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Immunohistochemical evidence for synaptic release of glutamate from orexin terminals in the locus coeruleus

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Abstract

Orexin (Orx or hypocretin) is critically important for maintaining wakefulness, since in its absence, narcolepsy with cataplexy occurs. In this role, Orx-containing neurons can exert their influence upon multiple targets through the brain by release of Orx but possibly also by release of other neurotransmitters. Indeed, evidence was previously presented to suggest that Orx terminals could utilize glutamate (Glu) in addition to Orx as a neurotransmitter. Using fluorescence and confocal laser scanning microscopy, we investigated whether Orx varicosities contain the presynaptic markers for synaptic release of Glu or GABA and come into contact with postsynaptic markers for excitatory synapses within the locus coeruleus of the rat brain. We found that a proportion of the Orx+ varicosities were immunostained for the vesicular transporter for Glu, VGluT2. None were immunostained for VGluT1 or VGluT3 or for the vesicular transporter for GABA, VGAT. Among the Orx+ varicosities, 4% of all and 28% of large varicosities contained VGluT2. A similar proportion of the large Orx+ varicosities contained synaptophysin, a presynaptic marker for synaptic vesicles. Orx+ varicosities also contacted elements immunostained for PSD-95, a postsynaptic marker for glutamatergic synapses. We thus conclude that synaptic release of Glu occurs from Orx terminals within the locus coeruleus and can thus be important for the engagement of noradrenergic neurons in stimulating and maintaining arousal.

Keywords

GABA; PSD-95; rat; synaptophysin; VGluT; VGAT

INTRODUCTION

Orexin (Orx or hypocretin) is a neuropeptide produced and released from neurons whose cell bodies are localized in the lateral hypothalamus (LH) and whose axons project in a diffuse manner through the brain and spinal cord (Peyron et al., 1998). Degeneration of Orx-containing neurons results in narcolepsy with cataplexy, a disorder characterized in humans by excessive sleepiness with sudden loss of muscle tone and onset of rapid eye movement (REM) sleep (Peyron et al., 2000; Thannickal et al., 2000). Although the principal symptoms

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of human narcolepsy are apparent in transgenic mice or dogs that lack only the Orx peptide or its receptors (Chemelli et al., 1999; Lin et al., 1999), a more complete array of symptoms is evident in mice in which the Orx-containing neurons degenerate through targeted genetic ablation (Hara et al., 2001). It is thus possible that other neuromodulators, such as peptides which are co-localized with Orx, might contribute to the role of Orx neurons in the brain (Crocker et al., 2005). In addition to neuropeptides, glutamate (Glu) might be released from Orx nerve terminals, since Glu immunostaining was found over small synaptic vesicles (SSVs) near the active zone along with Orx immunostaining over large dense core vesicles (LDCVs) deeper within the terminals in electron microscopic studies (Horvath et al., 1999; Torrealba et al., 2003). In addition, Orx terminals formed synaptic specializations, which were in the majority of the asymmetric type, typical of excitatory synapses, with a small number of the symmetric type, typical of inhibitory synapses (Horvath et al., 1999; Torrealba et al., 2003; Balcita-Pedicino and Sesack, 2007).

To establish whether Orx neurons utilize Glu as a neurotransmitter, we examined dual fluorescent immunohistochemical staining for Orx and specific pre- or postsynaptic markers for glutamatergic synapses in the rat brain. We investigated such staining in noradrenergic neurons of the locus coeruleus (LC), which are densely innervated and potentially excited by Orx and which in turn, participate in promoting waking (Horvath et al., 1999). Using dual-immunostaining with Orx, we examined whether the Orx terminals contain the vesicular transporters for Glu, VGluT1, 2 or 3 (Fremeau et al., 2001; Fremeau et al., 2002) or for GABA, VGAT (Chaudhry et al., 1998). To determine whether they also contain proteins for synaptic release, we subsequently examined whether the Orx+ terminals contain synaptophysin (Syp), a protein contained in SSVs of glutamatergic terminals (Navone et al., 1986; Bragina et al., 2007). To assess whether they form excitatory synapses, we also examined whether the Orx+ terminals contact elements containing PSD-95, a scaffolding protein and postsynaptic marker of glutamatergic synapses (Sheng and Pak, 2000; Sassoe-Pognetto et al., 2003).

