Pergamon

www.neuroscience-ibro.com

PII: S0306-4522(02)00017-9

Neuroscience Vol. 111, No. 2, pp. 379–387, 2002

0306-4522/02 $22.00+0.00

ANATOMICAL SUBSTRATES OF OREXIN–DOPAMINE INTERACTIONS: LATERAL HYPOTHALAMIC PROJECTIONS TO THE VENTRAL TEGMENTAL AREA

J. FADEL* and A. Y. DEUTCH

Departments of Psychiatry and Pharmacology and Center for Molecular Neuroscience, Vanderbilt University Medical Center, PHV Suite 313, 1601 23rd Avenue South, Nashville, TN 37212, USA

Abstract—Dopaminergic projections to the forebrain arising from the mesencephalic ventral tegmentum modulate information processing in cortical and limbic sites. The lateral hypothalamus is crucial for the coordination of behavioral responses to interoceptive cues. The presence of a hypothalamic input to the ventral tegmental area has been known for some time, but the organization of this pathway has received little attention. Among the neuropeptides found in the hypothalamus are the orexins, which are selectively expressed in the lateral hypothalamus and adjacent perifornical area and are critically involved in homeostatic regulatory processes, including arousal and feeding. We examined the anatomical relationships between orexin and dopamine neurons in rats, with particular attention to characterizing the lateral hypothalamic projection to midbrain dopamine neurons.

Iontophoretic deposits of the retrograde tracer FluoroGold into the ventral tegmental area revealed a large number of retrogradely-labeled cells that formed a band extending from the medial perifornical area arching dorsally over the fornix and then ventrolaterally into the lateral hypothalamus; approximately 20% of these cells expressed orexin A-like immunoreactivity. Moreover, axons that were anterogradely labeled from the lateral hypothalamus were seen throughout the ventral tegmental area, and were often in close proximity to the dendrites and somata of dopamine neurons. Dopamine and orexin fibers were found to codistribute in the medial prefrontal cortex; orexin fibers were present in lower density in the medial shell of the nucleus accumbens, and the central and posterior basolateral nuclei of the amygdala.

We conclude that the lateral hypothalamic/perifornical projection represents an anatomical substrate by which interoceptive-related signals may influence forebrain dopamine function.

Key words: amygdala, hypocretin, lateral hypothalamus, nucleus accumbens, prefrontal cortex, striatum.

The A10 dopamine (DA) neurons in the ventral tegmental area (VTA) project to cortical and limbic forebrain regions and receive afferents from a large number of sites. These afferents critically regulate the activity of VTA DA neurons (Deutch and Roth, 1990; Kalivas, 1993). The most comprehensive study of VTA afferents was published a generation ago (Phillipson, 1979). Although the broad scope of Phillipson's study precluded a detailed description of each source of afferents, it suggested that the single largest source of inputs was derived from the hypothalamus, noting a large input originating in the lateral hypothalamus (LH) as well as projections from other hypothalamic nuclei.

The orexins, also known as hypocretins, are two alternatively-spliced peptides that are expressed solely in neurons of the LH and adjacent perifornical area (PFA) (de Lecea et al., 1998; Sakurai et al., 1998). The orexins appear to be critically involved in both arousal and feeding, two behavioral states long linked to corticolimbic DA function (Terry et al., 1995; Robbins et al., 1998). The two orexin peptides (A and B) act at distinct G protein-coupled receptors known as the orexin 1 (OX1R) and orexin 2 (OX2R) sites (Sakurai et al., 1998). Activation of these receptors by central administration of orexin A (which binds with high affinity to both receptors) or B (which binds with high affinity only to OX2R) elicits feeding as well as locomotor activity and arousal (Hagan et al., 1999), and OX2R is present in high density in the VTA (Lu et al., 2000). These data suggest a functional interplay between orexin and corticolimbic DA neurons.

We therefore examined the organization of LH projections to the VTA using contemporary tract-tracing methods, with a particular emphasis on delineating the contribution of orexin-containing neurons to the VTA. Since orexin may also regulate DA through interactions at the mesocorticolimbic terminal fields, we examined the relationship between orexin and dopaminergic axons in the forebrain, including the prefrontal cortex (PFC), nucleus accumbens, and amygdala.

*Corresponding author. Tel.: +1-615-327-7092; fax: +1-615-322-1901.

E-mail address: jim.fadel@mcmail.vanderbilt.edu (J. Fadel).

Abbreviations: BDA, biotinylated dextran amine; DA, dopamine; FG, FluoroGold; LH, lateral hypothalamus; LI, like immunoreactivity; OX1R/2R, orexin- 1/2 receptors; PFA, perifornical area; PFC, prefrontal cortex; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

EXPERIMENTAL PROCEDURES

Animals and surgery

Adult male Sprague–Dawley rats (Harlan; Birmingham, AL, USA) were kept on a 12 h light–dark cycle and allowed free access to food and water. Experimental procedures were conducted in accord with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Rats were deeply anesthetized with pentobarbital and glass pipettes (18–24 μm outer diameter) filled with a 3% solution of the retrograde tracer FluoroGold (FG; Fluorochrome, Inc., Englewood, CO, USA) in 0.1 M cacodylic acid were stereotaxically guided to the VTA. FG was deposited iontophoretically using pulsed +2.5 μA current (7 s on/off) for 5–10 min. In other animals, a 10% solution of the anterograde tracer biotinylated dextran amine (BDA; 10 000 MW; Molecular Probes, Eugene, OR, USA) was deposited into LH sites using +5.0 μA pulsed for 10–15 min.

Immunohistochemistry

Seven to 14 days post-operatively, animals were deeply anesthetized and perfused transcardially with 4% paraformaldehyde; the brains were then removed and cryoprotected in 30% sucrose in 0.1 M phosphate buffer. Coronal sections (40 μm) were cut through the neuraxis and incubated for 24 h at room temperature in a mixture of guinea-pig anti-FG (1:3000; a gift from Dr. Lothar Jennes, University of Kentucky) and rabbit anti-orexin A (1:4000; Oncogene Research Products, Cambridge, MA, USA). Sections were then washed extensively and incubated for 2 h in Cy2-conjugated donkey anti-rabbit (1:1250) and Cy3-conjugated donkey anti-guinea-pig (1:1500) secondary antibodies (Jackson Immunoresearch Laboratories; West Grove, PA, USA). Sections were mounted and examined using epifluorescent and confocal fluorescent microscopy.

