Published in final edited form as:

J Chem Neuroanat. 2009 December ; 38(4): 273–281. doi:10.1016/j.jchemneu.2009.05.005.

Locally Collateralizing Glutamate Neurons in the Dorsal Raphe Nucleus Responsive to Substance P Contain Vesicular Glutamate Transporter 3 (VGLUT3)

K.G. Commons, Ph.D.

Department of Anesthesiology, Perioperative, and Pain Medicine, Children's Hospital Boston;
Department of Anaesthesia, Harvard Medical School

Abstract

The serotonergic dorsal raphe nucleus (DR) is an area enriched with cell bodies and axons containing the third vesicular glutamate transporter, VGLUT3. However, the role of VGLUT3-containing axons in modulating activity of serotonin (5-HT) neurons within the DR remains poorly understood. In this study, neurochemical features and topography of VGLUT3-containing cell bodies and axons in the DR were examined. Since many 5-HT cells have been reported to express VGLUT3, the distribution of dually labeled axons was examined within the DR. Axons containing both VGLUT3 and 5-HT immunolabeling had a topographic distribution: they innervated the ependyma and ramified within the caudal DR at the base of the aqueduct, an area known to give rise to ependymal innervation. Thus VGLUT3 is only present in a specific subcomponent of recurrent 5-HT axon collaterals. Remaining VGLUT3 axons were only rarely dually-immunolabeled for markers of monoamines, GABA, or acetylcholine, suggesting these axons contain a predominance of glutamate-filled vesicles. Since the substance P receptor, neurokinin 1 (NK1), has previously been associated with local glutamate neurons in the DR, the relationship between NK1, 5-HT and VGLUT3 cells was examined using triple-immunolabeling. Results indicate that the majority of non-5-HT VGLUT3-containing cell bodies in the DR often contain NK1 immunolabeling. Taken together, these findings indicate locally collateralizing glutamate neurons responsive to substance P contain VGLUT3.

Keywords

serotonin; substance P; neurokinin 1; feedback

Introduction

Three vesicular glutamate transporter isoforms (VGLUT1-3) have the capacity to fill synaptic vesicles with the neurotransmitter glutamate. VGLUT1 is highly expressed in the cerebral and cerebellar cortices, whereas subcortical areas predominantly utilize VGLUT2 (Freneau et al., 2001; Herzog et al., 2001 Varoqui et al 2001). VGLUT3 is distinguished from VGLUT-1 and -2 in its' localization to brain areas and neurons that are not thought to use glutamate as a neurotransmitter. For example, VGLUT3 is found in interneurons of the hippocampus,

Correspondence to: Kathryn G. Commons, Ph.D., Children's Hospital, 300 Longwood Ave., Enders 1206, Boston, MA, Telephone: 617-919-2220, FAX: 617-730-0967, Kathryn.Commons@Childrens.harvard.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

cholinergic striatal interneurons and within the serotonergic dorsal and median raphe nuclei (Freneau *et al.*, 2002; Gras *et al.*, 2002; Hioki *et al.*, 2004; Schafer *et al.*, 2002; Shutoh *et al.*, 2008). Thus there is a trend for VGLUT3 to localize in axons that may use multiple fast neurotransmitters. However, in some cases VGLUT3 is required for glutamate neurotransmission, most notably at the synapse of the ear's inner hair cell (Seal *et al.*, 2008).

Within the dorsal raphe nucleus (DR), one of the major sources of forebrain serotonin (5-HT), the relationship between VGLUT3 and 5-HT neurons has been examined in rat (Gras *et al.*, 2002; Shutoh *et al.*, 2008) and Syrian hamster (Mintz and Scott, 2006). These studies have consistently reported co-existence of VGLUT3 with markers for 5-HT within cell bodies and, to a varying extent, the existence of neurons that contain VGLUT3 in the absence of 5-HT and vice versa. Examination of VGLUT3 with respect to 5-HT axons in the forebrain reveal that some, but not all, forebrain 5-HT axons also contain VGLUT3 (Boulland *et al.*, 2004; Hioki *et al.*, 2004; Shutoh *et al.*, 2008).

5-HT neurons within the DR are not homogeneously distributed, rather they can be divided into distinct subgroups based upon their cytoarchitecture and neurochemical features (reviewed by (Jacobs and Azmitia, 1992; Michelsen *et al.*, 2007; Steinbusch, 1984)). However, it has never been examined if VGLUT3 axons, with or without 5-HT, have any sub-region specific characteristics within the DR. In fact, little is known about the role of VGLUT3 in DR circuits. Therefore, we examined the topographic distribution VGLUT3 containing axons with respect to potential co-transmitters. In addition, non-5HT VGLUT3-containing cell bodies were characterized for both their distribution and relationship to the receptor for substance P (neurokinin 1, NK1), which has previously been associated with glutamate neurotransmission in the DR (reviewed by Valentino and Commons, 2005).