EXPERIMENTAL PROCEDURES

All procedures conformed to the guidelines of the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee. Six male, young adult Long-Evans rats (~250 g, Charles River Canada, St. Constant, Quebec, Canada) were employed and maintained on a 12/12 hour light/dark cycle with ad libitum food and water in the animal facility. For the immunohistochemical study, they were killed during the day or light part of their cycle. For this purpose, they were deeply anaesthetized with sodium pentobarbital (100 mg/kg) and perfused transcardially with 250 ml of saline, followed by ~500 ml of 3 or 4% paraformaldehyde fixative solution. Brains were removed, placed in a 30% sucrose solution at 4° C for two days and then frozen to -50° C for storage at -80° C.

Coronal sections were cut on a freezing microtome (at 20 or 25 µm thickness) and collected (in 10 or 8 adjacent series) through the brain. Individual series of sections were collected through the LC region and processed using dual fluorescent immunostaining for Orx and one of the pre- or postsynaptic markers. Free-floating sections were washed in Trizma saline (TS) buffer (0.1 M, pH 7.4) and then in TS with 6% normal donkey serum (NDS) and 0.3% Triton X-100 (TX) for 30 min. Sections were then incubated overnight in TS containing 1% NDS and 0.3% TX with two primary antibodies. In each series, an antibody against Orx-A was combined with an antibody against one of the following markers: VGluT1, 2 or 3, VGAT, Synaptophysin (Syp) or PSD-95 (see Tables 1 and 2). The following day, sections were rinsed for 30 min and subsequently incubated for 2.5 h in TS containing 1% NDS and 0.3% TX with Cy5-conjugated or Cy3-conjugated anti-Gt (in Donkey [Dky], 1:800, Jackson ImmunoResearch Laboratories, West Grove, PA) for Orx-A detection and appropriate Cy2-

conjugated anti-Ms, anti-Rb or anti-GP (in Dky, 1:800, Jackson) for the vesicular transporters, Syp or PSD-95. After one hour washing in TS, sections were mounted out of Trizma water, dehydrated through alcohols and xylene and coverslipped with Permount (Fisher, Fair Lawn, NJ).

For Orx/VT or Syp dual-immunostained material, sections were viewed and analyzed by epifluorescence microscopy. For this purpose, a Nikon Eclipse E800 or Leica DMLB microscope equipped with x-y-z motorized stage, digital camera and filters for Cy2, Cy3 or Cy5 fluorescence detection were employed together with StereoInvestigator software (MicroBrightField [MBF], Williston, VT). For first assessment of co-localization in Orx/VT series, pairs of fluorescent images were obtained for the Cy2 and Cy5 emissions using a 100x oil objective (1.40 NA). Sampling was performed in one section through the center of the LC. A contour was traced around the LC and three different sites were selected randomly for acquisition with use of the Optical Fractionator probe. Thus for each Orx/VT series, three pairs of images per LC per brain were acquired to yield an average of ~350 Orx + varicosities per Orx/VT series per brain or a total of ~1400 varicosities examined in 4 brains. The image pairs were superimposed and analyzed using Adobe Photoshop Creative Suite (CS2, Adobe System, San Jose, CA). The total number of Orx+/VT- and Orx+/VT+ varicosities were counted in image pairs of each series to provide a total across 4 brains.

For the Orx/VGluT2 and Orx/Syp series, stereological analysis of the number and proportion of double-labeled varicosities was performed using the Optical Fractionator probe in StereoInvestigator on the Leica DMLB microscope. Analysis was performed through three levels (separated by 400 μm intervals) of the LC. For stereology, paired fluorescent (Cy2/Cy3) image stacks (9 image pairs, separated by 0.3 μm and located at least 2 μm below the surface) were acquired under a 100x oil objective (with 1.40 NA) with a digital camera within LC contours using a sampling grid of 200 $\mu\text{m} \times 100 \mu\text{m}$. Counts were then performed offline from the image stacks within a counting frame 20 $\mu\text{m} \times 20 \mu\text{m}$, and a dissector height 2.4 μm . In each location, all Orx+/VGluT2- (or Orx+/Syp-) and Orx+/VGluT2+ (or Orx+/Syp+) varicosities were marked. In this manner, approximately 200 Orx + varicosities were sampled per brain for each series.