Similar procedures were used to visualize the relationship between orexin-like immunoreactivity (-LI) axons and dopaminergic neurons, using the orexin A antibody and a mouse monoclonal tyrosine hydroxylase (TH) antibody (1:3000; Diasorin, Inc., Stillwater, MN, USA) to stain DA neurons. Adjacent or near-adjacent sets of sections were processed to reveal orexin- and TH-LI neurons using previously described dual immunoperoxidase and immunofluorescent methods (Deutch et al., 1991).

Anterogradely-labeled BDA fibers were visualized by incubating overnight in horseradish peroxidase-conjugated streptavidin (1:1600; Jackson Immunoresearch) and then developed in a nickel/cobalt-intensified diaminobenzidine solution. Adjacent sets of sections were processed to reveal both BDA-labeled fibers and TH-LI neurons to show the relationship between LH-derived afferents and DA neurons in the VTA.

Line drawings were made from video images captured using NIH Image 1.6 software (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image) and labeled cells or fibers plotted on outlines of the sections.

RESULTS

Retrograde labeling in the LH/PFA

FG injections of the VTA resulted in retrograde labeling that extended from the preoptic regions to the most caudal portions of the LH. The retrogradely-labeled cells in the LH and surrounding nuclei were organized as a dense band of neurons, which extended from the lateral border of the paraventricular hypothalamic nucleus and arched dorsally over the fornix before curving ventrolat-

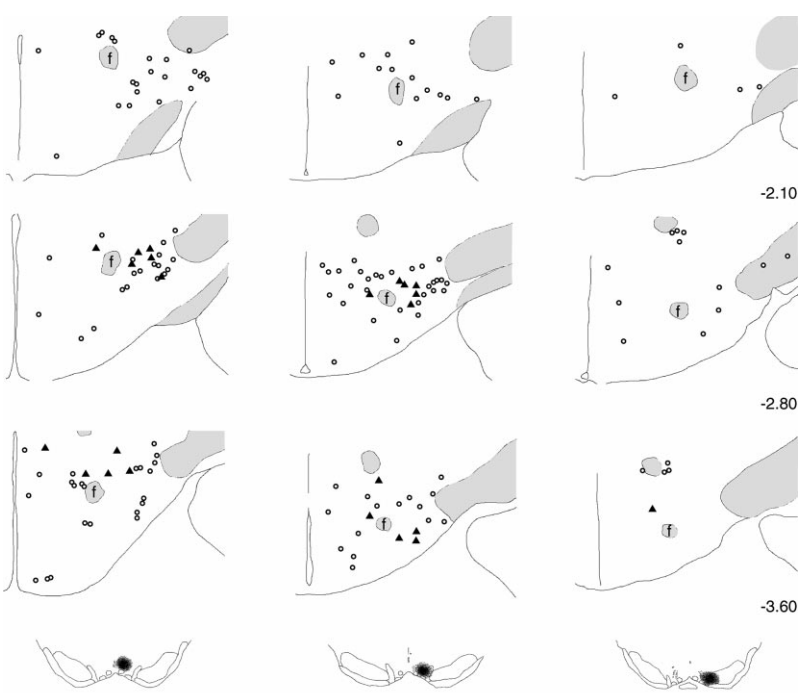

Fig. 1. The distribution of hypothalamic neurons that are retrogradely labeled from the VTA and express orexin-LI. The location of retrogradely-labeled cells at three different anteroposterior levels is shown; numbers refer to the approximate level in Paxinos and Watson (1986). Orexin- (triangles) and non-orexin- (open circles) containing retrogradely-labeled neurons are charted. The FG injection sites for the three cases shown (bottom) primarily involved the parabrachial (left column) or paranigral (middle column) nuclei of the VTA, or the medial substantia nigra (right column); these FG deposits were all placed at anteroposterior: −5.4–5.5 (Paxinos and Watson, 1986), but different deposits at other anteroposterior levels did not result in significantly different hypothalamic labeling patterns. f, fornix.

erally into the juxtacapsular LH (see Fig. 1). Retrograde labeling was predominantly ipsilateral, but a small number of retrogradely-labeled cells was also observed in the contralateral LH/PFA.

Very few retrogradely-labeled cells were seen in the LH/PFA after FG deposits involving the medial substantia nigra (Fig. 1). In contrast, FG deposits centered primarily in different parts of the VTA, including the interfascicular, parabrachial, and paranigral regions (see Phillipson, 1979) resulted in extensive LH/PFA labeling (see Fig. 1). However, we did not observe a clear topographical organization of LH projections onto the VTA, with similar patterns of retrograde labeling in the LH/PFA seen after tracer deposits into different parts of the VTA. However, we did not systematically explore the A10dc region, which includes the rostral part of the dorsal raphe, in our retrograde tracer studies.

Dual retrograde-tracing immunohistochemistry revealed that a substantial number of LH/PFA retrogradely-labeled cells exhibited orexin-LI (see Figs. 1 and 3); approximately 20% of the retrogradely-labeled neurons in the LH and adjacent PFA were orexin positive. Double-labeled cells were seen in both the LH and PFA, again without any clear topography (Fig. 1). The range of FG labeling within the entire hypothalamus greatly exceeded the restricted extent of orexin expression.

Double-labeled (FG-orexin) hypothalamic neurons were interspersed among single-labeled cells (Fig. 3), and were typically medium in size and multipolar; occasional elongated bipolar orexin-LI cells were seen.

