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Distribution of orexin/hypocretin immunoreactivity in the brain of a male songbird, the house finch, *Carpodacus mexicanus*

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Abstract

Previous research has shown orexin/hypocretin immunoreactive (orexin-ir) neurons in domesticated Galliformes. However, these findings may not be representative of other birds and these studies did not include a distribution of orexin-ir projections throughout the brain. The present study was carried out in a wild-caught passerine, the house finch, *Carpodacus mexicanus*, and includes a detailed description of orexin-ir neurons and their projections. Orexin A and B-ir neurons were located in a single population centered on the paraventricular nucleus of the hypothalamus extending into the lateral hypothalamic area, consistent with other studies in birds. Orexin A and B-ir fibers were similarly visible across the brain, with the highest density within the preoptic area, hypothalamus and thalamus. Orexin-ir projections extended from the paraventricular nucleus rostrally to the preoptic area, laterally towards the medial striatum, nidopallium, and dorsally along the lateral ventricle towards the mesopallium. Caudally, the highest densities of orexin-ir fibers were found along the third ventricle. The periaqueductal grey, substantia nigra pars compacta and the locus coeruleus also showed a high density of orexin-ir fibers. This study showed a detailed fiber distribution previously unreported in birds and showed that orexin-ir neurons were located in similar areas regardless of phylogeny or domestication in birds. The apparently conserved neural distribution of orexins suggests that these peptides play similar roles among birds. The widespread distribution of the projections in brain areas serving various roles indicates the potential involvement of these peptides in multiple behavioral and physiological functions.

Keywords: Birds; Hypothalamus; Comparative neuroanatomy; Paraventricular nucleus; Neuropeptide

1. Introduction

Orexin (hypocretin) is an orexigenic neuropeptide in the incretin peptide superfamily (Alvarez and Sutcliffe, 2002). Orexin is thought to be involved in feeding behaviors as well as arousal and the sleep–wake cycle in mammals (de Lecea et al., 1998; Sakurai et al., 1998; Dube et al., 1999; Karteris et al., 2005). Two forms of the peptide, orexin A and orexin B are cleaved separately (de Lecea et al., 1998; Sakurai et al., 1998) from a single precursor prepro-orexin. Interestingly, the amino

Abbreviations: A, arcopallium; APH, area parahippocampalis; BST, bed nucleus of stria terminalis; Cb, cerebellum; CoA, anterior commissure; CP, commissura posterior; DL, nucleus dorsolateralis anterior thalami; DMP, nucleus dorsomedialis posterior thalami; E, entopallium; EM, nucleus ectomammillaris; FA, fronto-arcopallial tract; FLM, fasciculus longitudinalis medialis; GP, globus pallidus; H, hyperpallium; HA, hyperpallium accessorium; Hb, habenula; HD, hyperpallium densocellularum; Hp, hippocampus; ICo, nucleus intercollicularis; III, third ventricle; IM, nucleus isthmi, pars magnocellularis; IPC, nucleus isthmi, pars parvocellularis; LFB, lateral forebrain bundle; LHN, lateral hypothalamic nucleus; LHy, lateral hypothalamus; LRF, lateral reticular formation; LoC, locus coeruleus; LSt, lateral striatum; LV, lateral ventricle; M, mesopallium; Mn V, nucleus mesencephalicus nervi trigemini; MRF, medial reticular formation; MSt, medial striatum; MStv, ventral medial striatum; N, nidopallium; NIII, nervus oculomotorius; n IV, nucleus nervi trochlearis; OC, optic chiasm; OM, occipito-mesencephalic tract; PAG, periaqueductal grey; PM, nucleus pontis medialis; POA, preoptic area; PPT, nucleus pedunculopontinus tegmenti; PSL, pallial subpallial lamina; PVN, paraventricular nucleus; Rt, nucleus rotundus; Ru, red nucleus; SCN, suprachiasmatic nucleus; SGP, substantia grisea et fibrosa periventricularis; SL, lateral septum; SM, medial septum; SPM, nucleus spiriformis medialis; SNc, substantia nigra; pars compacta; TeO, optic tectum; TFM, tractus thalamo-frontalis et frontalis-thalamicus medialis; TSM, tractus septopallio-mesencephalicus; TrO, optic tract; VH, ventral hypothalamus; VMH, ventromedial hypothalamus; VTA, ventral tegmental area

