PLSD) revealed a significant difference between Ucn 1 mRNA between PND 4 and PND 45 ($p = 0.0003$) and a difference between PND 12 and PND 45 ($p = 0.0007$). In contrast, the relative level of CART mRNA did not significantly change between these postnatal ages ($F(2,21)=0.36$, $p=0.70$). This difference between developmental expression profiles of Ucn 1 and CART was reflected in a significant difference in the ratio of Ucn1/CART mRNA levels across ages ($F,21)=64.9$, $p<0.0001$). Specifically, Fisher's PLSD revealed that the Ucn 1 mRNA/CART mRNA level ratio at PND 4 was 2.7 times lower than at PND 12 ($p = 0.03$) and 12.7 times lower than at PND 45 ($p < 0.0001$), and the Ucn 1 mRNA/CART mRNA level ratio at PND 12 was 4.7 times lower than at PND 45 ($p < 0.0001$).

3. Discussion

Our study for the first time established an ontogenetic postnatal profile of Ucn 1 and CART in the perioculomotor region of the midbrain. The developmental profile of these peptides was investigated simultaneously to compare the timing of their appearance. We observed the

absence of Ucn 1 –ir in pIIIu in B6 mice at birth and the progressive development of Ucn 1 peptide and mRNA in these mice through infancy until adulthood. We also did not observe Ucn 1 –ir in any other brain region in the slices stained laterally from the midline at the ages examined during the first postnatal week. Theoretically, the absence of immunoreactivity for a peptide during development can be interpreted as lack of expression of this peptide or as absence of neurons expressing this peptide. However, since Ucn 1 co-localizes with CART in pIII (Kozicz, 2003; Lazar et al., 2004; Lima et al., 2008; Xu et al., 2009), and CART –ir is already present at PND 1, our studies clearly indicate that pIIIu neurons are already present in this brain area at this developmental stage, but Ucn 1 is not expressed in them. Our findings suggest that it is unlikely that Ucn 1 plays a role in pIII or other brain areas during the first postnatal week. Our finding is in agreement with an increase of Ucn 1 – immunoreactive fibers observed in rat cerebellum at PND 8 and PND 12 (Swinny et al., 2004a). As described previously for adult mice (Weitemier et al., 2005), we also did not observe Ucn 1 –ir in the cerebellum, suggesting that postnatal appearance of Ucn 1 in this brain region is a species-specific effect. The fact that Ucn 1 –immunoreactive cells were detected in the inferior olive at PND 3 by Swinny et al (2004a), but not by us in this study suggests that developmental expression of Ucn 1 in the inferior olive is also species-specific. The existence of species-specific expression in the Ucn 1 neurocircuit has been documented earlier (Lim et al., 2006; Weitemier et al., 2005).

It can be theorized that although we did not detect Ucn 1 by immunohistochemistry at PND 1 and detected Ucn 1 –ir only in one out of five animals at PND 4, the peptide is still present and active but not detected at these early postnatal ages (for example, due to active release). This idea is contradicted by observations that when Ucn 1 is reported in terminal fibers of adult animals, the Ucn 1 –ir in positive cells appears more intense, suggesting that more peptide is produced than released (Bachtell et al., 2003; Bittencourt et al., 1999; Weitemier et al., 2005). It seems unlikely that in neonatal animals the release is more efficient than in adults. However, a more definitive confirmation could be obtained by future studies simultaneously examining the presence of different CRF-like peptides, as well as receptors and CRF-binding protein in cells and terminals during development.

The patterns of Ucn 1 –ir and Ucn 1 mRNA levels largely showed a parallel development, although it appeared that Ucn 1 mRNA levels continue to increase further into adulthood. This however, could be due to technical issues because on one hand qRT-PCR is more quantitative than immunohistochemical detection, but on the other hand, qRT-PCR relies on correct dissection of the pIIIu, which is difficult at early postnatal ages. Importantly both methods show that Ucn 1 levels in pIII increase during postnatal development by more than an order of magnitude, while levels of CART stay mostly unchanged.

It is worth noting that the very low levels of Ucn 1 during early postnatal development coincide with the hyporesponsive period of the hypothalamic-pituitary-adrenal axis (Levine, 1994; Sapolsky and Meaney, 1986). Although pIIIu neurons are activated by stress, it has been shown that this activation is independent of glucocorticoids (Gaszner and Kozicz, 2003; Kozicz et al., 2004; Weninger et al., 2000). However, the postnatal development of Ucn 1 expression and stress sensitivity is in agreement with the contribution of Ucn 1 to regulation of stress-related functions (Kozicz, 2007).