Methods

The Animal Care and Use Committee at The Children's Hospital, Boston approved the animal procedures used. Fifteen male Sprague-Dawley rats (250-500 g) were anesthetized with sodium pentobarbital (75 mg/kg i.p.) to minimize pain and transcardially perfused. The perfusate consisted of 10 ml of saline followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. After perfusion, brains were removed and left in the same fixative overnight. Brains were then saturated in 30% sucrose, frozen in crushed-dry ice and sectioned 40 μ m thick using a rotary microtome with a chuck cooled with a Peltier device. All sections were collected in 0.1 M PB with 0.9% saline (PBS) and processed free-floating.

Table 1 summarizes the antisera used. Overall, the strategy used to confirm specificity of all of the antisera was to characterize the immunolabeling attained in comparison to the known distribution of each of the antigens in the DR or other regions where they have been described. Since in each case, individual descriptions of gene expression and protein localization are well established, appropriate distribution in other regions provides a strong line of evidence to support specificity particularly when supported by additional evidence from the literature. Using the VGLUT3 antisera the distribution of VGLUT3 immunolabeled cell bodies was identical to prior descriptions in the ventral forebrain and striatum and the hippocampus. In addition, the distribution of VGLUT3 immunolabeled axons was consistent with previous descriptions, including for example localization to stratum pyramidale in the CA1 region of the hippocampus. This is consistent with the observations of others who compared immunolabeling with this antisera to that of others (Gabellec *et al.*, 2007) and with the pattern of source neurons expressing the mRNA for this transporter (Gras *et al.*, 2002; Schafer *et al.*, 2002). In addition for VGLUT3, previous studies have reported that VGLUT3 immunolabeling is lost when the VGLUT3 antiserum is preadsorbed to the antigenic peptide (Gabellec *et al.*, 2007; Shutoh *et al.*, 2008)). Immunolabeling for 5-HT and SERT was definitive for the known

distribution of 5-HT cells and axons, that is, cell bodies immunolabeled for these antigens were located in the raphe nuclei and absent from other areas of the brain including other monoamine nuclei. For VMAT2 the antibody is specific for 88 kDa protein by Western blot (Sandoval *et al.*, 2002) and identifies neurons in the raphe nuclei as well as neurons in all other monoamine nuclei. Immunolabeling for GABAergic axons was determined using a well-characterized monoclonal antibody, GAD6 that recognizes GAD-65 (Chang and Gottlieb, 1988), obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA, as well as with a GAD-67 antiserum obtained from Millipore/Chemicon (Varea *et al.*, 2005). These antisera were tested on sections containing the hippocampal formation, and there distribution was characteristic for the known localization of these GAD isoforms. The antiserum used to detect VACHT has previously been shown to identify cholinergic cell bodies and axons with high sensitivity (Arvidsson *et al.*, 1997), and in our tests immunolabeling with this antisera defined both ventral forebrain and pontine cholinergic cell groups. A rabbit antiserum to detect NK1 receptor was raised against a C-terminal portion of the rat NK1 receptor (Vigna *et al.*, 1989), which yields a pattern of immunolabeling identical to known descriptions in the hippocampus and striatum, as well as the DR (Commons and Valentino, 2002). An affinity purified anti-tryptophan hydroxylase (TPOH) polyclonal antisera raised in sheep was used to label the somatodendritic extent of 5-HT neurons. This TPOH antiserum identifies all 5-HT labeled cells in the raphe nuclei and exhibits variable cross-reactivity to neurons in catecholamine containing nuclei probably due to some similarity between TPOH and tyrosine hydroxylase.

Immunohistochemical processing took place with continuous light agitation. Between incubations the tissue was rinsed several times with PBS. For immunolabeling, antisera were diluted in PBS and 0.5% bovine serum albumin and tissue was incubated either overnight at room temperature, or 2-3 days in the coldroom with constant mild agitation. For visualization of axons 0.1% TRITON X-100 was added to the buffer but was excluded to maximize cell body labeling for VGLUT3. Fluorophore-conjugated secondary antisera raised in donkey were purchased from Jackson ImmunoResearch (CY3 conjugated) or Molecular Probes (Alexa-488 and Alexa-647 conjugated). Tissue was incubated with secondary antisera diluted 1:100 for 90 minutes at room temperature. After rinsing, immunolabeled tissue was mounted on glass slides from 0.5 M PB, dried and cover-slipped using mounting medium containing 90% glycerol and 10% PBS pH 8.5-9.