Sections were analyzed by confocal laser scanning microscopy using either a Zeiss LSM 510 or LSM 700. The Zeiss LSM 510 was equipped with Argon 488 nm, helium-neon 543 nm and helium-neon 633 nm lasers for excitation of Cy2, Cy3 and Cy5 respectively and with appropriate filters for detection of Cy2 (bandpass 500–530 nm, green), Cy3 (bandpass 565–615 nm, red) and Cy5 (bandpass 697–719 nm, infrared). The LSM 700 was equipped with solid state laser diodes emitting at 488 nm and 555 nm, respectively. Separation of the channels was achieved by way of a variable dichroic mirror as secondary beam splitter and appropriate optical filters in front of the detectors (shortpass 545 nm and longpass 560 nm, respectively). Orx+ axons were localized and image stacks acquired using a Plan-Apochromat 100x (with 1.40 NA) objective in the Zeiss LSM 510 microscope and a 63x objective (with 1.46 NA) in the LSM 700. Stacks consisted of images taken through the z-axis at 0.30–0.50 μm step size using the resident acquisition software in each microscope. Pinhole size was adjusted to the diffraction limit of 1.0 Airy units for each channel on the LSM 510 and to 1.0 and 1.08 Airy units for the red and green channel, respectively, on the LSM 700. For Orx/VTP, Orx/Syp and Orx/PSD-95 dual-immunostained material, orthogonal single optical plane images were obtained using the LSM 510 image browser for assessment of co-localization or apposition. For Orx/PSD-95 dual-immunostained material, in addition, rendered 3D reconstructions of the image stacks were analyzed in the image software Volocity 3.5.1 (Improvision, Lexington, MA, www.improvision.com) for interactive visualization, magnification, and rotation of the 3D images.

All images and figures were prepared using Adobe Illustrator Creative Suite and employing simple linear contrast adjustments for each color channel.

RESULTS

In material dual-immunostained for Orx-A and the vesicular transporters for Glu, VGluT1, 2 or 3, or GABA, VGAT, evidence for co-localization with Orx was only detected for VGluT2, under fluorescence (Fig. 1) or confocal microscopy (Fig. 2). With VGluT1, a relatively small number of varicosities was evident in the LC, but nonetheless well stained with the antibodies tested (from Rb and GP, Table 1). With neither of these antibodies were Orx+ terminals found to be double-labeled for VGluT1 under either fluorescence (0/1227 Orx+ varicosities, systematically examined in 4 brains using Rb antibody; Fig. 1 A) or confocal microscopy (Fig. 2 A). Similarly, with VGluT3, a small number of varicosities were apparent in the LC but also well stained with the antibodies tested (from Rb and GP, Table 1). With neither of the antibodies were Orx+ terminals found to be double-labeled for VGluT3 by fluorescence (0/1513 Orx+ varicosities systematically examined in 4 brains using Rb antibody, Fig. 1 C) or confocal microscopy (not shown). In material dual-immunostained for Orx-A and the vesicular transporter for GABA, a large number of VGAT + terminals were evident in the LC (Fig. 1 D) but virtually none showed co-localization of Orx and VGAT in fluorescence (1/1383 varicosities systematically examined in 4 brains, Fig. 1 D).