The role of Ucn 1 in pIIIu in sensitivity to ethanol and addictive drugs, hypothermic responses, and food and ethanol intake in adult B6 mice has been extensively documented (reviewed in (Ryabinin and Weitemier, 2006)). For example, Ucn 1 immunoreactivity in pIIIu is higher in two replicate lines of mice selectively bred for high alcohol preference versus two replicate control lines (Bachtell et al., 2003), in two replicate lines of mice selectively bred for high alcohol-induced hypothermia versus two replicate control lines (Bachtell et al., 2002b), in mice

selectively bred for high alcohol conditioned place preference versus its control line (Kiianmaa et al., 2003), and in four lines of rats selectively bred to prefer alcohol versus corresponding low alcohol preferring control lines (Fonareva et al., 2009; Turek et al., 2005). In agreement with these genetic studies, lesions of pIIIu significantly decrease alcohol preference and food consumption in mice (Bachtell et al., 2004; Weitemier and Ryabinin, 2005). However, little is known regarding the potential role of this neuropeptide during development. Since we observe that Ucn 1 –ir in pIIIu develops at later postnatal stages, it is unlikely that Ucn 1 plays a role in these behaviors prior to PND 8. Nevertheless, it is remarkable that this time course parallels the development of several relevant behaviors. Specifically, previous findings showed that sensitivity to the hypothermic effect of ethanol in mice, a response regulated by Ucn 1 (Bachtell et al., 2002b; Turek and Ryabinin, 2005), develops around PNDs 12–15 (French et al., 1995; Wood et al., 1999). On the other hand, sensitivity to the locomotor-stimulating effects of ethanol in mice, a behavior that is likely not influenced by Ucn 1 (Bachtell et al., 2003), develops around PND 30 (Wood et al., 1999), an age when Ucn 1 –ir in pIIIu in the present study reached levels close to those observed in adults. While alcohol intake has not been studied in mice at early postnatal ages, it has been shown that rats consume high amounts of ethanol before PND 8 (Sanders and Spear, 2007) and that intake of ethanol is higher during PND 9–14 than in adults (Hall, 1979; McKinzie et al., 1999), a finding in agreement with inhibitory effects of centrally-administered Ucn 1 on ethanol intake (Ryabinin et al., 2008). Finally, it should be noted that the development of Ucn 1 expression clearly coincides with the development of independent feeding in pups (Hall and Browde, 1986). The parallel development of Ucn 1 –ir with developmental changes in these behaviors is in agreement with the importance of this peptide for regulation of food and alcohol self-administration.

It has also been shown that administration of exogenous Ucn 1 can have neuroprotective and neurotrophic effects (Abuirmeileh et al., 2007; Brar et al., 2000; Calle et al., 2005; Choi et al., 2006; Facci et al., 2003; Gounko et al., 2005; Swinny et al., 2004b). Therefore, it was theoretically possible that Ucn 1, as a peptide released from pIII, could play a trophic or guiding role for other neurons or their processes. Since Ucn 1 appears to be almost absent during the first postnatal days, our results argue against such a role for Ucn 1 during early postnatal development.

Additionally, our results indicate that there is certain degree of variability when Ucn 1 –ir starts to appear in pIII. Thus, while six of seven mice examined prior to PND 8 using brightfield and fluorescent immunohistochemistry did not show Ucn 1 –ir, one PND 4 mouse did. This occurred despite the fact that these mice are inbred, and are at an age when social hierarchy in a cage is unlikely to play a role. It is also unlikely that this observation is due to sex differences because it appears much earlier than gonadal maturation. While the reason for a different start of Ucn 1 expression in pIIIu deserves future investigations, our findings indicate that in a majority of animals Ucn 1 –ir is absent prior to PND 8.