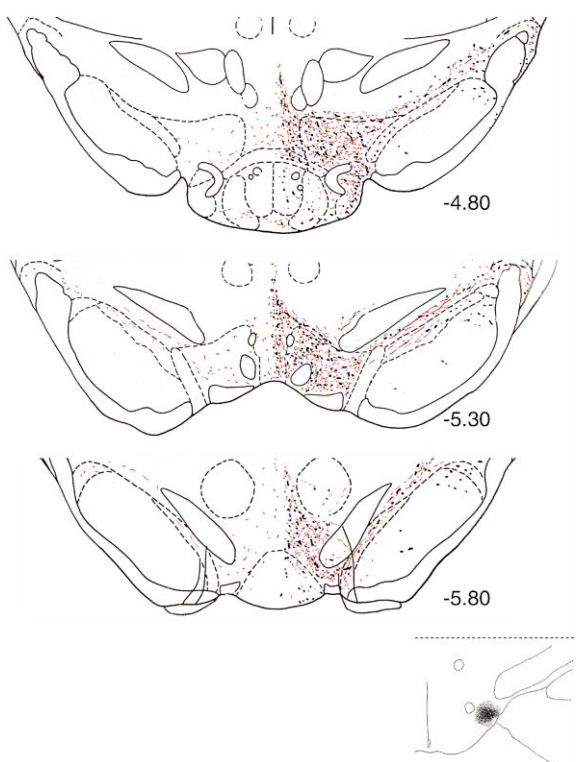

Fig. 2. A case in which the anterograde tracer BDA was injected into the LH (injection site depicted at bottom right) and the resultant pattern of anterograde labeling at different anteroposterior levels of the ventral mesencephalon; numbers refer to corresponding plates in the atlas of Paxinos and Watson (1986). The overall distribution of BDA-labeled fibers in the VTA is depicted in red, and the distribution of orexin-LI axons in the same sections is shown in black. The hypothalamic projection to the VTA is quite dense but neither anterogradely-labeled (BDA) fibers nor orexin-LI axons show a preferential labeling of any subnucleus within the VTA.

Anterograde labeling from the LH/PFA

A dense plexus of anterogradely-labeled fibers was observed throughout the VTA after BDA deposits into the LH (see Figs. 2 and 3). Anterogradely-labeled fibers from the LH entered the VTA both from a rostral medial stream of axons in the median forebrain bundle and from a lateral stream of fibers that appeared to enter the lateral substantia nigra and traverse the region immediately dorsal to the pars compacta. Virtually the entire VTA received a rich LH/PFA-derived innervation (Fig. 2). The majority of BDA-labeled fibers were relatively thin varicose axons. A group of BDA-labeled fibers coursed mediodorsally through the VTA toward the aqueduct. The density of anterogradely-labeled fibers decreased at progressively more caudal midbrain levels, suggestive of axons terminating in the midbrain rather than simply coursing toward caudal sites. BDA deposits into the LH/PFA resulted in a more extensive pattern of VTA labeling than revealed by the distribution of orexin-LI fibers, consistent with the observation that orexin-LI neurons comprise a minority of the retrogradely-labeled cells seen in the LH/PFA after FG injections of the VTA. Orexin-LI axons were seen throughout the VTA, but the density of these fibers appeared to increase in the caudal midline areas of the VTA, including the caudal extension into the dorsal raphe (the A10dc region of Hokfelt et al., 1984).

Relationship between orexin- and DA-containing neurons in the midbrain tegmentum

The VTA contained numerous orexin-LI fibers, which were distributed across the different subdivisions of the VTA in roughly comparable density, the exception being the caudal and dorsal VTA (A10dc region), in which a higher density of orexin-LI axons was seen. The orexin-LI axons in most of the rostral VTA were mostly fine caliber fibers with numerous varicosities, giving them a 'beaded necklace' appearance.

Within the VTA, orexin-LI fibers were extensively intermingled with TH-positive neurons (Fig. 3). Orexin-LI axons were frequently in close relation to the soma and dendrites of dopaminergic neurons, and occasionally appeared to be apposed to A10 DA neurons (see Fig. 3). The orexin fibers, as noted above, were more dense in the caudal VTA and the A10dc region, which extends from the caudal linear subnucleus into the dorsal raphe. In the caudal VTA and its dorsal extension into the raphe relatively large axons with large varicosities and short intervaricose segments were present, from which emanated very fine varicose axons that appeared to be apposed to DA somata and their dendrites.