Data Analysis

To estimate colocalization between immunolabeled structures, seven subregions of the DR were sampled (Fig. 1). Three rostrocaudal divisions of the DR were made: caudal was -8.54 to -9.26 mm; mid was -7.73 to -8.45 mm; rostral DR was -6.92 to -7.64 mm, coordinates measured from Bregma using the atlas by Paxinos and Watson (1998). These three levels were then divided into dorsal and ventral halves by estimating the midpoint between the base of the aqueduct and the ventral extent of TPOH containing cells yielding six groups. The lateral wings of the DR comprised the remaining group and was sampled in sections that also contained mid levels of the DR. For evaluation of colocalization of labeling, sections from at least four rats were processed in multiple immunolabeling runs. For quantitative analysis sections were selected where the intensity of immunolabeling was high, yet the pattern of labeling was representative of all the tissue viewed. For sampling, the relevant area was illuminated for each fluorophor and a single optical plane was digitally photographed using a spinning-disc confocal fluorescence microscope (Olympus DSU) and a 60× objective NA 1.42. At least three different fields were photographed for each area. An average of 3 non-overlapping 625 square micron areas were arbitrarily selected within each photograph and all profiles that were between 0.3 μ m and 1.5 μ m in diameter were counted within these areas while illuminating for VGLUT3

independently. Sizes were measured using Slidebook software. Subsequently, similar puncta singly labeled for the second immunolabel and those dually labeled were counted by visualizing the second fluorophor independently, and toggling between a merged image of the two fluorophors. For mapping single-labeled VGLUT3 cells with respect to single-labeled 5-HT neurons in the DR, photomontages of representative sections were created using Adobe Photoshop. When examining images at high magnification, each instance a cell was detectably labeled a color-coded dot was plotted over an outline of the nucleus in an overlying layer. For analysis of colocalization of VGLUT3 with NK1 in non-5-HT cell bodies, areas were sampled using conventional epi-fluorescence microscope using a 20× objective N.A 0.9. Sampled sections were arbitrarily selected among those where immunolabeling for both antigens was most visible within somata. Within these areas, all labeled cell bodies were counted by visualizing each fluorophor independently. Subsequently, dually labeled cell bodies were counted by comparing images of each fluorophor independently with a merged image.

Preparation of Figures

Images were captured using a digital camera and Slidebook software package and figures were assembled in Adobe PhotoShop. All of the images were adjusted for brightness and contrast. On some panels, sharpness was adjusted using the “unsharpen mask” filter at 50% with a 2-4 pixel diameter.

Results

VGLUT3 immunolabeling overall appeared enriched within the DR in comparison to the neighboring periaqueductal gray (Fig. 1). Examination at higher magnification revealed that immunolabeling for VGLUT3 identified a population of intensely labeled axonal boutons as well as less intense and more diffuse labeling within cell bodies (Fig. 2). Since previous studies have reported that 5-HT and VGLUT3 colocalize, mapping the colocalization of VGLUT3-immunolabeled axons with 5-HT was the first focus of our study.

In most subregions of the DR only a few axons appeared to be double labeled for 5-HT and VGLUT3 (Figs. 3, 4A). This lack of co-localization was confirmed by dual labeling of VGLUT3 with SERT (not shown). However, at all levels 5-HT axons within the ependyma were obviously dually labeled for VGLUT3 (Fig. 5). Innervation of the ependyma arises from clusters of cells at the base of the aqueduct in the caudal DR and additional cells located ventral to these (Mikkelsen *et al.*, 1997; Simpson *et al.*, 1998). The caudal dorsal clusters of 5-HT cells were densely surrounded by axons containing both VGLUT3 and 5-HT (Fig. 5). In addition, the caudal ventral DR had a higher level of colocalization between VGLUT3 and 5-HT than other regions of the DR.

VGLUT3 immunolabeling was next examined with respect to that for VMAT2 in axons to evaluate if VGLUT3 could be colocalized with another monoamine in the remainder of the DR. VMAT2 immunolabeling yielded clearer definition of axon-like boutons with less neuropil labeling when compared to 5-HT (Fig. 4). However, estimates of co-localization between VGLUT3 and VMAT2 were just slightly higher than those for 5-HT and VGLUT3 in all DR subregions (Figs. 3-4).

To determine if VGLUT3 colocalized with GABA within axon terminals, tissue was dually labeled for VGLUT3 and GAD65 or separately for VGLUT3 and GAD67. Immunolabeling for either GAD isoform resulted in abundant labeling of axonal boutons throughout the DR (Fig. 4). Co-localization was studied throughout the rostral-caudal extent of the nucleus. Only in the caudal ventral DR was there a trend for increased co-localization for VGLUT3 and GAD65, however these accounted for a small fraction of VGLUT3 labeled axons.

To determine if VGLUT3 colocalized with acetylcholine within axon terminals, tissue was dually labeled for VACHT. VACHT positive cells and axons were found in the lateral dorsal tegmental nucleus and elsewhere within the same tissue sections. However, VACHT-immunolabeling yielded sparse axonal labeling through the DR, and these were not detectably dually labeled for VGLUT3 in any subregion (Fig. 3-4).