In contrast to Ucn 1, CART –ir has been shown to migrate to the perioculomotor region prenatally and occupy the location of pIII already at embryonic day 16 in rats. In fact, CART may be one of the earliest neuropeptides with a neuromodulatory role that is expressed in the brain (Brischoux et al., 2002; Risold et al., 2006). Therefore, while we argue that a developmental role of Ucn 1 is unlikely, it is quite possible that CART from pIIIu neurons can have such a role. Moreover, finding that expression of CART and Ucn 1 appears in this brain region at such different ages, indicates differential regulation of genes expressed in pIII neurons during development. The pIIIu shows preferential expression of several additional genes involved in regulation of food consumption, stress and energy homeostasis, such as cholecystokinin, nesfatin and the growth hormone secretagogue receptor (also known as ghrelin receptor) (Brailoiu et al., 2007; Innis and Aghajanian, 1986; Maciewicz et al., 1984;

Xu et al., 2009). It would be important to comparatively investigate developmental profiles of different behaviorally-important genes in this brain region.

Taken together, our studies for the first time show a differential expression of genes (Ucn 1 versus CART) within a specific subregion of pIII during postnatal development. This population of pIII neurons has been shown previously to play a role in regulation of stress-related behaviors, alcohol sensitivity, alcohol drinking, and food consumption. Future studies should pay attention to age of animals when elucidating roles of genes expressed in pIII in these behaviors. We suggest that Ucn 1's role in these behaviors is age-dependent.

4. Experimental Procedures

Subjects

Breeding pairs were established from C57BL/6J (Jackson Laboratories, Bar Harbor, Maine), and neonatal mice were collected on postnatal days (PND) 1, 4, 8, 12, 16 and 24. Adult mice were collected at PND 45. Throughout the course of the study, mouse chow and water was available ad libitum. All animal procedures were in accordance with the Oregon Health & Science University and the National Institutes of Health guidelines for the care and use of laboratory animals.

Preparation of Tissue for Immunohistochemistry

For PND 1, PND 4, and PND 8 mice, brains were extracted after decapitation. For PND 12 and PND 16, half of the mice were euthanized by CO_2 inhalation prior to brain extraction and the other half by direct decapitation. This was done since it is at this stage that mice become susceptible to the effects of CO_2, and the effect of CO_2 on Ucn 1 and CART release and immunoreactivity is not known (upon examination of results, no obvious difference in Ucn1 –ir was observed between animals euthanized by CO_2 euthanasia or decapitation). PND 24 mice were all sacrificed by CO_2 inhalation prior to brain extraction. All brains were collected in 2% paraformaldehyde for 24 hours before being cryoprotected in 20% sucrose in 1% sodium azide (NaN_3)/phosphate-buffered saline (PBS) solution. After sinking, brains were transferred to a 30% sucrose in 1% NaN_3/PBS solution and stored at 4°C until slicing. All brains were frozen and sliced at 30 μm on a CM1850 cryostat (Leica Microsystems, Inc., Deerfield, IL.) at −20°C, and slices were placed in 1% NaN_3/PBS. The majority of brains were sliced in the sagittal plain, except for brains of animals of PND 4 and PND 24 used in double fluorescence immunohistochemistry.

Immunohistochemistry for Ucn 1

The staining protocols were modifications of ones previously reported by our laboratory (Bachtell et al., 2003; Weitemier et al., 2005). Brightfield immunohistochemistry was first used to characterize Ucn 1 –ir of ages: PND 1 (n=2), PND 4 (n=3), PND 8 (n=3), PND 12 (n=5), PND 16 (n=4), and PND 24 (n=2). For PND 1 and PND 4, an average of 25 slices was stained sagittally in each hemisphere laterally from the midline. This was done to determine if Ucn 1 –ir could be seen in other brain areas in addition to pIII at an early postnatal age, and because Bregma levels would differ for each postnatal age. Mice aged PND 8 and older were stained only for sagittal slices that included the pIII, where the majority of Ucn 1 –ir is seen in adults (Bittencourt et al., 1999; Kozicz et al., 1998; May et al., 2008; Ryabinin et al., 2005; Ryabinin et al., 2008; Spangler et al., 2009; Vaughan et al., 1995; Weitemier et al., 2005). Endogenous peroxidase activity was inhibited with 0.3% H_20_2 followed by several PBS rinses prior to blocking in 2% bovine serum albumin (BSA) in 1% PBS/Triton X-100 for five hours. Brain slices were incubated in anti-Ucn 1 primary antibody (Sigma-Aldrich, St. Louis, MO; catalog No. U4757) using a 1:5000 dilution in 1% PBS/Triton X-100 for two nights at room temperature. The specificity of this antibody was confirmed by incubation with Ucn 1 and

