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Identification of a Novel Vamp1 Splice Variant in the Cochlear Nucleus

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Abstract

Cochlear nucleus neurons propagate auditory impulses to higher brain stem centers at rapid firing rates with high fidelity. Intrinsic to synaptic transmission are the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins engaged in vesicle fusion, release and recycling. Herein we report a novel splice variant of the SNARE protein Vamp1 (vesicle-associated membrane protein 1) within the cochlear nucleus. We previously demonstrated, through serial analysis of gene expression and microarray studies, that Vamp1 is differentially expressed among the subdivisions of the rat cochlear nucleus. The 3′ end of this transcript, however, was poorly characterized and we could not initially confirm our findings. In this study, we designed RT-PCR primers using conserved 5′ regions and the mouse 3′ domain to validate the expression of Vamp1. Several species of Vamp1 were subsequently amplified from a rat brain cDNA library including a full length clone of Vamp1as and a novel splice variant we termed Vamp1nv. Using regional brain libraries Vamp1nv showed expression in the medulla and lack of expression in the cortex, cerebellum and thalamus. Expression of Vamp1nv was further confirmed and characterized by RT-PCR and real-time PCR in each of the cochlear nucleus subdivisions. The predicted protein sequence for Vamp1nv demonstrates a unique modification of the carboxy-terminal end of the protein as compared to known variants. This includes the appearance of two intra-vesicular serine residues with high predicted potential as kinase phosphorylation sites. Such splice variants of Vamp1 may alter the kinetics of SNARE complex formation and vesicle release and impart unique features to expressing neurons. This may be important for central auditory function and contribute to the distinct physiological properties observed in auditory neurons.

Keywords

cochlear nucleus; synaptic vesicle; Vamp1; splice variant; auditory

1. INTRODUCTION

The cochlear nucleus is the first central nervous system processing center for all information encoded in auditory nerve fibers returning from the peripheral cochlea. The auditory nerve distributes fibers in tonotopic distribution to three subdivisions of the cochlear nucleus. These subdivisions, the anterior-ventral (AVCN), posterior-ventral (PVCN) and dorsal (DCN)

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cochlear nuclei, have unique classes of neurons which filter information from the incoming auditory nerve and distribute fibers to multiple higher brain stem centers (Cant and Benson, 2003). Many of the contacts between cochlear nucleus neurons and other auditory centers are characterized by morphologically distinct synaptic endings. These include the large endbulbs of Held on AVCN bushy cells and the calyces of Held where cochlear nucleus bushy cells contact superior olivary neurons (Oleskevich et al., 2004). These endings provide high fidelity and rapid transmission of auditory signals along the sound localization pathway. The molecular machinery necessary for such transmission includes synaptic proteins that ensure rapid and repetitive neurotransmitter release (Sakaba et al., 2005). We postulate that the characteristic firing patterns of cochlear nucleus neurons are determined, in part, by differential expression of synaptic vesicle proteins and isoforms.

Neurotransmitter release at the synaptic ending requires the coordinated interaction of multiple vesicle and cell membrane SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins (Sudhof, 2004). The neurotransmitter containing vesicles dock along the synaptic zone and are primed to respond to an action potential. The influx of Ca^{2+} as the action potential reaches the axon terminal induces fusion of the vesicle with the cell membrane and release of neurotransmitter. The vesicle is recycled through endocytosis, filled with neurotransmitter and moved to the reserve pool of vesicles to await docking and re-release. Among the proteins important in the priming and fusion of synaptic vesicles are the Vamps (vesicle-associated membrane proteins) and synaptotagmins on the vesicle side, and syntaxin1A/B and SNAP-25 on the plasma membrane side.

Vamp is often referred to as synaptobrevin of which there are 2 synapse associated forms: Vamp1 and Vamp2. Vamp1 and Vamp2 are differentially distributed throughout the brain suggesting that each isoform imparts specific synaptic properties to their respective neurons (Raptis et al., 2005, Elferink et al., 1989). Vamp1 is less common than Vamp2 but they are similar in amino acid sequence with differences most notable in the amino-terminus proline rich region and the c-terminus hydrophobic region. Vamp1 interacts with SNAP-25 in the formation of SNARE complexes during vesicle priming and fusion. There is a conserved hydrophobic region that spans the vesicle membrane and a short intravesicular c-terminus that differs among known splice variants (Berglund et al., 1999). Vamp1 has been reported in association with the cochlear nucleus (Trimble et al., 1990).