Therefore with the exception of the caudal dorsal DR, VGLUT3 did not apparently colocalize with another neurotransmitter within axons. VGLUT3-containing cell bodies were investigated to further understand their identity. Since the colocalization of VGLUT3 in 5-HT cells has previously been evaluated using both immunohistochemistry and in situ hybridization techniques (Gras *et al.*, 2002; Mintz and Scott, 2006; Shutoh *et al.*, 2008), we focused on VGLUT3 cell bodies that lacked 5-HT immunoreactivity. Cell bodies immunolabeled for VGLUT3-only were mapped with respect to 5-HT at different levels, revealing there were present along the entire rostral-caudal axis of the DR (Fig. 5). They appeared most prevalent at a mid-dorsoventral and mid-rostrocaudal location (Fig. 5B) although they were spread along the entire rostrocaudal extent of the DR. Typically cells single labeled for VGLUT3 favored the midline (Fig. 5).

Previously glutamate-immunoreactive cells containing the substance P receptor, neurokinin 1 (NK1), were described in the DR (Commons, and Valentino, 2002), therefore it was determined if non-5-HT cells with VGLUT3 had immunolabeling for the NK1 receptor. For this, 5-HT cells were identified by the presence of the synthetic enzyme tryptophan hydroxylase (TPOH). NK1 distribution was identical to that previously published in the DR (Barbaresi, 1998; Commons and Valentino, 2002). Dually labeled cells were commonly found, particularly at mid-rostrocaudal levels of the DR, and appeared to account for the majority of non-5-HT VGLUT3 containing cells (Fig. 6). In an arbitrary selection of fields with triple labeling, 88 of 122 VGLUT3 labeled cells were also labeled for NK1 (72%). VGLUT3- and NK1-dually labeled cells were smaller than TPOH labeled cells, and were approximately 14 μ m in diameter vs. average of 21 μ m in diameter for TPOH cells. NK1 immunolabeling was almost entirely associated with the plasma membrane of cells and therefore was easily distinguished from overlying cytoplasmic VGLUT3 labeling within cell soma.

Discussion

The DR is enriched with VGLUT3-containing cell bodies and axons, yet the relationship of these neurons to 5-HT cells has remained incompletely understood. Immunolabeling and fluorescence microscopy revealed in this study that VGLUT3-containing axons in the DR are comprised of at least two populations. VGLUT3 colocalizes with 5-HT in some axons, and these preferentially innervate the caudal dorsal portion of the DR. Many VGLUT3 axons lacked 5-HT or another obvious co-transmitter, and at the same time many VGLUT3-labeled cell bodies had the receptor for substance P. This evidence suggests that non-5-HT VGLUT3 cells comprise a population of locally collateralizing glutamate neurons. Both of these populations likely play a role regulating excitation of 5-HT neurons within the DR.

All of the antigens examined in this study have well known independent patterns of distribution, providing evidence for the specificity of their immunolabeling and colocalization. However, the differing propensity of antigens to be enriched in distinct subcellular compartments could influence the accuracy of quantifying colocalization. Although VGLUT3 immunolabeling in cell bodies can be diffuse and much less intense than axon terminal labeling, the different subcellular distribution of labeling for VGLUT3 and NK1 receptors facilitated estimating colocalization between these antigens. With respect to colocalization of VGLUT3 and 5-HT in cell bodies, the technical approach used in the current study did not allow further insight beyond previously published careful descriptions using both immunohistochemistry or dual in

situ hybridization (Gras *et al.*, 2002; Mintz and Scott, 2006; Shutoh *et al.*, 2008). With respect to axons, individual 5-HT boutons were partially obscured by subcellular punctate labeling of 5-HT in soma and dendrites combined with a high degree of neuropil immunolabeling. However, the lack of co-localization of 5-HT and VGLUT3 was confirmed by both SERT and VMAT2. That is, VMAT2 colocalized with VGLUT3 in only a slightly greater population of axons than 5-HT either because of the greater efficiency of detecting VMAT2 within axons and/or the additional fractional colocalization of VGLUT3 with another monoamine labeled by VMAT2.

The results of this study show that within different DR subregions there is a differing extent of colocalization of VGLUT3 and 5-HT within axons. This result parallels previous descriptions of the relationship between VGLUT3 and 5-HT in the forebrain. VGLUT3 and 5-HT colocalize in axons in the ependyma (Shutoh *et al.*, 2008), and this innervation is sometimes referred to as the supraependymal plexus. These axons are well characterized to arise from the caudal DR, both from clusters of cells at the base of the aqueduct and additional ventral cells (Mikkelsen *et al.*, 1997; Simpson *et al.*, 1998). The density of 5-HT innervation of the supraependymal plexus correlates with ciliated regions (Mikkelsen *et al.*, 1997) and may function in regulating the beating rate of cilia and subsequently the flow of cerebral spinal fluid (Nguyen *et al.*, 2001). In addition, prominent colocalization between VGLUT3 and 5-HT in forebrain exists in the dorsal part of the lateral septum (Shutoh *et al.*, 2008) and projections to this area could also arise from the caudal DR (Vertes, 1991; Waselus *et al.*, 2006). Thus the high level of colocalization in these projection areas correlates with the high level of colocalization within the caudal DR, particularly dorsally. The selective colocalization of VGLUT3 and 5-HT in this area gives insight into the organization of re-current axon collateral innervation of the DR. That is, this caudal area that provides dually labeled innervation to the ependyma and/or the septum likely provides recurrent feedback innervation that is also dually labeled. At the same time, these dual 5-HT-VGLUT3 labeled axons provide only limited innervation of other subregions of the DR. These observations provide circumstantial evidence for region-autonomous feedback regulation, at least for this group of cells.

