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Coincidence of Neurokinin 1 Receptor with the Vesicular Glutamate Transporter 3 (VGLUT3) in the Rat Forebrain

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Abstract

The third vesicular glutamate transporter (VGLUT3) is expressed in a subset of cholinergic and GABAergic neurons in the forebrain. In this study the distribution of VGLUT3 was mapped in relation to the receptor for substance P, neurokinin 1 (NK1), which has been independently reported within cholinergic and GABAergic neurons in a similar distribution. Dual immunofluorescence labeling techniques were used, sometimes in combination with triple labeling for the vesicular acetylcholine transporter (VACHT), to identify cholinergic cells. Virtually all cells immunolabeled for VGLUT3 in the nucleus accumbens core and shell regions, ventral pallidum, olfactory tubercle and caudate putamen were cholinergic and also contained immunolabeling for the NK1 receptor. In the hippocampal formation where VGLUT3 has been described in GABAergic neurons, colocalization between NK1 and VGLUT3 was also common but less complete. Cells double labeled for NK1 and VGLUT3 were most prevalent in stratum radiatum in the CA1 subfield. In the habenula VGLUT3 was also found within NK1 receptor immunolabeled neurons. However, there were some areas where neurons containing these two proteins were separate populations including the cerebral cortex and median raphe nucleus. These results reveal a trend for VGLUT3 to localize within neurons containing the NK1 receptor in several areas of the forebrain.

Keywords

substance P; acetylcholine; hippocampus; neurokinin 1; nucleus accumbens

Introduction

VGLUT3 is one of three transporter isoforms that fills synaptic vesicles with glutamate (reviewed in [6]). While VGLUT1 and VGLUT2 are expressed in the majority of glutamatergic cortical and subcortical neurons, respectively [5,10,12,13,29], VGLUT3 is expressed in neurons and brain regions that were not previously thought to use glutamate as a neurotransmitter [4,9,25]. For example, VGLUT3 is present in a subset of cholinergic neurons in the basal forebrain and caudate putamen [4,9,20,25]. Recent studies have raised the possibility that VGLUT3, because of its ionic balance, helps to load synaptic vesicles with acetylcholine [8]. In addition, VGLUT3 is highly expressed in hippocampal interneurons where it often colocalizes with the neurotransmitter GABA [4,9,11]. However, the function of potential co-neurotransmission of acetylcholine or GABA with VGLUT3-transported glutamate remains to be fully elucidated.

VGLUT3 is also highly expressed in the dorsal raphe nucleus where it is present in both serotonergic and non-serotonergic cell bodies [9,19,26]. Recently, VGLUT3 has been localized to neurons that contain the receptor for substance P (SP), neurokinin 1 (NK1), in the dorsal raphe nucleus [3]. Previously cholinergic interneurons in the caudate putamen have been shown to contain VGLUT3, and independently, NK1 receptors [14,23,24]. Taken together these observations raise the possibility that colocalization of NK1 and VGLUT3 may occur within many different brain areas. To address this possibility, in this study the extent of colocalization between NK1 and VGLUT3 was examined in the forebrain using dual-immunofluorescence microscopy, sometimes in combination with triple immunolabeling for the vesicular acetylcholine transporter (VACHT).

Materials and Methods

To perform dual or triple immunolabeling for VGLUT3, NK1 and VACHT, rats were perfused with 4% paraformaldehyde while anesthetized with Nembutal (100 mg/kg i.p.). These procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Children's Hospital, Boston. The brains were removed, equilibrated in 30% sucrose, frozen and sectioned 40 μ m thick using a rotary microtome. Sections were collected and processed free-floating. Primary antisera included anti-VGLUT3 raised in guinea pig (Chemicon International/Millipore 1:2000) [3] and anti-NK1 raised in rabbit (Novus biologicals, 1:1000) [3], sometimes in combination with an anti-VACHT antisera raised in goat (Chemicon International/Millipore 1:2000) [2]. Primary antisera were diluted together in a 0.1 M phosphate buffer containing saline pH = 7.4, bovine serum albumin (0.5%), triton (0.1%) and sodium azide (0.05%) over night at room temperature or for two days at 4°C. Secondary antisera diluted 1:200 were conjugated to AlexaFluor 488, AlexaFluor 647 (Molecular Probes) or CY3 (Jackson ImmunoResearch). Secondary antisera were raised in donkey, diluted together and had no cross-reactivity to other relevant species. Sections through the forebrain were examined for the coincidence of labeling within cell bodies and were photographed using epifluorescence or spinning-disc confocal illumination (Olympus IX81 DSU). Images were adjusted for brightness and contrast using Adobe Photoshop.

