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INDIRECT PROJECTIONS FROM THE SUPRACHIASMATIC NUCLEUS TO MAJOR AROUSAL-PROMOTING CELL GROUPS IN RAT: IMPLICATIONS FOR THE CIRCADIAN CONTROL OF BEHAVIOURAL STATE

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Abstract—The circadian clock housed in the suprachiasmatic nucleus (SCN) controls various circadian rhythms including daily sleep–wake cycles. Using dual tract-tracing, we recently showed that the medial preoptic area (MPA), subparaventricular zone (SPVZ) and dorsomedial hypothalamic nucleus (DMH) are well positioned to relay SCN output to two key sleep-promoting nuclei, namely, the ventrolateral and median preoptic nuclei. The present study examined the possibility that these three nuclei may link the SCN with wake-regulatory neuronal groups. Biotinylated dextran-amine with or without cholera toxin B subunit was injected into selected main targets of SCN efferents; the retrograde labeling in the SCN was previously analyzed. Here, anterograde labeling was analyzed in immunohistochemically identified cholinergic, orexin/hypocretin-containing and aminergic cell groups. Tracer injections into the MPA, SPVZ and DMH resulted in moderate to dense anterograde labeling of varicose fibers in the orexin field and the tuberomammillary nucleus. The locus coeruleus, particularly the dendritic field, contained moderate anterograde labeling from the MPA and DMH. The ventral tegmental area, dorsal raphe nucleus, and laterodorsal tegmental nucleus all showed moderate anterograde labeling from the DMH. The substantia innominata showed moderate anterograde labeling from the MPA. These results suggest that the MPA, SPVZ and DMH are possible relay nuclei for indirect SCN projections not only to sleep-promoting preoptic nuclei as previously shown, but also to wake-regulatory cell groups throughout the brain. In the absence of major direct SCN projections to most of these sleep/wake-regulatory regions, indirect neuronal pathways probably play an important role in the circadian control of sleep–wake cycles and other physiological functions.

Key words: dual tract-tracing, immunohistochemistry, cholinergic, orexin/hypocretin, aminergic.

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Abbreviations: A, anterior; BDA, biotinylated dextran-amine; CTB, cholera toxin B subunit; DAB, diaminobenzidine; DMH, dorsomedial hypothalamic nucleus; HDB, horizontal limb of the diagonal band of Broca; HDC, histidine decarboxylase; ir, immunoreactive; LD, light–dark; MPA, medial preoptic area; MS-VDB, medial septum-vertical limb of the diagonal band complex; NPY, neuropeptide Y; P, posterior; PH, posterior hypothalamic area; REM, rapid eye-movement; SCN, suprachiasmatic nucleus; SPVZ, subparaventricular zone; TH, tyrosine hydroxylase; VAChT, vesicular acetylcholine transporter.

The circadian clock housed in the suprachiasmatic nucleus (SCN) of the hypothalamus governs daily rhythms of sleep–waking and other behavioral and physiological functions (Rusak and Zucker, 1979; Moore and Leak, 2001). SCN lesions in rodents abolish daily sleep–wake rhythms; interestingly, however, the amounts of sleep or wakefulness are not affected (Ibuka et al., 1977; Wurts and Edgar, 2000). These findings suggest that the SCN is not responsible for maintaining behavioral states, but influences their timing in a circadian manner. Behavioral states are, in fact, controlled by a neural system that networks across widely distributed brain regions (Steriade and McCarley, 1990; Lydic and Baghdoyan, 1999). Some of the circadian influence on the sleep/wake-regulatory system is likely to be mediated by humoral factors (LeSauter and Silver, 1998; Kramer et al., 2001; Cheng et al., 2002). However, "hard-wired" synaptic pathways certainly play a role as well (Swanson, 1987; Buijs and Kalsbeek, 2001; Pace-Schott and Hobson, 2002), and little is known about these pathways.