related peptide in our previous experiments (Bachtell et al., 2003), by incubation of slices from one of the animals without the primary antibody revealing no staining in pIII. After several rinses in PBS, anti-rabbit made in goat (Vector Laboratory Inc., Burlingame, CA, USA) was used as the secondary antibody for a one and a half hour incubation the following morning. Slices were once again rinsed in PBS and peptide expression was detected by amplifying signal with the Vectastain ABC kit (Vector Laboratory Inc.). Following additional PBS rinses, enzymatic development was performed with Metal Enhanced Diaminobenzamidine (DAB) (Pierce, Rockford, IL, USA). Slices were mounted onto gelatin-coated slides, dried overnight and dehydrated the following morning in 70% EtOH, 95% EtOH, and 100% EtOH for 10 minutes each and placed in xylene for 12 minutes after which they were immediately coverslipped with DPX mounting glue.

Immunohistochemistry for CART

Immunohistochemical detection of CART neuropeptide was performed on PND 1, 4, 8, 12, 16, and 24 (n=2/age). As with Ucn 1, endogenous peroxidase activity was inhibited with 0.3% H_20_2 followed by several PBS rinses prior to blocking in 4.5% goat serum in 1% PBS/Triton X-100 for five hours. Brain slices were incubated in anti-CART primary antibody (Phoenix Pharmaceuticals, Burlingame, CA; catalog No. H-003-62) using a 1:30,000 dilution for two nights at room temperature. The specificity of this antibody was confirmed previously by preincubation experiments (Dun et al., 2000), and here by incubation of slices from one of the animals without the primary antibody revealing no staining in pIII. After several rinses in PBS, anti-rabbit made in goat (Vector Laboratory Inc.) was used as the secondary antibody for a one and a half hour incubation the following morning. Slices were once again rinsed in PBS and peptide expression was detected by amplifying signal with the Vectastain ABC kit (Vector Laboratory Inc.). Following additional PBS rinses, enzymatic development was performed with Metal Enhanced DAB (Pierce, Rockford). Slices were mounted onto gelatin-coated slides, dried overnight and dehydrated the following morning in 70% EtOH, 95% EtOH, and 100% EtOH for 10 minutes each and placed in xylene for 12 minutes after which they were immediately coverslipped with DPX mounting glue.

Double fluorescence immunohistochemistry for Ucn 1 and CART

Four additional mice at PND 4 and PND 24 (N= 2 per age) were analyzed for simultaneous presence of both Ucn 1 and CART through the following procedure. Slices were blocked with 2% BSA, 7.5% 10,000 U per ml heparin, and 0.3% Triton-X 100 in PBS for 1 hour. The Ucn 1 antibody was incubated overnight at a 1:5,000 dilution in 2% BSA and 0.3% Triton-X 100 in PBS. This was followed by a 2-hour incubation with 0.5% AlexaFluor 555 anti-rabbit (raised in goat) (Invitrogen, Carlsbad, CA), 0.1% BSA, and 0.3% Triton-X 100 in PBS. After thorough washes with PBS, slices were blocked a second time in 2% BSA, and 0.3% Triton-X 100 in PBS for 2 hours. Slices were then incubated with the CART antibody at a 1:30,000 dilution overnight. The CART antibody was visualized with a 2-hour incubation with 0.5% AlexaFluor 488 anti-rabbit (raised in chicken) (Invitrogen) in PBS with 0.1% BSA and 0.3% Triton-X 100. Slices were washed with PBS, mounted on gelatinized slides, coverslipped with VectaShield mounting medium for fluorescence with DAPI (Vector Laboratory Inc.) and sealed with clear nail polish.

Immunohistochemistry analysis

Leica DM4000 microscope (Bartels & Stout Inc., Bellevue, WA) was used for visualization of stained sections. Ucn 1 and CART -positive cells stained above background were manually counted through all slices at 20x objective. Results for total cell counts are presented as mean ± standard error of the mean (SEM). For illustrations, images were taken using Qcapture 2.7 software (QImaging, Surrey, BC, Canada).