In contrast, colocalization of VGLUT3 and 5-HT is sparse or negligible in subregions of the amygdala, which are innervated by cells located at mid-levels of the DR (Imai *et al.*, 1986; Vertes, 1991), and the striatum, which is preferentially innervated by the rostral DR (Steinbusch *et al.*, 1981; Vertes, 1991; Brown and Molliver, 2000; Waselus *et al.*, 2006). Consistent with these observations, this study showed that 5-HT axons which innervate the mid and rostral DR, and in part could be composed of recurrent axon collaterals, have little colocalization with VGLUT3. However, at the level of cell soma many 5-HT cells have consistently been reported to contain VGLUT3 (Gras *et al.*, 2002; Hioki *et al.*, 2004; Mintz and Scott, 2006; Shutoh *et al.*, 2008). Evidently low expression of VGLUT3, or expression that is post-translationally regulated, does not fill the axonal arbor of these cells. Consistent with this conclusion, a previous study describing the colocalization of VGLUT3 and 5-HT in the forebrain noted that even within a single 5-HT fiber, some, but not all, varicosities may have detectable VGLUT3 (Shutoh *et al.*, 2008).

Within the DR, axons that colocalize 5-HT and VGLUT3 appear to represent a minority population, with single-labeled axons predominating in most areas. This partial colocalization distinguishes the relationship between VGLUT3 and 5-HT in the DR from that between VGLUT3 and acetylcholine in the striatum, which appears to be a more complete co-existence (Gras *et al.*, 2002). The perception that VGLUT3 is widespread in 5-HT axons is created by the high expression of VGLUT3 in the DR, however non-5-HT cells may be a major contributor to this expression.

In this study, it was demonstrated that many non-5-HT cells that contained VGLUT3 were dually labeled for the NK1 receptor. Previous studies have shown that application of substance P in a DR slice preparation increases glutamatergic postsynaptic potentials in 5-HT neurons (Liu *et al.*, 2002). Parallel anatomic studies confirmed that the NK1 receptor is present on glutamate-immunolabeled DR neurons (Commons and Valentino, 2002). Finally, using in vivo single-unit recordings the effects of substance P in the DR are blocked by a non-selective glutamate receptor antagonists, kynurenatate (Valentino *et al.*, 2003; Valentino and Commons, 2005). Thus the colocalization between NK1 and VGLUT3 provides strong evidence that VGLUT3 containing neurons within the DR have local axon collaterals that release glutamate onto resident neurons. Consistent with this conclusion, VGLUT3-containing axons do not prominently colocalize with other monoamines, GABA or acetylcholine.

In this study it was observed that 5-HT and VGLUT3 immunolabeling tend to colocalize within axons only in some regions of the DR, and these areas correspond to origins of dually-immunolabeled projections. VGLUT3 and 5-HT more commonly represent distinct populations of axons in the DR. Non-5-HT VGLUT3 containing cells were often immunolabeled for the NK1 receptor, and the circuitry underlying effects of activating NK1 receptors has been well studied (reviewed by (Valentino and Commons, 2005)). Specifically, activation of NK1 receptors within the DR leads to activation of local glutamatergic axons. Thus the results of this study place VGLUT3 within a predefined functional context in the DR nucleus.

Acknowledgments

Supported by The National Institute on Drug Abuse grant DA-021801; The National Institute for Child Health HD-036379 and the Mary E. Groff Charitable Trust. The assistance of Robin Sperling in this work is appreciated.