Following the general organizational principle of the hypothalamus (Swanson, 1987), the SCN does not project abundantly beyond the hypothalamus (Watts et al., 1987). No region in the sleep–wake regulatory system receives direct projections from the SCN, with the exception of the medial preoptic area (MPA), which receives strong direct SCN projections (Watts et al., 1987; Leak and Moore, 2001), as well as the ventrolateral preoptic nucleus (Novak and Nunez, 2000; Chou et al., 2002) and the area containing orexin (also known as hypocretin)-containing neurons in the posterior hypothalamus (Abrahamson et al., 2000), which both receive sparse direct SCN projections. Indirect pathways are certainly conceivable, however, and we have recently shown, using a dual tract-tracing method, that several known targets of direct SCN projections are well placed to relay SCN output to the ventrolateral (Deurveilher et al., 2002) and median preoptic nuclei (Deurveilher and Semba, 2003), two key structures involved in the generation of sleep (McGinty and Szymusiak, 2001; Saper et al., 2001). These potential relay nuclei are the MPA, subparaventricular zone (SPVZ), and dorsomedial hypothalamic nucleus (DMH). The synaptic connections that form the relays in each of these nuclei remain to be investigated. However, in the absence of major direct projections to the sleep–wake regulatory system, these potential indirect pathways may play an important role in circadian regulation of sleep.

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In the present study, using the same dual tract-tracing approach, we investigated whether the previously identified potential relay nuclei to the ventrolateral and median preoptic nuclei may also relay SCN output to major arousal/wake-promoting neuronal groups, including cholinergic neurons in the basal forebrain and mesopontine tegmentum; orexin-containing neurons in the posterior hypothalamus; and aminergic neurons in the tuberomammillary nucleus, ventral tegmental area, substantia nigra pars compacta, dorsal raphe nucleus and locus coeruleus. All of these neuronal populations are thought to play important roles in the control of cortical and behavioral arousal, and some are also implicated in the mechanisms of rapid eye-movement (REM) sleep (see above). The density of anterogradely labeled varicose fibers was analyzed in each of these wake-promoting structures. Preliminary results have been reported in abstract form (Deurveilher et al., 2001a,b,c).

EXPERIMENTAL PROCEDURES

As a follow up to two previous studies from our laboratory (Deurveilher et al., 2002; Deurveilher and Semba, 2003), we performed additional histological processing of brain sections from the animals used in these previous studies. The details of animals used, tracer injections, perfusion, and immunohistochemical procedures were described in Deurveilher et al. (2002). Briefly, adult male Wistar rats were injected, under anesthesia (60 mg/kg ketamine, 3.2 mg/kg xylazine, and 0.6 mg/kg acepromazine, i.m.), with a mixture of biotinylated dextran-amine (BDA; 10% in saline) and cholera toxin subunit B (CTB; 0.25 or 0.5%), or BDA alone (10%), into the MPA (n=10), SPVZ (n=11), DMH (n=8), or posterior hypothalamic area (PH; n=7). Postinjection survival periods ranged from 8 to 11 days (mostly either 9 or 11 days) after co-injections of BDA and CTB, and from 4 to 10 days (mostly either 5 or 6 days) after single injections of BDA. No obvious differences were noted among the cases with different survival periods. All experiments were performed in compliance with the guidelines established by the Canadian Council on Animal Care and by the Dalhousie University Committee on Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

The injection sites and the retrograde labeling in the SCN in relation to the vasopressin-rich shell, and neuropeptide Y (NPY)-rich core regions have been reported previously in detail (Deurveilher et al., 2002). For convenience, some of these results are included in the present study, with reference to the original source.

To examine anterograde BDA labeling in the wake-regulatory structures, a series of 40-μm coronal sections from each case was reacted with a standard avidin–biotin–horseradish peroxidase complex method, using diaminobenzidine (DAB) and nickel ammonium sulfate to produce a black–purple reaction product (Deurveilher et al., 2002). These sections were then reacted immunohistochemically with a peroxidase anti-peroxidase method and DAB without nickel to produce a brown reaction product. The following primary antibodies were used: rabbit anti-preprohypocretin antibody (1:1000; Chemicon International, Temecula, CA, USA) to visualize orexin/hypocretin-containing neurons in the posterior hypothalamus; rabbit anti-histidine decarboxylase [HDC; 1:1600; kindly provided by Dr. N. Inagaki, Osaka University, Osaka, Japan] to visualize histaminergic neurons in the tuberomammillary nucleus; rabbit anti-tyrosine hydroxylase (TH; 1:6000; Pel-Freez, Rogers, AR, USA) to visualize dopaminergic neurons in the ventral tegmental area and substantia nigra pars compacta, and noradrenergic neurons in the locus coeruleus; rabbit anti-serotonin (1:20,000; Diasorin, Stillwater, MN, USA) to visualize serotoninergic neurons in the dorsal raphe nucleus; and rat anti-choline acetyltransferase (1:50; Boehringer, Laval, Quebec, Canada) or rabbit anti-vesicular acetylcholine transporter (VAChT; 1:15,000; Chemicon International) to visualize cholinergic neurons in the basal forebrain [the medial septum–vertical limb of the diagonal band complex (MS-VDB), horizontal limb of the diagonal band of Broca (HDB), magnocellular preoptic nucleus, and substantia innominata] and in the mesopontine tegmentum (the pedunculopontine and laterodorsal tegmental nuclei).