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acid sequences for orexin A and B are highly conserved across vertebrates (Alvarez and Sutcliffe, 2002; Ohkubo et al., 2002). In most studies orexin immunoreactive (-ir) neurons have been located in the hypothalamus, although in various nuclei depending on the species considered. In fish, orexin-ir neurons were found in two populations: the anterior hypothalamus and along the third ventricle within the preoptic area (POA) and suprachiasmatic nucleus (SCN) (Kaslin et al., 2004). In amphibians, the distribution of these neurons varies between suborder of frogs. In *Xenopus laevis* of the Mesobatrachia suborder, orexin-ir neurons were predominantly found in the ventral hypothalamus (VH). In *Rana ridibunda* and *Hyla cinerea* of the Neobatrachia suborder, the neurons were centered on the SCN (Shibihara et al., 1999; Galas et al., 2001; Singletary et al., 2005). In reptiles, the orexin-ir cells were located along the third ventricle within the periventricular nucleus (Farrell et al., 2003). Studies in mammals showed a single population of orexin-ir neurons in the caudal parts of the lateral hypothalamus (LHy) (Peyron et al., 1998; Cutler et al., 1999; Nambu et al., 1999; McGranaghan and Piggins, 2001; Mintz et al., 2001).

The distribution of orexin-ir cells in birds has been described in chicken (Ohkubo et al., 2002) and Japanese quail (Phillips-Singh et al., 2003). Orexin-ir neurons and orexin mRNA in these species are primarily located in the paraventricular nucleus (PVN) and LHy. However, these studies did not determine the distribution of orexin-ir fibers throughout the brain. Furthermore, the Galliformes used in the studies were domesticated and it could be argued that domesticated animals have distributions of neuropeptides that are not representative of those in wild animals. In addition, Galliformes are phylogenetically separate from the majority of birds such as passerines (Groth and Barrowclough, 1999). As mentioned earlier, studies in phylogenetically different amphibians show dissimilar orexin-ir neuron location. Given these considerations, it is unclear whether observations from Japanese quail and chicken can be generalized to other birds. To address these issues, we determined the distribution of orexin in a wild-caught Passeriform, the house finch, *Carpodacus mexicanus*. The house finch was chosen over other Passeriformes because of the large number of house finches readily available. The present study provides a better understanding of the orexin system in birds and a comparative approach to other vertebrates.

2. Materials and methods

Adult male house finches, *C. mexicanus* ($n = 5$) were caught in Tempe, Arizona in September (latitude: 33.414N; longitude: 111.908W). They were housed in an outdoor aviary exposed to ambient temperature and natural photoperiod (10-h light/14-h dark at time of sacrifice), and received food and water ad libitum. Finches were all sacrificed on the same day in December. The Arizona State University Institutional Animal Care and Use Committee preapproved all experimental procedures.

Each bird received an intramuscular injection of 0.2 ml anesthetic solution (0.9% NaCl in water containing 20 mg/ml xylacine and 100 mg/ml ketamine) followed with Metofane (Schering-Plough Animal Health Corp., Union, NJ, USA) inhalation. Birds were perfused transcardially with 35 ml of wash solution (0.9% NaCl and 0.1% $NaNO_2$ in 0.1 M phosphate buffer (PB)), followed by 35 ml of fixative (4% paraformaldehyde and 0.1% $NaNO_2$ in 0.1 M PB). They were then decapitated, skulls were opened to expose the brain, and heads were placed in fixative overnight at 4 °C. Brains were dissected from the skull, placed in 0.1 M PB overnight at 4 °C, and followed by immersion in a 4% gelatin solution for 30 min. They were then embedded in an 8% gelatin mold and allowed to solidify overnight. After this, brains were immersed in 10 and 20% sucrose solutions for 24 h each, ending with 48 h in 30% sucrose solution. They were then frozen on dry ice and cut on a cryostat at 50 μm. Three parallel sets of sections were collected for each brain.