In sections dual-immunostained for Orx-A and VGluT2, co-localization was apparent in a proportion of Orx+ varicosities. With all three antibodies tested (Table 1), a moderate number of VGluT2+ varicosities, exceeding that of VGluT1 or VGluT3, was evident in the LC (Fig. 1 B). Although co-localization of Orx and VGluT2 was only apparent in a few varicosities with two antibodies tested from Rb, it was apparent in a significant number of varicosities immunostained with antibody from GP (Fig. 1 B). For systematic and quantitative analysis of this material, the Orx+ and double-labeled Orx+/VGluT2+ varicosities were examined under fluorescence microscopy with the StereoInvestigator Optical Fractionator probe. Among all varicosities immunostained for Orx in the LC (estimated at ~685,000), less than 5% were double-labeled for VGluT2 (Table 3). Yet it appeared that the varicosities which were double-labeled were large, whereas those which were not double-labeled were small (Fig. 1 B). By distinguishing varicosities as large vs. small (as those less than 0.5 μm in diameter), it was found that more than one fourth ($27.6 \pm 17.1\%$, Table 3) of the large Orx+ varicosities, were dual-immunostained for VGluT2, whereas barely 1% of the small Orx+ varicosities were so (Table 3). The co-localization of VGluT2 with Orx in varicosities was confirmed with confocal microscopy by examining orthogonal views of image stacks (Fig. 2 B).

In material dual-immunostained for Orx-A and the presynaptic marker, Syp, a significant number of Orx+ varicosities were seen to contain Syp under both fluorescence (not shown) and confocal microscopy (Fig. 2 C). In a quantitative assessment by stereological analysis of fluorescent material (not shown), approximately one fourth ($23.0 \pm 10.5\%$ in 3 brains) of large Orx+ varicosities were double-labeled with Syp. The co-localization of Orx and Syp in varicosities was confirmed with confocal microscopy in orthogonal views of image stacks (Fig. 2 C).

In material dual-immunostained for Orx-A and the postsynaptic marker, PSD-95, Orx+ varicosities were often seen in close proximity to PSD-95+ elements when viewed by confocal microscopy as single plane images (not shown) and 3D reconstructions of stacked images (Fig. 3 A–C). Using magnification and rotation through three orthogonal planes of rendered image stacks, many of the Orx+ terminals were judged to be in contact with

PSD-95+ puncta, since the two elements were distinct yet in apposition through 3 different image planes. These appositions were seen in the LC of all brains examined (n = 4).

DISCUSSION

The present results provide immunohistochemical evidence by immunostaining of VGluT2, Syp and PSD-95 for synaptic release of Glu from Orx terminals in the LC.

Systematically analyzed using fluorescence microscopy and confirmed by confocal laser scanning microscopy, the Orx+ varicosities did not appear to be immunostained for the vesicular transporters for VGluT1 or VGluT3, which were evident in scattered Orx-varicosities in the LC. In a previous study using *in situ* hybridization, a small percentage (13%) of Orx neurons were reported to express mRNA for VGluT1 (Rosin et al., 2003), suggesting the possibility for VGluT1 to be present in a small proportion of varicosities, which was however not affirmed here.

No Orx terminals were found to be immunostained for the vesicular transporter for GABA, VGAT. That Orx terminals might contain an inhibitory amino acid vesicular transporter was thought possible since not all Orx terminals were seen to make asymmetric synapses in electron microscopic studies within the ventral tegmental area (Balcita-Pedicino and Sesack, 2007). Moreover, a small number of Orx terminals were seen to make symmetric synapses and to be immunostained for GABA in the latter studies (Balcita-Pedicino and Sesack, 2007). Yet, GABA was not detected in electron microscopic studies of Orx terminals in the hypothalamus (Torrealba et al., 2003), and mRNA for the synthetic enzyme for GABA, glutamic acid decarboxylase (GAD), was also not detected by *in situ* hybridization in Orx neurons (Rosin et al., 2003), substantiating our results.