To quantify the amount of co-existence between VGLUT3 and NK1 immunolabeling in the hippocampal formation, 5-15 sections with clear labeling were counted per region. For each region, sections were visualized directly with epifluorescence illumination and cells containing VGLUT3 alone or both NK1 and VGLUT3 were counted and tabulated. Colocalization was judged by visualizing each fluorophore first independently and then together using a dual-band pass filter. Schematic representation was made by placing filled or open circles to represent the abundance of single- or double-labeled cells within the appropriate lamina in proportion to their sampling frequency. Since each region was sampled independently, mediolateral location and between subfield comparisons are not valid.

Results

Immunolabeling for VGLUT3 often consisted of diffuse perisomatic labeling combined with punctate axon-like labeling. In contrast, NK1 receptor immunoreactivity appeared to be associated with the plasma membrane of cells, outlining their somatic and dendritic profiles. VGLUT3 immunolabeled cell bodies were found scattered throughout the ventral forebrain within the nucleus accumbens core and shell region, ventral pallidum, olfactory tubercle and striatum. Virtually all VGLUT3 immunolabeled cells in these areas were dually labeled for NK1, although NK1 was also found in the absence of VGLUT3 immunolabeling (Fig. 1). Triple immunolabeling for VGLUT3, NK1 and VACHT revealed that VGLUT3 cells with NK1 receptor in these areas were cholinergic (Fig. 2). In the basal forebrain and striatum,

NK1 cells that lacked VGLUT3 immunolabeling also lacked VACHT labeling. In addition, no VGLUT3 cells that lacked VACHT were detected. VACHT labeling precisely overlay cell body labeling for VGLUT3. However, the presence of single labeled cells for VACHT in the nucleus of the diagonal band and single labeled cells for VGLUT3 in the cortex and hippocampus within the same tissue sections demonstrates that there was no cross-detection of antigens.

In forebrain areas where VGLUT3 does not colocalize with VACHT, somatic labeling for VGLUT3 colocalized with that for the NK1 receptor. A high density of cells dually labeled for VGLUT3 and NK1 were detected in the hippocampal formation, and the amount of colocalization varied with subfield and lamina (Fig. 3). When VGLUT3 was present in cholinergic cells, virtually all of these contained NK1 immunolabeling, however in the hippocampus VGLUT3 cells that lacked NK1 were also detected. The area with the highest colocalization between NK1 and VGLUT3 was the CA1 in stratum radiatum (89% of VGLUT3 cells were dually labeled for NK1). In other lamina of CA1 dually labeled cells accounted for 55-62% of total VGLUT3 labeled cells. In the CA3 region, 71% of VGLUT3 cells in stratum radiatum had NK1 immunolabeling, while co-localization in other lamina ranged from 44-50%. Within the dentate gyrus, almost all VGLUT3 cells were located either in the subgranular zone of the granule cell layer or within the hilus and 47% and 48% of cells in these areas respectively were dually labeled for NK1. The total number of VGLUT3 labeled cells counted to determine the percent colocalization with NK1 was 96 in CA1, 19 in CA3 and 120 in the dentate gyrus. Additional cells containing immunolabeling for NK1 in the absence of VGLUT3 were present but not quantified.

VGLUT3 cells were detected in the basolateral amygdala (BLA) and habenula at a low density. In each of these areas as well VGLUT3 occasionally co-existed with NK1 immunolabeling, but not VACHT. Consistent with previous reports, we found the BLA was densely innervated by cholinergic axons dually labeled for VGLUT3 [20].

In the cortex, the frequency of coexistence between VGLUT3 and NK1 was negligible. However, sparse cells containing immunolabeling for each antigen alone were observed (not shown). We previously reported that VGLUT3 was present in NK1-immunolabeled non-serotonin cells in the dorsal raphe nucleus [3]. In contrast to this observation there was negligible colocalization between VGLUT3 and NK1 in the median raphe nucleus (Fig. 3D).