RNA Isolation

For qRT-PCR studies, 10 mice were euthanized by decapitation on PND 4, 10 mice were euthanized by decapitation on PND 12, and 4 male mice were sacrificed on PND 45 by a CO_2 inhalation (punch dissections of pIIIu from PND 1 brain were also attempted, but were considered technically not feasible and therefore not continued). Only male mice were used at PND 45 but previous studies revealed no difference between male and female mice in Ucn 1 expression (Bachtell et al., 2002a). Brains were immediately sliced on a pre-chilled brain matrix (Research Instruments Inc., Corvallis, OR), and a 1 mm slice containing pIIIu was isolated. Punches were taken using a chilled 18-gage blunt needle, and the punch was pushed out of the needle with a 6 ml syringe into 100 μl RNA Later solution (Qiagen, Valencia, CA). Tissue was stored at 4°C overnight. RNA Later was subsequently removed, and tissue punches were stored at −80°C.

RNA was isolated using the Arcturus PicoPure RNA Isolation Kit (Molecular Devices, Sunnyvale, CA) using a protocol adapted from the Kit manual. Briefly, 150 μl Extraction buffer was added to tubes containing brain punches and incubated at 42°C for 3 hours. RNA Purification Columns were conditioned with 250 μl Conditioning buffer for 5 minutes at room temperature. One hundred fifty μl of 70% EtOH was added to each tube containing cell extract and mixed. The entire mixture (300 μl) was pipetted onto a conditioned column and the column was centrifuged to collect RNA. Columns were washed in Wash Buffer #1, and DNAse was performed using Qiagen's RNase-Free DNase Set. Each sample received a mixture of 5 μl DNase I stock and 35ul Buffer RDD. The columns were incubated for 15 minutes at room temperature at which point 40 μl Wash Buffer #1 was added to each column, and the columns were centrifuged to clear out the liquid. The columns were washed twice with 100 μl Wash Buffer #2. Each column was transferred to a new microcentrifuge tube and the RNA was eluted using 15 μl Elution buffer.

qRT-PCR

qRT-PCR was performed using Quantitect SYBR Green RT-PCR Kit (Qiagen). Commercially-predesigned QuantiTect primers (Qiagen) for Ucn 1, CART and 18S were used in the assays. Spectrophotometer readings were taken of each RNA sample and the concentrations of each sample were diluted to be equal to that of the least concentrated sample for reaction with Ucn 1 and CART primers. A 10000-fold dilution of each sample was used with primers for the housekeeping gene 18S. Five μl of each sample was added to each well. Master Mixes consisted of 3 μl of 10X Primer, 15 μl 2X Master Mix, 0.3 μl Reverse Transcriptase and 6.7 μl water. Reactions were performed using the MX3000P RT-PCR Machine (Stratagene, La Jolla, CA). Reverse transcription was performed at 50°C for 30 minutes. The polymerase chain reaction thermal profile consisted of 15 minutes at 95°C followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C. The fluorescence data were collected at the end of each annealing step. Dissociation curve data were collected at the end of all reactions, and the resulting melting curves were identical for each sample of the three analyzed genes. The relative levels of RNA were calculated as the exponential of cycle threshold difference for each of the corresponding genes. While ratios of Ucn 1/18S, CART/18S and Ucn 1/CART were calculated this way, it should be noted that only ratios of Ucn1/CART should be used as a reliable measure of Ucn1 expression in pIIIu because each dissection can contain a variable amount of pIIIu neurons. The resulting relative measures of expression were used in statistical analysis using ANOVA.

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Abbreviations

ANOVA	analysis of variance
BSA	bovine serum albumin
CART	cocaine- and amphetamine-regulated transcript
CRF	corticotropin releasing factor
DAB	diaminobenzidine
EtOH	ethanol
ir	immunoreactivity
NaN_3	sodium azide
npEW	non-preganglionic Edinger Westphal nucleus
pIII	perioculomotor area
pIIIu	periocolumotor urocortin-containing neurons
PBS	phosphate-buffered saline
PLSD	Protected Least Significant Difference
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
Ucn 1	urocortin 1