Although found in a relatively small proportion, VGluT2 was clearly contained in Orx varicosities in the LC. These results would substantiate the original indication from *in situ* hybridization for VGluT2 mRNA in half of Orx neurons, that this vesicular transporter would be utilized by at least a proportion of Orx neurons (Rosin et al., 2003). Here, in our quantitative stereological examination, we found <5% of all Orx+ varicosities were double-labeled for VGluT2, yet more than one fourth of large Orx+ varicosities were so. The restriction of immunostaining to large varicosities could be due to a technical limitation whereby only in those varicosities with large numbers of vesicles and thus amounts of VGluT2, was the threshold for detection reached. It is also possible that VGluT2 is present only in varicosities which form synapses, since in electron microscopic studies of cholinergic or monoaminergic terminals, junctional varicosities have been found to be larger than non-junctional varicosities (Umbriaco et al., 1994). Indeed, VGluT2 was found in a proportion of dopaminergic varicosities in young animals, in which the double-labeled VGluT2+/tyrosine hydroxylase (TH)+ varicosities were on average larger in size than the single-labeled TH+ varicosities and in which all the VGluT2+/TH+ varicosities formed synapses, whereas only a minor proportion of the single labeled TH+ varicosities did so (Descarries et al., 2008; Berube-Carriere et al., 2009).

As also viewed by confocal microscopy, many Orx varicosities through the LC contain Syp, a protein present in SSVs of most glutamatergic terminals and proposed to be involved in neurotransmitter exocytosis at the synapse (Navone et al., 1986; Valtorta et al., 2004; Bragina et al., 2007). Here, interestingly the proportion of large Orx+ varicosities double-labeled with Syp was similar to that double-labeled with VGluT2 and thus likely also corresponding to the Orx varicosities which form glutamatergic synapses.

As visualized by confocal laser scanning microscopy and 3D rendering of images, many Orx + varicosities in the LC appeared to be in contact with PSD-95, a scaffolding protein

localized at the postsynaptic density of asymmetric synapses and interacting with NMDA receptors (Kornau et al., 1995; Sassoe-Pognetto et al., 2003). These results confirm the electron microscopic studies showing asymmetric synapses by Orx terminals in the locus coeruleus (Horvath et al., 1999) along with others in the tuberomammillary nucleus (Torrealba et al., 2003) and the ventral tegmental area (Balcita-Pedicino and Sesack, 2007). Although the incidence of contacts with PSD-95 elements could not be estimated quantitatively in the present study, the incidence of synapses for Orx+ terminals was estimated in the ultrastructural studies within the ventral tegmental area to be ~30% (Balcita-Pedicino and Sesack, 2007). Collectively, these findings indicate that a proportion of Orx+ terminals form glutamatergic synapses in target regions, including the locus coeruleus.

In summary, the present study establishes that a proportion of Orx terminals have the capacity to release Glu in addition to Orx and to act upon postsynaptic targets via glutamatergic synapses. Orx, contained in LDCVs, might be released from all Orx+ varicosities in a nonsynaptic manner, diffuse to reach Orx receptors distributed along the somato-dendritic membrane and excite or modulate through signal transduction, receptor-bearing cells in the terminal region (Horvath et al., 1999; Torrealba et al., 2003; Balcita-Pedicino and Sesack, 2007; Brischoux et al., 2008). Glu, contained in SSVs, would be released from a subset of the Orx+ varicosities into the synaptic cleft, bind to glutamate receptors anchored to the postsynaptic density and rapidly excite through direct ion channel opening, particular target cells (Sheng and Pak, 2000; Fremeau et al., 2001). Such fast glutamatergic transmission onto target neurons, including those in the LC, is likely important for the role of Orx neurons in eliciting and maintaining a waking state along with muscle tone, sympathetic activation and behavioral arousal. Conversely, the pathophysiology of the human sleep disorder, narcolepsy with cataplexy, is likely due to loss of the excitatory synaptic connections of the Orx neurons together with the loss of their peptide following their degeneration.

Abbreviations

Cy2	Cyanine
Cy3	Indocarbocyanine
Cy5	Indodicarbocyanine
Dky	donkey
Glu	glutamate
GP	guinea pig
Gt	goat
LC	locus coeruleus
LDCVs	large dense core vesicles
Ms	mouse monoclonal
NDS	normal donkey serum
NMDA	N-methyl-D-Aspartate
Orx	Orexin
PSD-95	postsynaptic density protein-95
Rb	rabbit

SSVs	small synaptic vesicles
Syp	synaptophysin
TS	trizma saline buffer
TX	triton x-100
VAcHT	vesicular acetylcholine transporter
VGAT	vesicular GABA transporter
VGluT	vesicular glutamate transporter
VT	vesicular transporter

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Fig. 1.

