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EFFERENT PROJECTIONS FROM THE MEDIAN PREOPTIC NUCLEUS TO SLEEP- AND AROUSAL-REGULATORY NUCLEI IN THE RAT BRAIN

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Abstract—The median preoptic nucleus (MnPO) has been implicated in the regulation of hydromineral balance and cardiovascular regulation. The MnPO also contains neurons that are active during sleep and in response to increasing homeostatic pressure for sleep. The potential role of these neurons in the regulation of arousal prompted an analysis of the efferent projections from the MnPO. Anterograde and retrograde neuroanatomical tracers were utilized to characterize the neural connectivity from the MnPO to several functionally important sleep- and arousal-regulatory neuronal systems in the rat brain. Anterograde terminal labeling from the MnPO was confirmed within the core and extended ventrolateral preoptic nucleus. Within the lateral hypothalamus, labeled axons were observed in close apposition to proximal and distal dendrites of hypocretin/orexin immunoreactive (IR) cells. Projections from the MnPO to the locus coeruleus were observed within and surrounding the tyrosine hydroxylase–IR cell cluster. Labeled axons from the MnPO were mostly observed within the lateral division of the dorsal raphé nucleus and heavily within the ventrolateral periaqueductal gray. Few anterogradely labeled appositions were present juxtaposed to choline acetyltransferase–IR somata within the magnocellular preoptic area. The use of retrogradely transported neuroanatomical tracers placed within the prospective efferent terminal fields supported and confirmed findings from the anterograde tracer experiments. These anatomical findings support the hypothesis that MnPO neurons function to promote sleep by inhibition of orexinergic and monoaminergic arousal systems and disinhibition of sleep regulatory neurons in the ventrolateral preoptic area. **© 2007 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: locus coeruleus, hypothalamus, dorsal raphé, magnocellular preoptic area, basal forebrain.

The preoptic area/anterior hypothalamus participates in the regulation of homeostatic processes, including blood pressure, thirst, salt appetite, thermoregulation, sexual behavior and arousal (Boulant and Silva, 1988; Everitt, 1990; McGinty and Szymusiak, 1990; Johnson et al., 1996). The median preoptic nucleus (MnPO) and the ventrolateral preoptic area (vlPOA) contain neurons that are activated during both slow-wave (non-rapid eye movement (REM)) and REM sleep, compared with waking (Sherin et al., 1998; Szymusiak et al., 1998, 2001; Gong et al., 2000; Suntsova et al., 2002; McGinty and Szymusiak, 2003).

MnPO neurons innervate the paraventricular nucleus of the hypothalamus (PVH) (Silverman et al., 1981; Tanaka et al., 1987) the thalamus, the perifornical lateral hypothalamus (pLHA), the bed nucleus of the stria terminalis (BNST) (Gu and Simerly, 1997; Uschakov et al., 2001, 2006; Yoshida et al., 2006b), the suprachiasmatic nucleus (Oldfield et al., 1991b; Moga and Moore, 1997), the supraoptic nucleus (SON) (Miselis et al., 1979; Oldfield et al., 1991b), the dorsal raphé nuclei (DRN), the locus coeruleus (LC), the pericoerulear area (Zardetto-Smith and Johnson, 1995) and the vlPOA (Chou et al., 2002; Thompson and Swanson, 2003; Uschakov et al., 2006). Efferent connectivity with these areas of the brain may, in part, mediate the homeostatic processes attributed to the MnPO (Jones, 1988; Fitzsimons, 1998).

Sleep-related suppression of neural activity in some of these regions of the brain may be mediated by projections from MnPO GABAergic neurons that are activated during sleep (Gong et al., 2004; Uschakov et al., 2006). Several identified and potential targets of the MnPO are prominently implicated in the regulation of sleep and arousal, including the LC, DRN, posterior hypothalamus (PH), pLHA and magnocellular basal forebrain (see Jones, 2003, 2005 for review). However, projections from the MnPO to neurochemically characterized cell types in these areas have not been previously quantified. Therefore, we utilized anterograde and retrograde neuroanatomical tracers and the immunohistochemical identification of noradrenalin-, 5-HT-, acetylcholine- and orexin/hypocretin-containing cells, to characterize the efferent neural projections from the MnPO to potential arousal regulatory cell groups.