References

- Arvidsson U, Riedl M, Elde R, Meister B. Vesicular acetylcholine transporter (VACHT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. *J Comp Neurol* 1997;378(4):454–467. [PubMed: 9034903]
- Barbaresi P. Immunocytochemical localization of substance P receptor in rat periaqueductal gray matter: a light and electron microscopic study. *Journal of Comparative Neurology* 1998;398(4):473–490. [PubMed: 9717704]
- Boulland JL, Qureshi T, Seal RP, Rafiki A, Gundersen V, Bergersen LH, Freneau RT Jr, Edwards RH, Storm-Mathisen J, Chaudhry FA. Expression of the vesicular glutamate transporters during development indicates the widespread corelease of multiple neurotransmitters. *J Comp Neurol* 2004;480(3):264–280. [PubMed: 15515175]
- Brown P, Molliver ME. Dual serotonin (5-HT) projections to the nucleus accumbens core and shell: relation of the 5-HT transporter to amphetamine-induced neurotoxicity. *J Neurosci* 2000;20(5):1952–1963. [PubMed: 10684896]
- Chang YC, Gottlieb DI. Characterization of the proteins purified with monoclonal antibodies to glutamic acid decarboxylase. *J Neurosci* 1988;8(6):2123–2130. [PubMed: 3385490]
- Commons KG, Valentino RJ. Cellular basis for the effects of substance P in the periaqueductal gray and dorsal raphe nucleus. *J Comp Neurol* 2002;447(1):82–97. [PubMed: 11967897]
- Freneau RT Jr, Burman J, Qureshi T, Tran CH, Proctor J, Johnson J, Zhang H, Sulzer D, Copenhagen DR, Storm-Mathisen J, Reimer RJ, Chaudhry FA, Edwards RH. The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. *Proc Natl Acad Sci U S A* 2002;99(22):14488–14493. [PubMed: 12388773]
- Gabellec MM, Panzanelli P, Sassoe-Pognetto M, Lledo PM. Synapse-specific localization of vesicular glutamate transporters in the rat olfactory bulb. *Eur J Neurosci* 2007;25(5):1373–1383. [PubMed: 17425564]

- Gras C, Herzog E, Belenchi GC, Bernard V, Ravassard P, Pohl M, Gasnier B, Giros B, El Mestikawy S. A third vesicular glutamate transporter expressed by cholinergic and serotonergic neurons. *J Neurosci* 2002;22(13):5442–5451. [PubMed: 12097496]
- Harkany T, Holmgren C, Hartig W, Qureshi T, Chaudhry FA, Storm-Mathisen J, Dobszay MB, Berghuis P, Schulte G, Sousa KM, Fremeau RT Jr, Edwards RH, Mackie K, Ernfors P, Zilberter Y. Endocannabinoid-independent retrograde signaling at inhibitory synapses in layer 2/3 of neocortex: involvement of vesicular glutamate transporter 3. *J Neurosci* 2004;24(21):4978–4988. [PubMed: 15163690]
- Herzog E, Gilchrist J, Gras C, Muzerelle A, Ravassard P, Giros B, Gaspar P, El Mestikawy S. Localization of VGLUT3, the vesicular glutamate transporter type 3, in the rat brain. *Neuroscience* 2004;123(4):983–1002. [PubMed: 14751290]
- Hioki H, Fujiyama F, Nakamura K, Wu SX, Matsuda W, Kaneko T. Chemically specific circuit composed of vesicular glutamate transporter 3- and preprotachykinin B-producing interneurons in the rat neocortex. *Cereb Cortex* 2004;14(11):1266–1275. [PubMed: 15142960]
- Imai H, Steindler DA, Kitai ST. The organization of divergent axonal projections from the midbrain raphe nuclei in the rat. *J Comp Neurol* 1986;243(3):363–380. [PubMed: 2419370]
- Jackson J, Bland BH, Antle MC. Nonserotonergic projection neurons in the midbrain raphe nuclei contain the vesicular glutamate transporter VGLUT3. *Synapse* 2009;63(1):31–41. [PubMed: 18925658]
- Jacobs BL, Azmitia EC. Structure and function of the brain serotonin system. *Physiol Rev* 1992;72(1):165–229. [PubMed: 1731370]
- Liu R, Ding Y, Aghajanian GK. Neurokinins activate local glutamatergic inputs to serotonergic neurons of the dorsal raphe nucleus. *Neuropsychopharmacology* 2002;27(3):329–340. [PubMed: 12225691]
- Michelsen KA, Schmitz C, Steinbusch HW. The dorsal raphe nucleus--from silver stainings to a role in depression. *Brain Res Rev* 2007;55(2):329–342. [PubMed: 17316819]
- Mikkelsen JD, Hay-Schmidt A, Larsen PJ. Central innervation of the rat ependyma and subcommissural organ with special reference to ascending serotonergic projections from the raphe nuclei. *J Comp Neurol* 1997;384(4):556–568. [PubMed: 9259489]
- Mintz EM, Scott TJ. Colocalization of serotonin and vesicular glutamate transporter 3-like immunoreactivity in the midbrain raphe of Syrian hamsters (*Mesocricetus auratus*). *Neurosci Lett* 2006;394(2):97–100. [PubMed: 16266785]
- Molliver ME. Serotonergic neuronal systems: what their anatomic organization tells us about function. *J Clin Psychopharmacol* 1987;7(6 Suppl):3S–23S. [PubMed: 3323265]
- Nguyen T, Chin WC, O'Brien JA, Verdugo P, Berger AJ. Intracellular pathways regulating ciliary beating of rat brain ependymal cells. *J Physiol* 2001;531(Pt 1):131–140. [PubMed: 11179397]
- Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*. San Diego: Academic Press; 1998.
- Sandoval V, Riddle EL, Hanson GR, Fleckenstein AE. Methylphenidate redistributes vesicular monoamine transporter-2: role of dopamine receptors. *J Neurosci* 2002;22(19):8705–8710. [PubMed: 12351745]
- Schafer MK, Varoqui H, Defamie N, Weihe E, Erickson JD. Molecular cloning and functional identification of mouse vesicular glutamate transporter 3 and its expression in subsets of novel excitatory neurons. *J Biol Chem* 2002;277(52):50734–50748. [PubMed: 12384506]
- Seal RP, Akil O, Yi E, Weber CM, Grant L, Yoo J, Clause A, Kandler K, Noebels JL, Glowatzki E, Lustig LR, Edwards RH. Sensorineural deafness and seizures in mice lacking vesicular glutamate transporter 3. *Neuron* 2008;57(2):263–275. [PubMed: 18215623]
- Shutoh F, Ina A, Yoshida S, Konno J, Hisano S. Two distinct subtypes of serotonergic fibers classified by co-expression with vesicular glutamate transporter 3 in rat forebrain. *Neurosci Lett* 2008;432(2):132–136. [PubMed: 18222609]
- Simpson KL, Fisher TM, Waterhouse BD, Lin RC. Projection patterns from the raphe nuclear complex to the ependymal wall of the ventricular system in the rat. *J Comp Neurol* 1998;399(1):61–72. [PubMed: 9725701]
- Steinbusch, H. Serotonin-immunoreactive neurons and their projections in the CNS. In: Bjorklund, A.; Hokfelt, T.; Kuhar, M., editors. *Handbook of Chemical Neuroanatomy*. Amsterdam: Elsevier Science Publishers; 1984. p. 68-121.