Discussion

The results of this study suggest that many cells that contain VGLUT3 in the forebrain also have the receptor for SP, NK1. These include all VGLUT3-containing cholinergic cells in the basal forebrain and striatum. Moreover, many VGLUT3-immunolabeled cells in the hippocampus and other areas have NK1 receptor immunolabeling. This colocalization suggests that activating these cells with SP may be a means to further understand the role of VGLUT3 in neurotransmission. Taken together with the previous observation that VGLUT3 is present in neurons immunolabeled for NK1 receptors in the dorsal raphe nucleus [3], these findings indicate that colocalization of this transporter and receptor repeats within several, but not all brain regions.

Methodological Considerations

The pattern of immunolabeling for VGLUT3 was identical to previous descriptions using other antibodies or using *in situ* hybridization to determine gene expression patterns. For example VGLUT3-immunolabeled cell bodies were identified in expected cholinergic cells in the basal forebrain [11,20,25], and VGLUT3 immunolabeling was present in axon arbors

that densely innervated the pyramidal layers of the hippocampus [27], and the BLA [20]. VGLUT3 immunolabeling tended to diffusely fill the soma of cells and was thus clearly distinguished from NK1-receptor immunolabeling, which outlined the plasma membrane. VGLUT3 and VACHT immunolabeling appeared to precisely overlap, which is consistent with the idea that these two transporters may reside on the same vesicle populations [8].

Coexistence of VGLUT3- and NK1- immunolabeling in Cholinergic Neurons

The finding that cholinergic cells containing VGLUT3 also have immunolabeling for NK1 receptor is consistent with the known distribution of each of the antigens individually to cholinergic cells. Specifically previous studies have consistently reported that VGLUT3 is present in a subpopulation of cholinergic neurons throughout the magnocellular basal forebrain; in the striatum, nucleus accumbens, ventral pallidum and olfactory tubercle [4,9,20,25]. In the striatum, NK1 receptors are present on aspiny cholinergic interneurons [7,14,23]. There are some NK1 positive cells in the striatum that lack acetylcholine and VGLUT3 in the striatum and these may contain somatostatin and neuronal nitric oxide synthase [14,17]. Cholinergic cells that contain VGLUT3 in the basal forebrain appear to selectively innervate the BLA, while there is a low incidence of VGLUT3 immunolabeling within cholinergic axons in many of the other areas [20].

VGLUT3 Hippocampal Interneurons

Interestingly VGLUT3- and NK1-immunolabeling also colocalize in non-cholinergic cells, particularly in the hippocampus. Previously NK1 receptors have been reported to localize to large multipolar cells in CA1 that contain cholecystokinin (CCK) [1]. In addition, NK1 receptors are found in pyramidal or fusiform cells in the dentate gyrus that contain parvalbumin, CCK or neuropeptide Y, each of these cell types innervates different target areas [1]. Taken together, these observations are consistent with a previous study that reported that VGLUT3 cells in the hippocampus form a subset of CCK-positive cells [27]. Thus indicating that NK1-immunolabeled cells with VGLUT3 are also likely to contain CCK. CCK-containing cells in the hippocampus have axons that ramify through the pyramidal cell regions forming axon-somatic synapses [15].

SP has a direct excitatory effect on neurons with NK1 receptors in part by suppressing a resting potassium conductance [28]. Existing studies of the electrophysiological effects of SP acting at the NK1 receptor drives inhibitory interneurons. More specifically, it has been reported that SP increases inhibitory postsynaptic potentials (IPSPs) in CA1 neurons that are likely mediated by GABA_A receptors while having no consistent effect on spontaneous or evoked excitatory postsynaptic potential (EPSPs) [21]. This study also reported that SP activates inhibition under conditions where excitatory neurotransmission is blocked. However, there have been links reported between NK1 receptors and glutamate neurotransmission in the hippocampus. For example, SP acting at NK1 receptors facilitates induction of status epilepticus, and this effect is accompanied by enhanced glutamate release [18]. Kouznetsova and Nistri [16] found that SP acting at NK1 receptors decreased evoked EPSPs and IPSPs, while increasing spontaneous GABAergic synaptic events in CA1, and although these events were thought to be inhibitory, there were greatly reduced by a non-selective glutamate receptor antagonist, kynurenic acid, suggesting they depended on an excitatory synapse. However there maybe multisynaptic mechanisms that could underlie this effect as well [22].