Fluorescent images of Orx/VGluT1, VGluT2, VGluT3 or VGAT dual-immunostaining in LC. (A-D) Orx+ varicosities (as labeled with Gt-anti-Orx-A primary and Cy5-conjugated secondary antibodies, A1, B1, C1 and D1), which were considered as small (less than approximately 0.5 μm in diameter, small solid arrowheads) or large (>0.5 μm diameter, large solid arrowheads), were examined for double-labeling for the vesicular transporters in fluorescence microscopy. Note all Orx+ varicosities were judged negative (open arrowheads) for VGluT1 (A2, labeled with the Rb-anti-VGluT1 primary and Cy2-conjugated secondary antibodies), VGluT3 (C2, labeled with the Rb-anti-VGluT3 primary and Cy2-conjugated secondary antibodies) and VGAT (D2, labeled with the Rb-anti-VGAT primary and Cy2-conjugated secondary antibodies). Although one small and one large Orx+ varicosity (B1) are also negative for VGluT2 (B2, open arrowheads, labeled with the GP-anti-VGluT2 primary and Cy2-conjugated secondary antibodies), one large Orx+ varicosity (B1) is positive for VGluT2 (B2, solid arrowhead). Note also that among the VT stained varicosities (indicated by small arrows for Orx-VT+), VGluT2+ varicosities are most numerous among the VGluT+ and similar in density to the VGAT+ varicosities in the LC. Scale bar = 2 μm .

Fig. 2.

Confocal images of Orx/VGluT1, VGluT2 or Syp dual-immunostaining in LC. (A1-3) An Orx+ varicosity (A1, large filled arrowhead, labeled with the Gt-anti-Orx-A primary and Cy5-conjugated secondary antibodies in red) is not immunostained for VGluT1 (A2, large open arrowhead, using GP-anti-VGluT1 and Cy2 in green). As clearly evident in the image stacks (A1-A3) and the corresponding three orthogonal views (xy, xz and yz sub-panels), the Orx+ varicosity is negative, despite robust staining for VGluT1 of isolated Orx-terminals within the LC (top right corner in A2 and A3). (B1-3) In contrast, multiple Orx+ varicosities (B1, solid arrowheads, labeled with the Gt-anti-Orx-A and Cy3 in red) are positively immunostained for VGluT2 (B2, filled arrowheads, labeled with GP-anti-VGluT2 and Cy2

in green), as evident in the merged image (B3, solid arrowheads, yellow). The co-localization of VGluT2 with Orx can be confirmed for one Orx+ varicosity (large solid arrowhead) in the three orthogonal views of the merged image (B3, yellow in xy, xz and yz sub-panels). (C1-3) An Orx+ varicosity (C1, large solid arrowhead, labeled with Gt-anti-Orx-A and Cy3 in red) is positively immunostained for Syp (C2, large solid arrowhead, labeled with the Ms-anti-Syp and Cy2 in green) as evident in the merged image (C3, large solid arrowhead, in yellow) and confirmed in the orthogonal views. An adjacent Orx+ varicosity (C1, small solid arrowhead) is not labeled for Syp (C2 and C3, small open arrowheads). All panels are based on image stacks of 20 or 28 optical sections of 300 or 200 nm thickness, respectively. The center images of each panel (xy view) represent single optical sections. The xz and yz views are orthogonal views of the 3D data volume. Scale bar = 2 μ m (for all panels and subpanels).

Fig. 3. Confocal images of Orx and PSD-95 dual-immunostaining in LC. (A) Rendered region of interest of a 3D confocal image stack from dual-immunostained material for Orx and PSD-95 in the LC, showing an Orx+ axon (labeled with the Gt-anti-Orx-A primary and Cy5-conjugated secondary antibodies shown in red) with varicosities (solid arrowheads), which appose PSD-95+ profiles (arrows, labeled with the Ms-anti-PSD-95 and Cy2 shown in green). (B1-B3) Zoomed and rendered image stack of the portion indicated by bracket B in A to show at higher magnification the close apposition between the Orx+ varicosities (B1 and B3, solid arrowheads) and the PSD-95+ elements (B2 and B3, arrows). (C1-C3) Zoomed and rendered image stack of the portion indicated by bracket C in A, to show in detail the apposition of a small PSD-95+ profile (C2 and C3, arrow) to a large Orx+ varicosity (C1 and C3, solid arrowhead). Scale bars = 5 μ m (A); 2 μ m (B and C).