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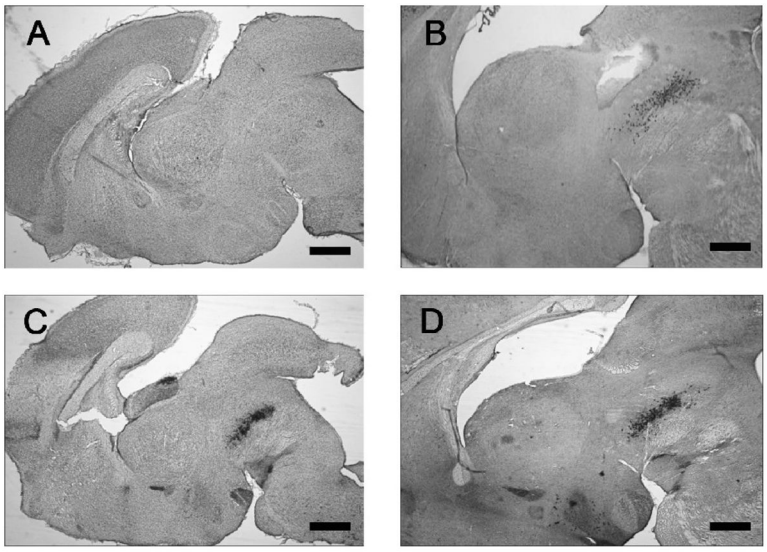

Fig. 1.

Representative sagittal sections of Ucn 1 –ir in pIII at PND 4 (A), and PND 24 (B) and CART –ir at PND 4 (C) and PND 24 (D) at low magnification. Ucn 1 – ir was largely absent at PND 4 and present only in pIIIu at PND 24. In contrast, CART is present in pIIIu and other brain regions at both ages. Scale bar = 500 μm.

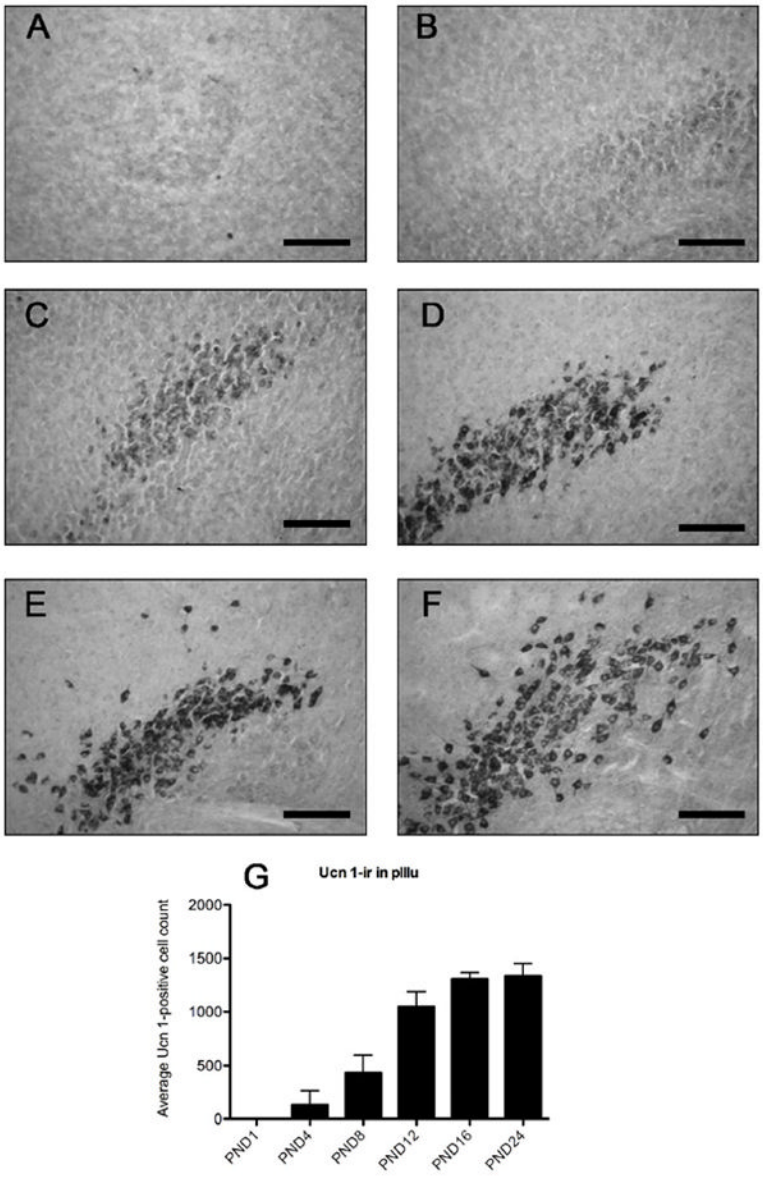

Fig. 2.