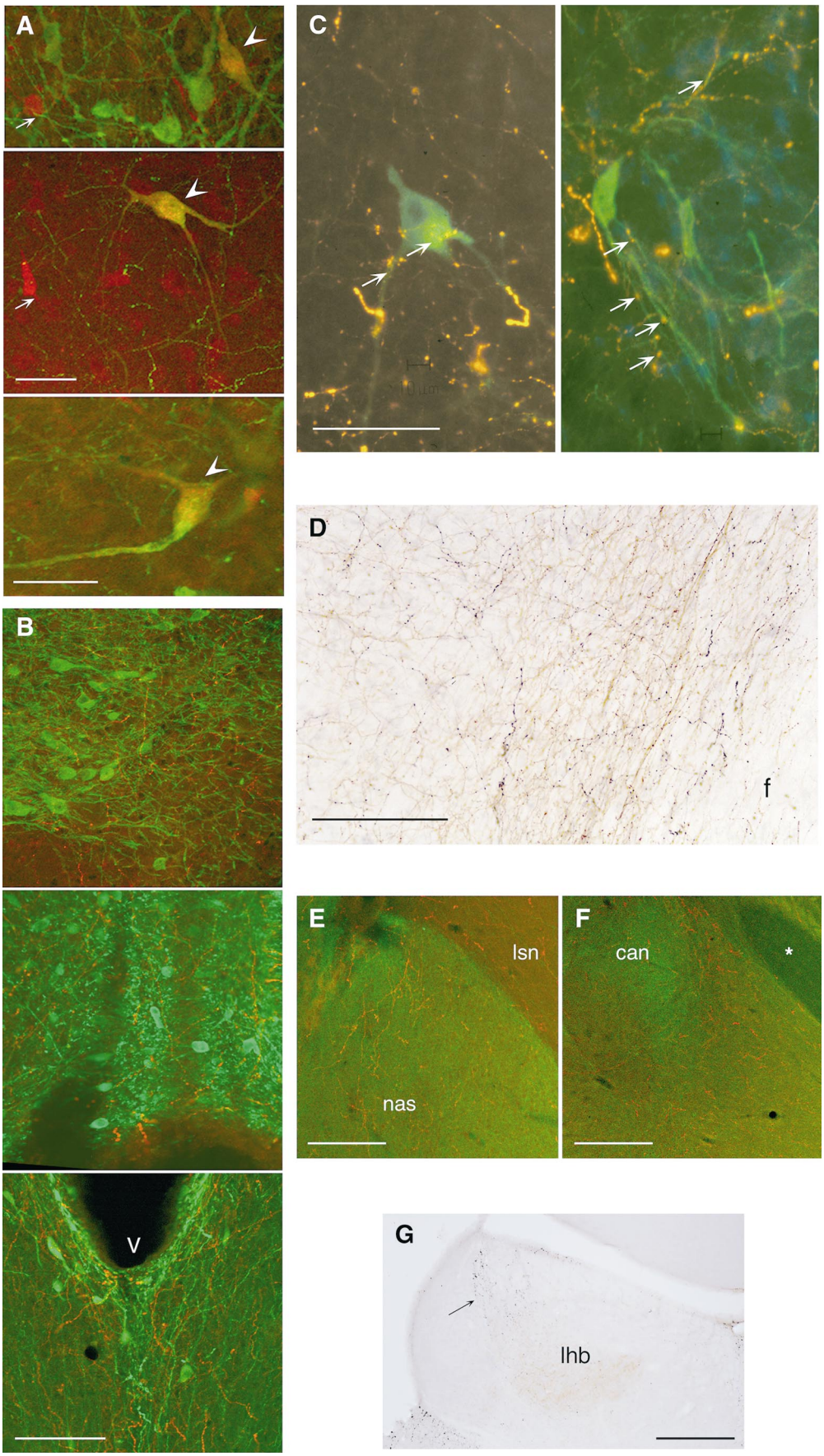

Fig. 3.

In addition to the presence of orexin-LI fibers in the VTA, orexin axons were also seen in the retrorubral field, particularly the medial aspects, where they overlapped the distribution of the medial A8 DA neurons.

Orexin and dopaminergic axons in the mesocorticolimbic terminal fields

Orexin-LI axons were associated with dopaminergic fibers in most mesocorticolimbic DA terminal fields. Within the forebrain DA terminal field areas, orexin-LI axons were most densely distributed in the medial PFC. In this region, orexin-LI fibers largely followed the distribution of DA axons. In the PFC, orexin-LI fibers were seen in the infralimbic, prelimbic, dorsal anterior cingulate, and medial precentral cortices. Orexin-LI axons ascended along the white matter of the forceps, with fibers ramifying in the deep layers, where the DA innervation is most dense (Fig. 3). The density of orexin-LI axons was greatest in the deep layers of the infralimbic and prelimbic cortices, and was less dense more dorsally in the pregenual dorsal anterior cingulate and medial precentral cortices. Orexin-LI axons traversed the cortical lamina in route to the superficial layers of the PFC, where they coursed with the noradrenergic TH-LI axons that run parallel to the pial surface. Only a few scattered orexin-LI axons were seen lateral to the medial wall of the PFC, with the exception of the suprarhinal cortex, where a moderately dense plexus of orexin-LI axons was in register with the dopaminergic innervation. Orexin-LI axons were also found in the supragenual (dorsal anterior cingulate) cortex, overlapping the DA innervation of this region.

The density of orexin-LI axons in the striatal complex was much lower than observed in the PFC. Orexin-LI fibers were scattered throughout ventral striatum, including the nucleus accumbens and olfactory tubercle. In the nucleus accumbens orexin-LI axons were most dense in the dorsomedial shell (septal pole region; see Fig. 3); fewer orexin-LI fibers were seen in rest of the medial shell, and still fewer in the lateral shell and core. Scattered orexin-LI axons were also seen in the olfactory tubercle. Few orexin-LI axons were in the precommissural dorsal striatum, where they were confined to the medial periventricular zone. Orexin-LI axons were more dense in the medial aspects of the post-commissural striatum than in the precommissural regions.

A relatively dense orexin-LI innervation of the lateral septal nucleus was observed. However, orexin-LI fibers were primarily observed in the ventrolateral and dorsal aspects of the septum, and along the ventricular border. Few orexin-LI axons were seen in the intermediate lateral septum to which most of the DA innervation is targeted.

Orexin-LI fibers were present in both the dorsal and ventral aspects of the bed nucleus of the stria terminalis, areas enriched with both dopaminergic and noradrenergic fibers. Scattered orexin-LI fibers were seen in the ventral pallidum, and were somewhat more dense in the interstitial nucleus of the anterior commissure. Scattered orexin-LI fibers were also present in the amygdalostriatal transition area.

In the body of the amygdala orexin-LI fibers were present in central nucleus, particularly the medial aspects, but mainly seemed to avoid the areas more densely invested with TH fibers. Very few orexin-LI axons were found in the lateral and anterior basolateral nuclei of the amygdala. However, orexin-LI axons formed a relatively dense plexus in the posterior-most aspects of the basolateral nucleus, where the DA innervation is more dense. Orexin-LI axons were also seen in the intercalated nuclei of the anterior amygdala, in which TH-LI axons were seen.

In the diencephalon, orexin-LI fibers were found in the medial habenula, but were sparse in the lateral habenula where the DA innervation is found (see Fig. 3). In the nearby paraventricular nucleus of the thalamus, which receives a DA innervation as well as a noradrenergic input, orexin-LI fibers densely defined the nucleus.