Primary antibodies

Table 1

Antigen	Host	Source	Cat. #	Immunogen	Specificity
Orx-A	Gt	Santa Cruz	1	Peptide mapping at the C-terminus of human Orx-A of the Orx precursor, identical to corresponding mouse sequence (AA 48 – 66)	AB reacts with Orx-A of mouse, rat and human by WB, immunostaining blocked with antigen peptide (sc-8070 P) and pattern of staining identical to that with Orx-(Rb) A H-003-30 (Phoenix)
PSD-95	Ms	ABR	4	MA1-045	By WB the AB recognizes a ~95 kDa protein and a slightly larger species from rat brain extracts
Syp	Ms	Sigma	6	S5768	By WB the antibody recognizes a 38 kDa band from rat brain extracts. Labels synaptophysin-1 in neurons and other tissues
VGAT	Rb	Millipore	8	AB5062P	By WB the AB recognizes a band at ~55–60 kD
VGluT1	GP	SY SY	11	135 304	By WB the AB recognizes expected protein band (~60 kD) from rat brain vesicle fraction. No band detected in WB from VGluT1 -/- K.O. mice crude synaptosomes. No homology with other proteins
VGluT1	Rb	Gift RHE	13	-	By WB the AB recognizes a ~62 kD band from rat brain
VGluT2	GP	SY SY	135 404	Purified recombinant protein of rat VGluT2 C-terminus (AA 510 – 582)	By WB the AB recognizes expected protein band (~60 kDa) from rat brain vesicle fraction. No homology with other proteins
VGluT2	Rb	Gift RHE	-	Synthetic peptide corresponding to 64 last AA of C-terminus of rat DNPi (VGluT2)	By WB the AB recognizes a ~50–62 kD band from rat brain
VGluT2	Rb	SY SY	135 403	Strep-Tag® fusion protein of rat VGluT2 C-terminus (AA 510 – 582)	By WB the AB recognizes expected protein band (below ~60 kDa) from rat brain vesicle fraction. No homology with other proteins
VGluT3	GP	Millipore	AB5421	Synthetic peptide from cloned rat VGluT3 protein (AA 569 – 588)	The AB labels VGluT3+ cells and fibers, in agreement with other VGluT3 antisera
VGluT3	Rb	SY SY	135 203	Recombinant C-terminus of mouse VGluT3 (AA 543 – 601)	Specific for mouse VGluT3. WB signal is efficiently and specifically blocked by preadsorption with control peptide, used for immunogenicity

Abbreviations: AA, amino acid; AB, antibody; BNPi, brain-specific Na⁺-dependent inorganic phosphate cotransporter; DNPi, differentiation-associated Na⁺-dependent phosphate transporter; GP, guinea pig; Gt, goat; Ms, mouse (monoclonal); Rb, rabbit; Syp, Synaptophysin; WB, western blot

¹ Santa Cruz Biotechnology, Santa Cruz, CA

² Information provided by Santa Cruz data sheet and technical service

³ Information on WB provided by Santa Cruz data sheet and technical service, blocking experiments carried out in our lab with the Santa Cruz antigen peptide and immunostaining of Orx cell population compared for two antibodies in our lab in this and previous studies (Modirrousta et al., 2005; Henny and Jones, 2006b)

⁴ ABR: Affinity BioReagents, Golden, CO

⁵ <http://www.bioreagents.com/>

⁶ Sigma-Aldrich Co., St Louis, MO

⁷ <http://www.sigmaaldrich.com/>

⁸ Millipore, Billerica, MA

⁹ Supplied from Millipore on request

¹⁰ <http://www.millipore.com/>

¹¹ Synaptic Systems, Goettingen, Germany

¹² <http://www.sysy.com/>

¹³ Gift from Edwards, R.H. and Freneau, R.T. Jr.