Representative sagittal sections showing postnatal development of Ucn 1 –ir in pIIIu at high magnification. Ucn 1 –positive cells in the pIIIu at PND 1 (A), PND 4 (B), PND 8 (C), PND 12 (D), PND 16 (E), and PND 24 (F). DAB staining of the pIIIu region revealed the absence of Ucn 1 –positive cells at PND 1. Scale bar = 100 μm. Quantification of Ucn 1 –positive cells across six postnatal time points (G).

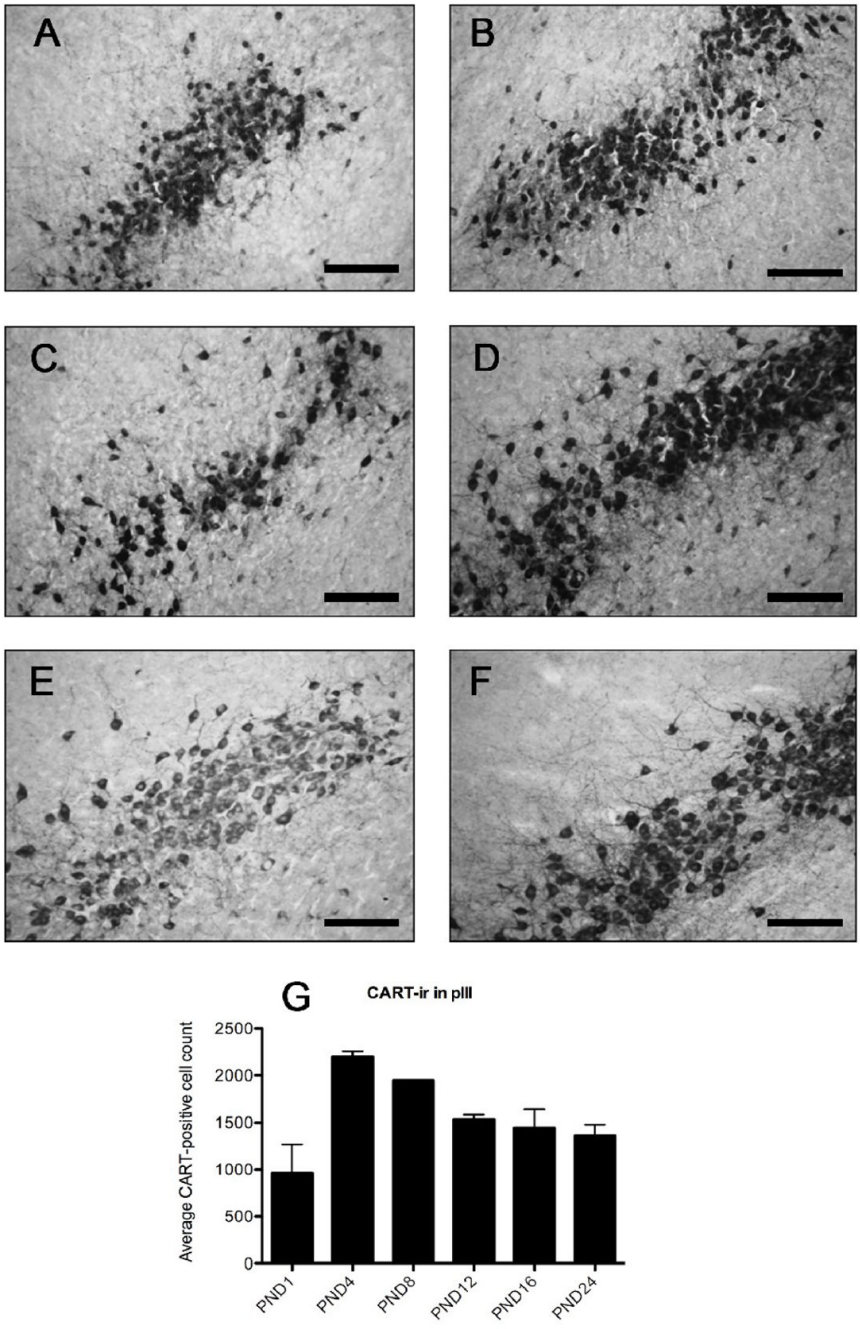

Fig. 3.