Finally, the distribution of orexin-LI neurons within the hypothalamus overlapped the medial forebrain bundle, embedded in which were orexin-LI perikarya. The

Fig. 3. Anatomical relationships of orexin and dopaminergic neurons. Images were obtained either digitally with laser-scanning confocal microscopy (A–B; E–F) or on 35 mm film under epifluorescent illumination (C–D; G). In both cases images were imported into Adobe Photoshop (v. 5.5) where minor contrast and brightness adjustments were made. (A) Confocal microscopic images of retrogradely-labeled (FG) neurons (red) and orexin-LI cells (green) in the LH after FG deposits into the VTA. Double-labeled cells (orange, arrowheads) are clearly seen intermingled with retrogradely-labeled but orexin-negative neurons (small arrows) and green orexin-LI neurons that are not retrogradely labeled. (B) The orexin-LI innervation (red) of the DA neurons (green) in the VTA is depicted at three different anteroposterior levels. The top part shows the rostral VTA, the middle part the VTA at level of the rostral interpeduncular nucleus where the interfascicular nucleus is seen, and the bottom part depicts the DA neurons of the A10dc region in the dorsal raphe. Orexin-LI axons are present in all parts of the VTA. (C) Small arrows point to areas where orexin-LI axons (red) are apposed to the somata and dendrites of VTA DA cells (green). (D–G) The relationship of orexin- and TH-LI axons in several areas is shown in the remaining panels. In the medial PFC (D), the brightfield photomicrograph shows a dense plexus of orexin-LI axons (black fibers) mixed with the DA innervation as revealed by TH-LI (brown fibers). (E) The orexin-LI innervation (red) of the dorsomedial accumbens (marked by the green dopaminergic fibers). (F) In the amygdala the DA innervation (green) of the central nucleus to some degree overlaps the orexin (red) fibers, but in the medial aspects rather than where the DA innervation is most dense. (G) Brightfield photomicrograph of the DA input to the lateral habenula (brown fibers), which does not overlap significantly with the orexin-LI innervation (black fibers, arrow). A dense orexin-LI innervation of the dorsal part of the thalamic paraventricular nucleus, ventrolateral to the habenula, can be seen. Abbreviations: can, central amygdaloid nucleus; f, forceps major; lhb, lateral habenula; lsn, lateral septal nucleus; V, ventricle; *, stria terminalis; nas, nucleus accumbens. Scale bars = 30 μm (A, C); 250 μm (E, F); 500 μm (B, D, G).

orexin-LI somata were not intermingled with the dopaminergic neurons on the zona incerta, nor were they in the dendritic fields of these neurons.

DISCUSSION

Orexin cells contribute to but are not the sole source of a dense projection from the LH to the VTA. The anatomical distributions of orexin and DA suggest that orexin regulates DA neurons via projections from the LH to the VTA and perhaps at the level of the mesocorticolimbic terminal fields.

Anatomical characteristics of the LH-VTA projection

As previously reported, orexin-LI cells were essentially restricted to the hypothalamic region between the lateral border of the paraventricular nucleus and the juxtacapsular LH (de Lecea et al., 1998; Peyron et al., 1998; Date et al., 1999). This area is also the region in which we observed the greatest number of retrogradely-labeled cells after FG deposits of the VTA. We were unable to differentiate reliably between orexin- and non-orexin-containing cells that project to the VTA on the basis of the size or morphology of FG-labeled cells.

We used iontophoretic FG deposits to label retrogradely the hypothalamus. This approach minimizes, but does not completely eliminate, uptake of the tracer by fibers of passage (Schmued and Fallon, 1986; Dado et al., 1990). We observed little or no retrograde labeling in the medial habenula after FG deposits that abutted the lateral aspect of the fasciculus retroflexus. It is therefore unlikely that FG uptake by fibers of passage contributed significantly to our findings. Moreover, the dense network of anterogradely-labeled fibers in the VTA after BDA deposits of the LH/PFA confirmed the hypothalamic projection to the VTA, consistent with the original report by Phillipson (1979).

The density of BDA-labeled fibers in the midbrain tegmentum decreased from rostral to more caudal aspects of the VTA. In contrast, the apparent density of terminal plexuses of orexin-LI axons was higher in the more caudal VTA, including the A10dc region, than the rostral part of the VTA. This suggests that the VTA is a major target of both non-orexin- and orexin-containing neurons of the LH/PFA, and that the orexin neurons that contribute to this descending hypothalamic projection innervate brainstem sites in addition to the VTA. This suggestion is consistent with the orexin innervation of the dorsal raphe and locus coeruleus (Peyron et al., 1998; Horvath et al., 1999b; Brown et al., 2001). Nonetheless, given the large hypothalamic projection onto the VTA, orexin neurons provide a major input to the VTA. The zone of BDA-labeled fibers completely encompassed the area of TH-LI cell bodies and orexin-LI fibers within the VTA.

Only about 20% of the retrogradely-labeled cells within the area of the LH/PFA that contains orexin neurons were immunoreactive for the peptide. The phenotype of non-orexin LH/PFA neurons that project to the VTA is unclear. Among the likely candidates are neurons expressing melanin-concentrating hormone (Broberger et al., 1998), the cocaine- and amphetamine-regulated transcript (Broberger, 1999), and galanin. It is also possible that amino acid-containing neurons and a few neurotensin neurons in the LH/PFA (Zahm et al., 2001) may project to the VTA. We have previously observed a very small number of leumorphin and orphanin FQ-LI neurons in the LH/PFA that were retrogradely labeled from the VTA (Fadel and Deutch, 1999). Studies are in progress to define the phenotype and topographic characteristics of projection neurons in the LH and other hypothalamic areas that innervate the VTA.

Functional implications of orexin–dopamine interactions

The intricate relationship between orexin-LI fibers and the A10 DA neurons of the VTA suggests that orexin regulates midbrain DA neurons at the somatodendritic level; the precise synaptic relationship between orexin axons and midbrain DA cells awaits electron microscopy studies. In addition to the orexin projection to the VTA, orexin and DA may interact at the level of the mesocorticolimbic terminal fields. Orexin-LI axons were found in most forebrain dopaminergic terminal fields, with the exception of the lateral septum and lateral habenula, in which only rare scattered fibers were seen. Orexin-LI axons typically avoid dorsal striatal areas, which receive most of their DA innervation from the substantia nigra rather than VTA. An exception to the very sparse orexin innervation of the dorsal striatum was the medial postcommissural striatum, which receives afferents from A8 (retrorubral field) and A10 (VTA) dopaminergic neurons (Deutch et al., 1991). Thus, orexin neurons project to A10 and medial A8 regions and to the forebrain areas that receive dopaminergic projections from these midbrain sites. Orexin neurons do not provide a significant innervation of the nigral A9 neurons or their major forebrain target, the dorsal striatum.