Free-floating sections were processed by immunocytochemistry for orexin A and B following a protocol adapted from other anatomical studies (Wommack and Delville, 2002; Singletary et al., 2005). After washing in 0.1 M phosphate buffer solution (PBS), sections were treated with 0.5% hydrogen peroxide to inhibit endogenous peroxidase activity. Sections were blocked in a 10% normal donkey serum solution and washed in a 0.1 M PBS/0.3% Triton X-100 solution to ensure permeabilization. Sections were successively incubated in the primary (orexin A or B antiserum, 1:4000, Santa Cruz Biotechnology, Santa Cruz, CA) and secondary antibodies (donkey anti-goat immunoglobulin, 1:400, Jackson Immunoresearch, West Grove, PA). The goat polyclonal orexin A and B antibodies are raised against an epitope within the last 100 amino acids of the C-terminus of prepro-orexin and affinity purified. The prepro-orexin epitope is of human origin and identical to the corresponding sequence of mouse origin. It is been shown that chicken orexin A and B are 85 and 65% homologous to human orexin A and B, respectively (Ohkubo et al., 2002). Sections were labeled with 0.5 μg/ml diaminobenzidine (Sigma, St. Louis, MO) after incubation in an avidin-biotinylated peroxidase conjugate (1:100, VectaStain ABC kit, Vector Labs, Burlingame, CA). Sections were dehydrated in gradient alcohols, defatted with xylene (Sigma, St. Louis, MO) and allowed to dry. Some series of orexin A and B slides were counterstained with thionin. Sections were then mounted on gelatin coated slides and coverslipped with Permount (Fisher Scientific, Hampton, NH). The distribution of immunoreactive neurons and fibers was mapped at 20× through a camera lucida attachment (Nikon Y-IDT) to the microscope (Nikon Eclipse E600). The quality of the labeling was similar for both orexin A and B. Photomicrographs were taken using a SPOT RT Slider CCD camera and SPOT 3.0.6 software (Diagnostic Instruments, Inc., Sterling Heights, MI). Brain regions were identified using avian brain atlases and previous descriptive studies (Stokes et al., 1974; Aste et al., 1998; Reiner et al., 2004; Krutzfeldt and Wild, 2004; Kuenzel, 2004). Adobe Photoshop 5.5 was used to add text to images.

The specificity of the immunostaining was tested through omission of primary antibodies and pre-absorption of the antibodies to orexin A and B. For this, orexin antibodies were incubated overnight in the presence of orexins (40 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) before application to tissue sections. Omission of the primary antibodies and peptide pre-absorption eliminated all immunostaining.

3. Results

Orexin A and B immunoreactive (orexin A and B-ir) perikarya had the same distribution, forming a discrete population centered on the PVN (Figs. 1 and 4). Because they are similar, only maps of orexin A are shown here to illustrate results (Fig. 4). Orexin-ir cells were seen extending from the PVN into the caudal POA. Here, orexin-ir cells were located mainly along the walls of the third ventricle, with a few seen just dorsal to the LHy. At the level of the PVN, the distribution of cells extended into the lateral edge of the LHy with a few seen ventrally towards the ventro-medial hypothalamus (VMH).

The distribution of immunoreactive fibers was also similar between orexin A and B (Figs. 1–3). Orexin-ir fiber innervation is illustrated here (Figs. 2 and 3). Orexin innervation was highest in the diencephalon and mesencephalon. A moderate to