Representative sagittal sections showing postnatal development of CART –ir in pIII at high magnification. CART –positive cells in pIII at PND 1 (A), PND 4 (B), PND 8 (C), PND 12 (D), PND 16 (E), and PND 24 (F). DAB staining of the pIII region revealed CART –positive cells at PND 1. Scale bar = 100 μm. Quantification of CART –positive cells across six postnatal time points (G).

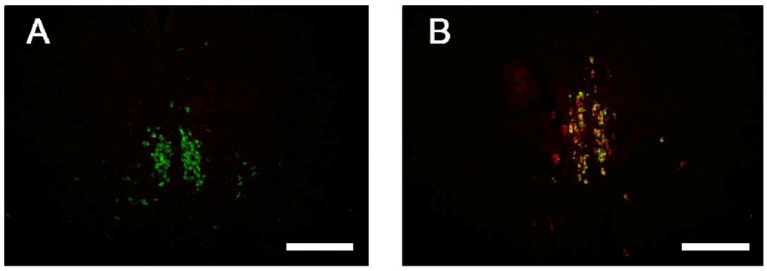

Fig. 4.

Representative coronal sections showing absence of Ucn 1 during the first postnatal week at high magnification. Double label immunofluorescence staining of Ucn 1 (red) and CART (green) at PND 4 (A) and PND 24 (B). Scale bar = 200 μm. Staining revealed absence of Ucn 1 –positive cells at PND 4, while both Ucn 1 and CART were present at PND 24 and are largely co-localized (yellow).

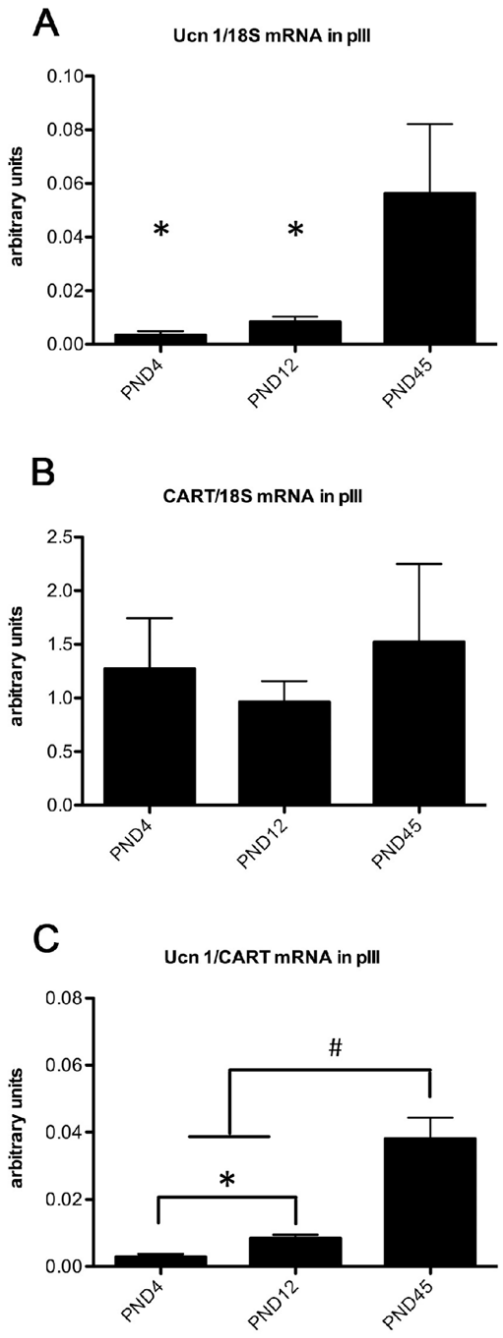

Fig. 5.

Relative Ucn 1 mRNA level (A), relative CART mRNA level (B) and ratio of Ucn 1 mRNA: CART mRNA (C) at PND 4, 12, and 45. In (A) * indicates a significant difference ($p < 0.05$) of Ucn 1 mRNA expression between PND 4 and PND 45 and between PND 12 and PND 45. In (B) there is no significant difference between CART mRNA levels on any days. In (C) * indicates a significant difference ($p < 0.05$) of Ucn 1:CART mRNA expression between PND 4 and PND 12, while # indicates PND 4 and PND 12 are both significantly different from PND 45 ($p < 0.0001$).