We used TH as a marker of the dopaminergic innervation. Since most of the mesocorticolimbic terminal fields receive rich dopaminergic but weak or absent noradrenergic innervations, TH is an appropriate marker for the dopaminergic innervations of most of these regions. In some areas, such as the PFC, both DA and norepinephrine inputs are present, but the laminar segregation of these innervations is distinct and thus one can distinguish between the two catecholamines (Lindvall et al., 1978; Lewis et al., 1979). Moreover, TH antibodies have been shown to preferentially label dopaminergic but not noradrenergic axons in the PFC (Lewis et al., 1987; Noack and Lewis, 1989; Sesack et al., 1995). Similarly, in most the amygdala, including the central nucleus, TH preferentially stains dopaminergic rather than noradrenergic fibers (Asan, 1993), consistent with fluorescent histochemical studies that indicate that the DA innervation of the central nucleus is much more dense than the noradrenergic input (Fallon et al., 1978). Studies on the distribution of DA β-hydroxylase- and TH-LI axons in the habenula also reveal that the

medial part of the lateral habenula, where we see orexin-LI fibers codistributing with TH-LI axons, predominantly receives a DA not noradrenergic input (Swanson and Hartman, 1975; Hokfelt et al., 1976). In some areas, however, such as the basal forebrain and the bed nucleus of the stria terminalis, there is a large noradrenergic component which is coextensive with and more dense than the dopaminergic innervation (Zaborszky and Cullinan, 1996). However, in most of the mesotelencephalic terminal fields of the A10 DA neurons, TH serves as a very good marker of DA axons.

Expression of the OX2R mRNA is abundant in the VTA (Lu et al., 2000), offering a means whereby orexin-containing afferents to the VTA might drive DA neurons. Although it remains to be determined if OX2R is expressed by A10 DA neurons, intraventricular orexin administration increases locomotor activity, an effect that is blocked by D2 receptor antagonists (Nakamura et al., 2000). Since locomotor activity is subserved by the accumbal DA innervation, it is possible that orexin regulates the activity of mesoaccumbens neurons by activating these cells at the midbrain level. Of the mesocorticolimbic DA terminal fields, the PFC has the most dense orexin innervation, which is largely in register with the DA input; the functional regulation of the mesoprefrontal cortical dopaminergic system remains to be demonstrated.

The LH, VTA, and orexin in arousal

Orexin has been the object of intense interest because of its involvement in arousal and narcolepsy (Hagan et al., 1999; Lin et al., 1999; Kilduff and Peyron, 2000; Thannickal et al., 2000; van den Pol, 2000). Mesocorticolimbic DA projections are critically involved in mediating the locomotor activation associated with arousal (Robbins et al., 1998). Our anatomical data indicate that the orexin projection to the VTA may integrate functions of the two regions. Orexin cells express Fos, a marker of neurons that are metabolically activated, during the awake period of the normal light–dark cycle or in response to the wakefulness-promoting drugs modafinil and methamphetamine (Scammell et al., 2000; Estabrooke et al., 2001); both modafinil and amphetamine increase striatal extracellular DA levels (Wisor et al., 2001). While these data suggest that orexin regulates DA neurons, the degree to which this regulation is direct or occurs indirectly through non-DA neurons of the VTA, or both, remains unclear and will require ultrastructural studies to clarify.

Orexin-containing LH projections to the VTA and feeding behavior

The LH has classically been considered a 'feeding center' (Bernardis and Bellinger, 1996). Mesolimbic DA function has also been related to feeding (Stellar et al., 1983; Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1999). The orexins functionally antagonize the food intake-suppressing effects of leptin (Horvath et al., 1999a; Shiraishi et al., 2000), and the hyperphagia and obese phenotype characteristic of the leptin knockout mouse depends upon the presence of DA (Szczypka et al., 2000). It is possible that hypothalamic neurons, including orexin-containing cells, regulate mesolimbic DA neurons and thereby influence appetitive behavior. Alternatively, descending accumbal projections to the LH may be critical to feeding behavior (Stratford and Kelley, 1999).

Orexin and brainstem monoaminergic neurons

We focused our attention on the anatomical relationships between orexin and DA, including the hypothalamic projection to the VTA. However, orexin-containing neurons project to brainstem serotonergic and noradrenergic as well as dopaminergic cells (Peyron et al., 1998; Horvath et al., 1999b; Brown et al., 2001). In the dorsal raphe we found that orexin fibers were often apposed to dopaminergic neurons; the DA neurons embedded in the raphe project to the PFC (Yoshida et al., 1989). Ultrastructural studies will be required to determine the degree to which orexin axons synapse with serotonergic and dopaminergic neurons in the dorsal raphe. In view of the orexin projections to brainstem regions in which DA, norepinephrine, and serotonin neurons are found, it seems likely that orexin influences arousal by the coordinate regulation of all three types of monoaminergic neurons.

Summary

We have demonstrated that the lateral hypothalamic region provides a dense innervation of the VTA that includes an orexin-containing component. The projection is essentially limited to the VTA and does not involve the substantia nigra. Orexin and DA axons are also found in close proximity in most mesocorticolimbic DA terminal fields, but not in the dorsal striatum, which receives a DA input from the substantia nigra. The apposition of BDA- or orexin-labeled axons and VTA DA neurons suggests that the LH projection to the midbrain may be critical for a number of behavioral states, ranging from appetitive behavior to arousal. Since these behaviors require the integration of interoceptive cues with activation of the forebrain via the reticular core, and because the LH is a conduit for information from corticolimbic sites (such as the PFC, nucleus accumbens, and amygdala) to brainstem areas, the LH projection to the VTA is likely to play a pivotal role in these behaviors. These observations suggest that hypothalamic orexin neurons are a locus wherein humoral and interoceptive cues gain access to and influence over mesocorticolimbic DA neurons.

Acknowledgements—We appreciate the assistance of Tamara Altman in the preparation of figures, and thank Dr. Lothar Jennes for his generous gift of the guinea-pig anti-FluoroGold antibody. This work was supported in part by a NARSAD Young Investigator Award (J.F.), NIH grants MH 45124 and MH 57995 (A.Y.D.), and the National Parkinson Foundation Center of Excellence at Vanderbilt University.