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Abbreviations: BDA, biotin–dextran lysine; BNST, bed nuclei stria terminalis; ChAT, choline acetyltransferase; c-MnPO, midline/central median preoptic nucleus; c-vlPOA, core ventrolateral preoptic area; DAB, diaminobenzidine; DRN and DR, dorsal raphé nucleus; ex-vlPOA, extended ventrolateral preoptic area; FITC, fluorescein isothiocyanate; IR, immunoreactive; LC, locus coeruleus; l-MnPO, lateral lamina median preoptic nucleus; LPO, lateral preoptic area; LS, lateral septal nucleus; MCP, magnocellular preoptic area; MnPO, median preoptic nucleus; MPO, medial preoptic area; NDB, nucleus of the diagonal band of Broca; PB, phosphate buffer; pLHA, perifornical lateral hypothalamic area; REM, rapid eye movement; SI, substantia innominata; SO or SON, supraoptic nucleus; TBS, tris-buffered saline; vlPAG, ventrolateral periaqueductal gray; vlPOA, ventrolateral preoptic area.

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EXPERIMENTAL PROCEDURES

All experiments were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals. All protocols described here were reviewed and approved by the Animal Care and Use Committee at the Veterans Administration of the Greater Los Angeles Health Care System. Every effort was made to minimize the number of animals used and their suffering.

Subjects were male, Sprague–Dawley rats, maintained on a 12-h light/dark schedule at an ambient temperature of 21±3 °C. Animals weighed 250–300 g at the time of anatomical tracer injection, consistent with the neuroanatomical maps used to determine stereotaxic coordinates (Swanson, 1998). Injections were performed during a single, aseptic surgery under ketamine/xylazine (80/8 mg^{-1} kg^{-1}) anesthesia.

Anterograde studies utilizing biotin–dextran amine

Borosilicate glass pipettes (O.D. 1.2 mm, I.D. 0.69 mm; tip diameter 10–15 μm) were filled with a 10% solution of biotin-dextran lysine (BDA-10,000, Sigma-Aldrich, St. Louis, MO, USA) in sterile isotonic saline. BDA was iontophoretically applied (10%, 7 s on 7 s off for 45 min, +5 μA) into the MnPO (A/P from bregma, 0.0 mm; lateral, 0.0 mm; depth from top of cortex, 6.5 mm). Animals were allowed to recover for 10 days after which they were killed with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and perfused through the heart with 100 ml isotonic saline followed by 300 ml of 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.2), then by 100 ml of 10% sucrose and 100 ml 30% sucrose in PB. Brains were removed and stored in 30% sucrose for 24–48 h. Serial coronal sections, 40 μm in thickness, were cut throughout the brain. A Vectastain 'Elite' biotin–avidin horseradish peroxidase conjugation followed by a nickel diaminobenzidine (Ni-DAB) visualization procedure was performed on all sections (Hsu and Soban, 1982).

Immunohistochemistry

Sections that had been processed for BDA staining were then separated into regions which are known to contain cholinergic neurons in the magnocellular preoptic area (MCP), orexin/hypocretin neurons in the pLHA, serotonergic neurons in the DRN, or noradrenergic neurons in the LC.

Choline acetyltransferase (ChAT). Sections collected through the MCP area were incubated for 48 h in monoclonal mouse anti-ChAT (1:500, Chemicon, Billerica, MA, USA) made up in tris buffered saline (0.05 M, pH 7.2, 15% NaCl; TBS) with 2% Triton X-100 and 4% normal goat serum. Tissue was then washed three times in TBS for 5 min. Following this, sections were incubated in the secondary antibody, biotinylated goat–anti-rat (mouse) IgG (1:200, Vector Laboratories, Burlingame, CA, USA) in TBS with 4% normal goat serum, for 2 h. After washing in TBS, sections were incubated with avidin peroxidase for 2 h (1:200 Vector 'Elite') and developed with DAB.

Orexin/hypocretin. Sections spanning the pLHA area were incubated for 48 h in polyclonal rabbit anti-orexin A (1:5000 AB-1, Oncogene, Cambridge, MA, USA) in TBS with 2% Triton X-100 and 4% normal goat serum. Sections were washed in TBS followed by 2 h incubation in biotinylated secondary antibody, goat anti-rabbit IgG (1:200 Vector 'Elite') in TBS with 4% normal goat serum. Sections were then incubated with avidin peroxidase for 2 h (1:200 Vector 'Elite') and developed with DAB.