In addition to the sections processed for double labeling as above, a second series of sections was reacted to visualize BDA singly for quantitative analysis of anterograde labeling in the areas mentioned above.

Data analysis

The density of anterograde labeling in the wake-related cell groups was assessed using either two or three sections for each cell group at the following anterior (A) to posterior (P) levels (mm to bregma) according to the brain atlas by Paxinos and Watson (1998): MS-VDB (A 0.7 and A 0.2); HDB (A 0.1 and P 0.3); magnocellular preoptic nucleus (P 0.3 and P 0.8); substantia innominata (P 1.3 and P 1.6); orexin cell group (P 2.8 and P 3.14); tuberomammillary nucleus (dorsal division: P 3.8 and P 4.16; ventral division: P 4.16 and P 4.3); ventral tegmental area (P 5.3 and P 5.6) and substantia nigra pars compacta (P 5.8 and P 6.04); dorsal raphe nucleus (P 7.8, P 8.2 and P 8.8); pedunculopontine (P 7.64 and P 8.3) and laterodorsal tegmental nuclei (P 8.8 and P 9.16); and locus coeruleus (dendritic region: P 9.16 and P 9.68; cell body region: P 9.68 and P 10.64). Note that the dendritic region of the locus coeruleus, which was delineated by a dense plexus of TH-immunoreactive (-ir) dendrites, overlaps with Barrington's nucleus at P 9.16 (Rizvi et al., 1994; Steininger et al., 2001). In addition to the above regions, the density of anterograde labeling was evaluated in regions of the mesencephalic reticular formation (P 6.72 and P 7.04), involved in arousal, and the pontine reticular formation (P 8.8 and P 9.3), involved in REM sleep generation (Steriade and McCarley, 1990).

As in our previous studies (Deurveilher et al., 2002; Deurveilher and Semba, 2003), anterograde labeling was analyzed using sections stained only for BDA, because the additional immunostaining often interfered with the evaluation of BDA-labeled elements. Transmitter-specific cell groups in BDA-labeled sections were identified by comparing with adjacent sections double-stained for BDA and a relevant transmitter marker. The density of anterograde labeling was assessed within a region that closely outlined the entire cluster of immunolabeled cell bodies and proximal dendrites for all the cell groups except the locus coeruleus, basal forebrain regions, orexin field, and mesencephalic and pontine reticular formation. For the locus coeruleus, dendritic and cell body regions were analyzed separately as the density of anterograde labeling appeared denser in the dendritic, than the cell body, region. For the three basal forebrain regions, the orexin field, and the mesencephalic and pontine reticular formation, density analysis boxes were used. For the MS-VDB, a box (0.4 mm in width×1.5 mm in height at A 0.7; 0.4 mm×2.5 mm at A 0.2) was placed so that its medial border was aligned with the midline of the brain section. For the HDB, a box (1 mm×0.4 mm) was positioned tangential to the ventral surface of the brain. For the substantia innominata, a box (1 mm×0.6 mm) was placed subjacent to the globus pallidus; no box was required for the magnocellular preoptic nucleus, which can be delineated easily in sections single-stained for BDA on the basis of a faint non-specific staining of its magnocellular neurons. For the orexin cell group, a box (1 mm×0.5 mm) was placed so that the midpoint of its ventral segment was centered at the fornix, thus encompassing the perifornical area where orexin neurons are most concentrated; the analysis box was then divided into two halves to analyze the medial and lateral portions separately, as anterograde labeling