REFERENCES

Asan, E., 1993. Comparative single and double immunolabelling with antisera against catecholamine biosynthetic enzymes: criteria for the identification of dopaminergic, noradrenergic and adrenergic structures in selected rat brain areas. Histochemistry 99, 427–442.

Bernardis, L.L., Bellinger, L.L., 1996. The lateral hypothalamic area revisited: ingestive behavior. Neurosci. Biobehav. Rev. 20, 189–287.

Broberger, C., 1999. Hypothalamic cocaine- and amphetamine-regulated transcript (CART) neurons: histochemical relationship to thyrotropin-releasing hormone, melanin-concentrating hormone, orexin/hypocretin and neuropeptide Y. Brain Res. 848, 101–113.

Broberger, C., De Lecea, L., Sutcliffe, J.G., Hokfelt, T., 1998. Hypocretin/orexin- and melanin-concentrating hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: relationship to the neuropeptide Y and agouti gene-related protein systems. J. Comp. Neurol. 402, 460–474.

Brown, R.E., Sergeeva, O., Eriksson, K.S., Haas, H.L., 2001. Orexin A excites serotonergic neurons in the dorsal raphe nucleus of the rat. Neuropharmacology 40, 457–459.

Dado, R.J., Burstein, R., Cliffer, K.D., Giesler, G.J., 1990. Evidence that Fluoro-Gold can be transported avidly through fibers of passage. Brain Res. 533, 329–333.

Date, Y., Ueta, Y., Yamashita, H., Yamaguchi, H., Matsukura, S., Kangawa, K., Sakurai, T., Yanagisawa, M., Nakazato, M., 1999. Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. Proc. Natl. Acad. Sci. USA 96, 748–753.

de Lecea, L., Kilduff, T.S., Peyron, C., Gao, X., Foye, P.E., Danielson, P.E., Fukuhara, C., Battenberg, E.L., Gautvik, V.T., Bartlett, F.S., Frankel, W.N., van den Pol, A.N., Bloom, F.E., Gautvik, K.M., Sutcliffe, J.G., 1998. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc. Natl. Acad. Sci. USA 95, 322–327.

Deutch, A.Y., Lee, M.C., Gillham, M.H., Cameron, D.A., Goldstein, M., Iadarola, M.J., 1991. Stress selectively increases fos protein in dopamine neurons innervating the prefrontal cortex. Cereb. Cortex 1, 273–292.

Deutch, A.Y., Roth, R.H., 1990. The determinants of stress-induced activation of the prefrontal cortical dopamine system. Prog. Brain Res. 85, 367–402.

Estabrooke, I.V., McCarthy, M.T., Ko, E., Chou, T.C., Chemelli, R.M., Yanagisawa, M., Saper, C.B., Scammell, T.E., 2001. Fos expression in orexin neurons varies with behavioral state. J. Neurosci. 21, 1656–1662.

Fadel, J., Deutch, A.Y., 1999. Topographic and phenotypic organization of hypothalamic inputs to the ventral tegmental area. Soc. Neurosci. Abstr. 25, 1926.

Fallon, J.H., Koziell, D.A., Moore, R.Y., 1978. Catecholamine innervation of the basal forebrain. II. Amygdala, suprarhinal cortex and entorhinal cortex. J. Comp. Neurol. 180, 509–532.

Hagan, J.J., Leslie, R.A., Patel, S., Evans, M.L., Wattam, T.A., Holmes, S., Benham, C.D., Taylor, S.G., Routledge, C., Hemmati, P., Munton, R.P., Ashmeade, T.E., Shah, A.S., Hatcher, J.P., Hatcher, P.D., Jones, D.N., Smith, M.I., Piper, D.C., Hunter, A.J., Porter, R.A., Upton, N., 1999. Orexin A activates locus coeruleus cell firing and increases arousal in the rat. Proc. Natl. Acad. Sci. USA 96, 10911–10916.

Hokfelt, T., Martensson, R., Bjorklund, A., Kleinau, S., Goldstein, M., 1984. Distributional maps of tyrosine-hydroxylase-immunoreactive neurons in the rat brain. In: Bjorklund, A., Hokfelt, T. (Eds.), Handbook of Chemical Neuroanatomy: Vol. 2: Classical Transmitters in the CNS, Part I. Elsevier, Amsterdam, pp. 277–379.

Hokfelt, T., Johansson, O., Fuxe, K., Goldstein, M., Park, D., 1976. Immunohistochemical studies on the localization and distribution of monoamine neuron systems in the rat brain. I. Tyrosine hydroxylase in the mes- and diencephalon. Med. Biol. 54, 427–453.

Horvath, T.L., Diano, S., van den Pol, A.N., 1999a. Synaptic interaction between hypocretin (orexin) and neuropeptide Y cells in the rodent and primate hypothalamus: a novel circuit implicated in metabolic and endocrine regulations. J. Neurosci. 19, 1072–1087.

Horvath, T.L., Peyron, C., Diano, S., Ivanov, A., Aston-Jones, G., Kilduff, T.S., van Den Pol, A.N., 1999b. Hypocretin (orexin) activation and synaptic innervation of the locus coeruleus noradrenergic system. J. Comp. Neurol. 415, 145–159.

Kalivas, P.W., 1993. Neurotransmitter regulation of dopamine neurons in the ventral tegmental area. Brain Res. Rev. 18, 75–113.

Kilduff, T.S., Peyron, C., 2000. The hypocretin/orexin ligand-receptor system: implications for sleep and sleep disorders. Trends Neurosci. 23, 359–365.

Lewis, D.A., Campbell, M.J., Foote, S.L., Goldstein, M., Morrison, J.H., 1987. The distribution of tyrosine hydroxylase-immunoreactive fibers in primate neocortex is widespread but regionally specific. J. Neurosci. 7, 279–290.

Lewis, M.S., Molliver, M.E., Morrison, J.H., Lidov, H.G., 1979. Complementarity of dopaminergic and noradrenergic innervation in anterior cingulate cortex of the rat. Brain Res. 164, 328–333.