5-HT. Sections spanning the DRN were incubated for 48 h in primary antibody, polyclonal rabbit anti-5-HT (1:20,000 Immunostar, Hudson, WI, USA), in TBS with 2% Triton X-100 and 4% normal donkey serum. Tissue was washed in TBS, and incubated in secondary antibody, donkey anti-rabbit-HRP (1:100, Jackson, West Grove, PA, USA) in TBS with 4% normal donkey serum, for 2 h. Sections were then incubated with avidin peroxidase for 2 h (1:200 Vector 'Elite') and developed with DAB.

Tyrosine hydroxylase. Sections spanning the LC (~8.5 mm caudal to 10.5 mm caudal to the optic chiasm) were incubated for 48 h in polyclonal rabbit anti-tyrosine hydroxylase (1:500 Chemicon) in TBS with 2% Triton X-100 and 4% normal goat serum. Following three washes in TBS, sections were incubated for 2 h in biotinylated secondary antibody, goat anti-rabbit IgG (1:200 Vector 'Elite') in TBS with 4% normal goat serum. Sections were then incubated with avidin peroxidase for 2 h (1:200 Vector 'Elite') and developed with DAB.

Retrograde tracer studies utilizing fluoro-gold

The retrograde tracer, fluoro-gold (Fluorochrome, Denver, CO, USA), was unilaterally pressure injected (0.2 μl of 4% Fluoro-Au, in isotonic saline) at a rate of 10 nl/min into the pLHA, (A/P 3 mm; lateral, 1.4 mm; depth, 7.8 mm), the LC (A/P, 10 mm; lateral, 1.4 mm; depth 7 mm), the DRN (A/P, 8 mm; lateral, 0.0 mm; depth, 6.4 mm) or the MCP (A/P, 0.1 mm; lateral, 1.2 mm; depth 7.0 mm). Following a 10-day survival period, animals were perfused with 100 ml PBS, 300 ml 4% paraformaldehyde made up in PB, 100 ml of 10% sucrose and 100 ml of 30% sucrose in PB and stored in 30% sucrose overnight. Coronal sections throughout the injection site and the preoptic area were cut at 40 μm. Collected sections were divided into two series; the first series were mounted onto gelatin-coated glass slides, air-dried and then defatted in xylene for 10 min. Following this the slides were coverslipped with a xylene-based medium (DPX). These sections were analyzed using a Nikon Eclipse E-600 fluorescent microscope. The second series were incubated for 24 h in primary antibody, polyclonal rabbit anti-Fluoro-Au (1:5000 Chemicon) in TBS with 2% Triton X-100 and 4% normal goat serum. Sections were washed three times in TBS and incubated for 2 h in secondary antibody, goat anti-rabbit IgG (1:200 Jackson) conjugated to either fluorescent Rhodamine Red or fluorescein isothiocyanate (FITC). Sections were then mounted on gelatin-coated glass slides and coverslipped with a water-based mounting medium (Fluromount).

BDA-10,000 analyses

The computer-aided plotting system Neurolucida (V. 6.0) with a Lucivid attachment (MicroBrightfield) and a Ludl motorized stage controller was used to analyze and represent the locations of injection sites and boutons/varicosities within terminal fields. Injection loci were defined as sites containing major deposits of Ni-DAB reaction product. Labeled neurons surrounding the main deposit of BDA were considered as part of the injection site. Sections spanning the rostral–caudal levels of the vlPOA, MCP, pLHA, DRN and LC were selected for analysis. BDA-labeled processes and the distribution of those processes with respect to immuno-labeled neurons were examined in each selected region.

BDA-labeled structures included 1) terminal boutons; those structures that displayed noticeable swellings with little or no apparent continuation of the axon fiber, 2) en passant fibers; axons with multiple swellings along the longitudinal axis and 3) fibers of passage; axons located within the plane of section with no apparent intumescence or nodular structures.

An initial graphical format is presented whereby rostral–caudal sections throughout the target areas display the locations of varicosities and/or boutons (diameter ≥1.2 μm) as determined by cursor size. Sections mapped were referenced to the standardized maps (adapted from Swanson, 1998) on which they were to be superimposed. Scaling, alignment and placement of anatomical profiles were guided by readily recognizable landmarks including magnocellular divisions (e.g. the SON) chiasms, fiber bundles, distinct nuclei and the ventricular system. Areas of the brain with

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