- Steinbusch HW, Nieuwenhuys R, Verhofstad AA, Van der Kooy D. The nucleus raphe dorsalis of the rat and its projection upon the caudatoputamen. A combined cytoarchitectonic, immunohistochemical and retrograde transport study. *J Physiol (Paris)* 1981;77(23):157–174. [PubMed: 6169825]
- Valentino RJ, Bey V, Pernar L, Commons KG. Substance P Acts through local circuits within the rat dorsal raphe nucleus to alter serotonergic neuronal activity. *J Neurosci* 2003;23(18):7155–7159. [PubMed: 12904475]
- Valentino RJ, Commons KG. Peptides that fine-tune the serotonin system. *Neuropeptides* 2005;39(1):1–8. [PubMed: 15627494]
- Varea E, Nacher J, Blasco-Ibanez JM, Gomez-Climent MA, Castillo-Gomez E, Crespo C, Martinez-Guijarro FJ. PSA-NCAM expression in the rat medial prefrontal cortex. *Neuroscience* 2005;136(2):435–443. [PubMed: 16216431]
- Vertes RP. A PHA-L analysis of ascending projections of the dorsal raphe nucleus in the rat. *J Comp Neurol* 1991;313(4):643–668. [PubMed: 1783685]
- Vigna SR, Mantyh CR, Soll AH, Maggio JE, Mantyh PW. Substance P receptors on canine chief cells: localization, characterization, and function. *J Neurosci* 1989;9(8):2878–2886. [PubMed: 2475593]
- Waselus M, Galvez JP, Valentino RJ, Van Bockstaele EJ. Differential projections of dorsal raphe nucleus neurons to the lateral septum and striatum. *J Chem Neuroanat* 2006;31(4):233–242. [PubMed: 16540283]

Figure 1.

Architecture of the DR revealed with immunolabeling for VMAT2, which detects 5-HT as well as dopamine neurons in the DR (**A-C**), and corresponding topography of VGLUT3 (**A'-C'**). Merged VMAT2 (green) and VGLUT3 (red) visible in panels **A''-C''**. **A-A''**. At rostral levels VGLUT3 is visible dorsal (RD boxes) and ventral (RV). **B-B''**. At mid-rostrocaudal levels three separate areas were analyzed, mid-dorsal (MD), mid-ventral (MV) and lateral wings (LW). The highest levels of overall VGLUT3 immunoreactivity occur at the center of the nucleus (C, in panel B'). LW area has comparatively less dense immunoreactivity. **C-C''**. Potential overlap between VGLUT3 and 5-HT or VMAT2 is seen caudal and dorsal (CD) particularly at the base of the aqueduct (arrows in C' and C''). CV= caudal ventral. All panels same scale, Bar in C=100 microns.

Figure 2.

Immunolabeling for VGLUT3 in representative DR subregions. **A.** and **B.** VGLUT3 labeling consists of axon-like punctate labeling abundantly distributed through the neuropil (arrowheads) as well as more diffusely labeled cell soma (arrows). **C.** The lateral wings of the DR shows less dense punctate labeling. Panels same scale, Bar=50 microns.

Figure 3.