Lin, L., Faraco, J., Li, R., Kadotani, H., Rogers, W., Lin, X., Qiu, X., de Jong, P.J., Nishino, S., Mignot, E., 1999. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell 98, 365–376.

Lindvall, O., Bjorklund, A., Divac, I., 1978. Organization of catecholamine neurons projecting to the frontal cortex in the rat. Brain Res. 142, 1–24.

Lu, X.Y., Bagnol, D., Burke, S., Akil, H., Watson, S.J., 2000. Differential distribution and regulation of OX1 and OX2 orexin/hypocretin receptor messenger RNA in the brain upon fasting. Horm. Behav. 37, 335–344.

Maldonado-Irizarry, C.S., Swanson, C.J., Kelley, A.E., 1995. Glutamate receptors in the nucleus accumbens shell control feeding behavior via the lateral hypothalamus. J. Neurosci. 15, 6779–6788.

Nakamura, T., Uramura, K., Nambu, T., Yada, T., Goto, K., Yanagisawa, M., Sakurai, T., 2000. Orexin-induced hyperlocomotion and stereotypy are mediated by the dopaminergic system. Brain Res. 873, 181–187.

Noack, H.J., Lewis, D.A., 1989. Antibodies directed against tyrosine hydroxylase differentially recognize noradrenergic axons in monkey neocortex. Brain Res. 500, 313–324.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates, 4th edn. Academic Press, London.

Peyron, C., Tighe, D.K., van den Pol, A.N., de Lecea, L., Heller, H.C., Sutcliffe, J.G., Kilduff, T.S., 1998. Neurons containing hypocretin (orexin) project to multiple neuronal systems. J. Neurosci. 18, 9996–10015.

Phillipson, O.T., 1979. Afferent projections to the ventral tegmental area of Tsai and interfascicular nucleus: a horseradish peroxidase study in the rat. J. Comp. Neurol. 187, 117–143.

Robbins, T.W., Granon, S., Muir, J.L., Durantou, F., Harrison, A., Everitt, B.J., 1998. Neural systems underlying arousal and attention. Implications for drug abuse. Ann. N. Y. Acad. Sci. 846, 222–237.

Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R.M., Tanaka, H., Williams, S.C., Richardson, J.A., Kozlowski, G.P., Wilson, S., Arch, J.R., Buckingham, R.E., Haynes, A.C., Carr, S.A., Annan, R.S., McNulty, D.E., Liu, W.S., Terrett, J.A., Elshourbagy, N.A., Bergsma, D.J., Yanagisawa, M., 1998. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92, 573–585.

Scammell, T.E., Estabrooke, I.V., McCarthy, M.T., Chemelli, R.M., Yanagisawa, M., Miller, M.S., Saper, C.B., 2000. Hypothalamic arousal regions are activated during modafinil-induced wakefulness. J. Neurosci. 20, 8620–8628.

Schmued, L.C., Fallon, J.H., 1986. Fluoro-Gold: a new fluorescent retrograde axonal tracer with numerous unique properties. Brain Res. 377, 147–154.
Sesack, S.R., Snyder, C.L., Lewis, D.A., 1995. Axon terminals immunolabeled for dopamine or tyrosine hydroxylase synapse on GABA-immunoreactive dendrites in rat and monkey cortex. J. Comp. Neurol. 363, 264–280.
Shiraishi, T., Oomura, Y., Sasaki, K., Wayner, M.J., 2000. Effects of leptin and orexin-A on food intake and feeding related hypothalamic neurons. Physiol. Behav. 71, 251–261.
Stellar, J.R., Kelley, A.E., Corbett, D., 1983. Effects of peripheral and central dopamine blockade on lateral hypothalamic self-stimulation: evidence for both reward and motor deficits. Pharmacol. Biochem. Behav. 18, 433–442.
Stratford, T.R., Kelley, A.E., 1999. Evidence of a functional relationship between the nucleus accumbens shell and lateral hypothalamus subserving the control of feeding behavior. J. Neurosci. 19, 11040–11048.
Swanson, L.W., Hartman, B.K., 1975. The central adrenergic system. An immunofluorescence study of the location of cell bodies and their efferent connections in the rat utilizing dopamine-beta-hydroxylase as a marker. J. Comp. Neurol. 163, 467–505.
Szczypka, M.S., Rainey, M.A., Palmiter, R.D., 2000. Dopamine is required for hyperphagia in Lep(ob/ob) mice. Nat. Genet. 25, 102–104.
Terry, P., Gilbert, D.B., Cooper, S.J., 1995. Dopamine receptor subtype agonists and feeding behavior. Obes. Res. Suppl. 3, S515–S523.
Thannickal, T.C., Moore, R.Y., Nienhuis, R., Ramanathan, L., Gulyani, S., Aldrich, M., Cornford, M., Siegel, J.M., 2000. Reduced number of hypocretin neurons in human narcolepsy. Neuron 27, 469–474.
van den Pol, A.N., 2000. Narcolepsy: a neurodegenerative disease of the hypocretin system? Neuron 27, 415–418.
Wisor, J.P., Nishino, S., Sora, I., Uhl, G.H., Mignot, E., Edgar, D.M., 2001. Dopaminergic role in stimulant-induced wakefulness. J. Neurosci. 21, 1787–1794.
Yoshida, M., Shirouzu, M., Tanaka, M., Semba, K., Fibiger, H.C., 1989. Dopaminergic neurons in the nucleus raphe dorsalis innervate the prefrontal cortex in the rat: a combined retrograde tracing and immunohistochemical study using anti-dopamine serum. Brain Res. 496, 373–376.
Zaborszky, L., Cullinan, W.E., 1996. Direct catecholaminergic-cholinergic interactions in the basal forebrain. I. Dopamine-beta-hydroxylase and tyrosine hydroxylase input to cholinergic neurons. J. Comp. Neurol. 374, 535–554.
Zahm, D.S., Grosu, S., Williams, E.A., Qin, S., Berod, A., 2001. Neurons of origin of the neurotensinergic plexus enmeshing the ventral tegmental area in rat: retrograde labeling and *in situ* hybridization combined. Neuroscience 104, 841–851.

(*Accepted* 21 *December* 2001)