¹⁴ Bellocchio et al., 1998

¹⁵ Freneau et al., 2001

Table 2

Combinations for dual-immunostaining with primary antibodies¹

Series ²	Antigen	Source	Host	Dilution	Cases ³
Orx/PSD-95	Orx-A	Santa Cruz	Gt	1:200	4
	PSD-95	ABR	Ms	1:100	
Orx/Syp	Orx-A	Santa Cruz	Gt	1:200	6
	Syp	Sigma	Ms	1:200	
Orx/VGAT	Orx-A	Santa Cruz	Gt	1:200	4
	VGAT	Millipore	Rb	1:250	
Orx/VGluT1	Orx-A	Santa Cruz	Gt	1:200	3
	VGluT1	SY SY	GP	1:2000	
Orx/VGluT1	Orx-A	Santa Cruz	Gt	1:200	4
	VGluT1	Gift RHE	Rb	1:1000	
Orx/VGluT2	Orx-A	Santa Cruz	Gt	1:200	3
	VGluT2	SY SY	GP	1:5000	
Orx/VGluT2	Orx-A	Santa Cruz	Gt	1:200	4
	VGluT2	Gift RHE	Rb	1:5000	
Orx/VGluT2	Orx-A	Santa Cruz	Gt	1:200	3
	VGluT2	SY SY	Rb	1:5000	
Orx/VGluT3	Orx-A	Santa Cruz	Gt	1:1000	4
	VGluT3	Millipore	GP	1:200	
Orx/VGluT3	Orx-A	Santa Cruz	Gt	1:200	4
	VGluT3	SY SY	Rb	1:800	

¹ For sources and specificity of primary antibodies see Table 1.

² Dual-immunostained sections were processed with combinations of appropriate secondary Dky-anti-(primary Host sp), Cy2-, Cy3- or Cy5-conjugated secondary antibodies from Jackson (Jackson ImmunoResearch Laboratories, West Grove, PA; <http://www.jacksonimmuno.com/>). Please refer to text and figure legends for specific combination.

³ Each case refers to one brain from which multiple series were processed.

Abbreviations: Dky, donkey; GP, Guinea Pig; Gt, goat; Ms, mouse (monoclonal); Rb, rabbit

Table 3

Estimates of Orx+ and double-labeled Orx+/VGluT2+ varicosities in the locus coeruleus

	Varicosities	Mean $\pm$ SD
	<i>N</i> ° Orx+ vars. *	685,783 $\pm$ 176,120
All vars.	<i>N</i> ° Orx+/VGluT2+ *	25,050 $\pm$ 16,537
	% double-labeled vars. **	3.7 $\pm$ 1.9
	<i>N</i> ° Orx+ vars. *	97,022 $\pm$ 54,607
Large vars.	<i>N</i> ° Orx+/VGluT2+ *	20,011 $\pm$ 12,305
	% double-labeled vars. **	27.6 $\pm$ 17.1[‡]
	<i>N</i> ° Orx+ vars. *	588,761 $\pm$ 129,759
Small vars.	<i>N</i> ° Orx+/VGluT2+ *	5,039 $\pm$ 6,016
	% double-labeled vars. **	0.8 $\pm$ 0.9[‡]

Varicosities were dual-immunostained for Orx-A and VGluT2 (from GP, SY SY) and judged as small vs. large in size if less than approximately 0.5 μ m in diameter within the fluorescent image.

* Mean $\pm$ SD of estimated varicosities from 3 brains (one side per brain) based upon stereological estimates using the StereoInvestigator optical fractionator probe

** Mean $\pm$ SD of the percentage (%) of double-labeled varicosities from 3 brains

[‡] p = 0.05 (t-test) for comparison of % of Large vs. Small double-labeled varicosities in Orx/VGluT2 series

Abbreviations: Orx, orexin; vars., varicosities; VGluT2, vesicular glutamate transporter 2