Percent of VGLUT3 axons double-labeled (DL) for other neurotransmitter markers in different DR subregions. Colocalization of VMAT2 and VGLUT3 is slightly greater than that for 5-HT and VGLUT3 in all subregions, with robust colocalization occurring caudally in the nucleus. A small percent of remaining axons are dually immunolabeled for markers of GABAergic or cholinergic neurotransmission.

Figure 4.

Colocalization of VGLUT3 (red) was quantified with respect to 5-HT, VMAT2, GAD65, GAD65, and VACHT (green in panels A-E respectively). For quantification, images representing each subregion of the DR were photographed and sampled by arbitrary placement of a 25×25 micron box (representative boxed areas are shown and enlarged in ' panels). **A.** Although 5-HT and VGLUT3 dually labeled axons are sometimes detected, the majority of VGLUT3 axons lack detectable 5-HT immunolabeling in most regions of the DR. An image from a rostral-ventral location is depicted. **B.** VMAT2 immunolabeling yields less somatodendritic labeling than 5-HT and more defined boutons, but similarly in most regions of the DR does not colocalize with VLGUT3, mid dorsal area depicted. Dual labeling for

GAD65 (**C**), GAD67 (**D**) and VACht (**E**) result in defined labeling of boutons that lack co-incident VGLUT3 immunoreactivity. In **C-E**, mid-ventral locations are depicted. Scale, boxed are in A-E is 25×25 μm .

Figure 5.

A-A'' A high degree of colocalization was observed between 5-HT (green) and VGLUT3 (red) in the axons that innervate the ependyma. A few of the many dually labeled varicosities are indicated with an arrowhead of the merged images **A**, and each fluorophor viewed independently **A'**, labeling for VGLUT3 and **A''**, labeling for 5-HT. Bar=10 microns. **B.** Neurons located caudally in clusters at the base of the aqueduct are known to give rise to innervation of the ependyma. In this area there is a high density of VMAT2 (green), which identifies all 5-HT axons as well as other monoamines, and VGLUT3 (red). Bar=100 microns. Panel **C.** is another focal plane from approximately the bracketed area in **B.** Panel **D.** is an enlarged view of the boxed area in **C.** The majority of varicosities in this area are dually labeled

for VGLUT3 (red) VMAT2 (green), paralleling colocalization of VGLUT3 and 5-HT. Arrowheads point to some of the many dually labeled boutons, although axons single labeled for VGLUT3 (red, arrows, **D'**) or VMAT2 (green, double arrowheads, **D''**) are still detectable. Bar in C=25 microns, bar in D=5 microns.

Figure 6.

Mapping of VGLUT3-immunoreactive cells that lack 5-HT in the DR from caudal (A) to rostral (D) distance from Bregma according to the atlas of Paxinos and Watson (1998) is noted. For each representative section every cell body with detectable VGLUT3 immunolabeling was marked with a black dot, and 5-HT immunolabeled cells were marked with a gray dot. Colocalization between VGLUT3 and 5-HT was not evaluated. VGLUT3 cells appear to have a preferential distribution toward the midline as well as toward a mid-dorsoventral location, and are present though the entire rostro-caudal extent of the nucleus. Bar = 500 microns.

Figure 7. VGLUT3 (green) labeled cell bodies have immunolabeling for the neurokinin 1 (NK1) receptor (red). 5-HT cells are identified by immunolabeling for tryptophan hydroxylase (TPOH, blue). **A** At a mid-dorsal location, VGLUT3 cells with membrane-associated NK1 labeling (arrows) are interspersed between larger TPOH containing cells (blue). **B**. At mid levels in the center of the nucleus many VGLUT3 containing cells are visible with NK1 immunolabeling (arrows). Arrowhead indicates a VGLUT3 cell lacking both detectable NK1 or TPOH, double-headed arrow indicates a TPOH labeled cell with detectable VGLUT3. **A' B'** show VGLUT3 and TPOH immunolabeling; **A'' B''** show TPOH and NK1 immunolabeling. A and B same scale, bar in A=20 microns.

Table 1

Antigen	Species	Company, Location	Catalog #	Dilution
VGLUT3	guinea pig	Millipore (Chemicon), Billerica, MA	AB5421	1:2000 or 1:4000
5-HT	rabbit	Immunostar, Hudson, WI	20080	1:10,000
SERT	rabbit	EMD (Calbiochem), Madison, WI	PC177L	1:1000
VMAT2	rabbit	Millipore (Chemicon)	AB1767	1:1000
GAD65	mouse	Developmental Studies Hybridoma Bank, Iowa City, IA	GAD6	1:100
GAD67	mouse	Millipore (Chemicon)	MAB5406	1:1000
VACTH	goat	Millipore (Chemicon)	AB1578	1:1000
TPH	sheep	Millipore (Chemicon)	AB1541	1:1000
NK1	rabbit	Novus Biologicals, Littleton, CO	NB 300-101	1:1000