Although the colocalization of VGLUT3- with NK1 receptor-immunolabeling repeats in several areas, it does appear to vary by region. For example, VGLUT3 cells in the cortex and the median raphe lack NK1 receptor immunolabeling. In addition, in all regions additional cells with NK1 receptor immunolabeling that lacked VGLUT3 were found.

Conclusion

The finding that VGLUT3 is present in NK1-immunolabeled neurons raises several new possibilities for the functional role of VGLUT3 within these neurons based upon prior knowledge of NK1 function. Likewise, the data suggest new avenues to explore to fully understand the role of VGLUT3 in filling vesicles with glutamate and for how that function contributes to behavior.

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Fig. 1.

Dual immunolabeling for VGLUT3 and NK1 in the basal forebrain nucleus accumbens core (AcbC) and shell (AcbSh) region show virtually all cells with immunolabeling VGLUT3 also contain immunolabeling for NK1 receptor, although additional cells with NK1 alone are present. **A.** VGLUT3 identifies scattered cells. **A'.** NK1 has a similar distribution. **A''.** Overlay of VGLUT3 (green) and NK1 (red). Boxed regions of core region shown at higher magnification in **(B)** and shell region shown in **(C)**. **B.** VGLUT3 immunolabeling diffusely fills perisomatic region of cells, whereas NK1 immunolabeling defines the overlaying cytoplasm in dually immunolabeled cells (arrowheads). Additional cells that contain NK1 immunolabeling alone are detected (arrow). **B'** and **B''** show labeling for each antigen individually. **C.** In the AcbSh region NK1- and VGLUT3-immunolabeling are similarly colocalized. Bar in **A''** = 250 μ m, **B** = 60 μ m, **B** and **C** same scale.

Fig. 2.

Cells dually labeled for NK1 and VGLUT3 are cholinergic in the basal forebrain and striatum. **A.** The same cells in each image (arrowheads) of the nucleus accumbens shell region have detectable VGLUT3 (**A**), NK1 (**A'**) and VAcHt (**A''**), overlay of all three fluorophores with VGLUT3 (green), NK1 (red), and VAcHt (fuchsia) in panel **A'''**. **B.** Similarly in the striatum, cholinergic cells with VGLUT3 also contain NK1 (arrowheads), while there are addition cells that contain NK1 alone (arrow in **B'** and **B'''**). All panels same scale, bar in **B'''** = 60 μ m.

Fig. 3.

NK1 and VGLUT3 were colocalized in the hippocampal formation (A-C) but not in the median raphe nucleus (D). **A.** A photomontage of three cells in the dentate gyrus that contain both NK1 outlining the cytoplasm (red) and VGLUT3 (green, arrow heads). The white line marks the junction between the granule cell layer (GCL) and the hilus, these cells are located just at the border in the subgranular layer. **B.** Two neighboring interneurons located in stratum radiatum of the CA1 region are dually labeled for NK1 and VGLUT3 (arrowheads). Both panels same scale bar = 20 μ m. **C.** Relative proportions of cells dually labeled for VGLUT3 and NK1 (filled circles) and cells containing only VGLUT3 (open circles) by laminar location and subfield, CA1, CA3 and dentate gyrus (DG). Several

sections were sampled and single VGLUT3 labeled and cells dually labeled for VGLUT3 and NK1 were counted in each lamina. Medio-lateral distribution of cells is compressed and between subfield comparisons are not quantitative since each region was sampled independently from a different number of sections. For the CA1 region, most cells containing VGLUT3 were located in stratum radiatum (86% or 66/77) and the majority of these also contained NK1 immunolabeling (89% or 59/66). In all the remaining areas, the percentage of VGLUT3 cells that also contained NK1 averaged 44-62%, except for stratum radiatum of the CA3 region where 71% were dually labeled. Total number of cells sampled in CA1=96, CA3=19, and DG=120. Lamina abbreviations are O, oriens; P, pyramidale; L, lucidum; R, radiatum; M, molecular layer; sG, subgranular layer; H, hilus. **D.** Some areas exhibited very little colocalization between NK1 and VGLUT3 including the median raphe nucleus. VGLUT3 is present in large cells (arrowheads) that are distinct from NK1 immunolabeled cells in the same field (arrows) that are typically smaller in diameter. Scale bar = 40 um.