In order to understand genetic determinants of auditory neuron function we performed serial analysis of gene expression (SAGE) and microarray analyses on the individual subdivisions of the cochlear nucleus (Friedland et al., 2006). Among the significantly differentially expressed genes between regions was Vamp1. Vamp1 expression was directly identified by microarray and indirectly indicated by a 10-base pair SAGE tag correlated with Vamp1 by the NCBI SAGEMap database. This 10-base pair nucleotide sequence, however, was not present in reported mRNA sequences for rat Vamp1 wild-type or two known splice variants, Vamp1as and Vamp1b. Additional RT-PCR experiments for Vamp1, to identify the mRNA sequence encoding this SAGE tag, subsequently identified a novel splice variant. This novel variant, Vamp1nv, differs significantly in the intra-vesicular c-terminus from other variants and contains two kinase recognition and phosphorylation sites. Real-time RT-PCR was performed with primer-probes specific for each splice variant to assess for differential expression of these species in the cochlear nucleus.

2. METHODS

This study was performed in accordance with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals, the *NIH Guide for the Care and Use of Laboratory Animals*, and the Animal Welfare Act (7 U.S.C. et seq.). The animal use protocol

was approved by the Institutional Animal Care and Use Committee (IACUC) of the Medical College of Wisconsin.

2.1 RNA extraction

Cochlear nuclei were dissected from 20 female Brown Norway rats of 6–8 weeks of age. Rats were anesthetized with Nembutal (50mg/kg IP), decapitated and the brains dissected. The cochlear nucleus, which forms a prominent bulge on the posterolateral brain stem, was dissected off and grossly separated into AVCN, PVCN and DCN (Friedland et al., 2006). Tissue from each subdivision was pooled. The tissues were homogenized in RNase-free Eppendorf tubes. RNA was extracted using the TRIzol protocol (Invitrogen, Carlsbad, CA) and precipitated in isopropyl alcohol, washed with 75% ethanol and then dissolved in RNase-free water. Purity and concentration of extracted RNA was determined by spectrophotometry. Integrity of the RNA was confirmed by gel electrophoresis by the presence of 28s and 18s ribosomal RNA bands. A minimum of 3 different pools of RNA (20 rats per pool) was used for each of the experiments in this report.

2.2 PCR, nested PCR and characterization of Vamp1 splice variants

Vamp1 was indicated as being present within the cochlear nucleus by our previous experiments utilizing serial analysis of gene expression (Friedland et al., 2006). Serial analysis of gene expression (SAGE) uses a 10 base pair sequence (i.e., tag) from the 3′ end of the transcript as a marker of the gene product. We identified multiple copies of tag TATTCTCAAC which mapped to Vamp1 using the NCBI SAGEMap reliable database (http://www.ncbi.nlm.nih.gov/SAGE/). Examination of all known rat Vamp1 sequences (i.e., Vamp1, Vamp1as, Vamp1b) in Genbank at the time failed to identify this tag but the sequence was identified in the rat genome just downstream of known exons for Vamp1. Primers were designed to amplify a sequence from the conserved 5′ region to the SAGE tag sequence. A forward primer based in exon 1, located upstream of the start codon, was used for these amplifications (For1-4). The reverse downstream primer was based upon the known SAGE tag (tagRev). For Vamp1b, which does not have a full length mRNA sequence, the reverse primer was based in its exon 4 downstream of a variable splice site (1Rev). This site is in an intron in all other Vamp1 variants. The sequence for determining full length Vamp1nv used For1-4 and tagRev to generate a full length transcript. Primer sets and their expected amplicon sizes are noted in Table 1 and their locations within Vamp1 in Figures 1 and 2. Since these experiments, a full length sequence for Vamp1 has been added to Genbank (BC092206) which does include the SAGE tag.

PCR reactions were performed in the three subdivisions of the cochlear nucleus, in whole rat brain library (Invitrogen, Carlsbad, CA), and in rat brain libraries of the cortex, thalamus, medulla and cerebellum (gift of Dr. M. Bento Soares, Children's Memorial Research Center, Chicago, Ill.). Approximately 0.5 to 2.0μg aliquots of total RNA were used to generate cDNA for these experiments. RNA was treated with 1U DNase I, Amp Grade (Invitrogen, Carlsbad, CA) in 1X DNase I Reaction Buffer (Invitrogen, Carlsbad, CA) and incubated for 15 minutes at room temperature. DNase I was inactivated by adding 1μl of 25 mM EDTA and heating for 20 minutes at 70°C. RNA was then reverse transcribed using oligo dT primers (Invitrogen, Carlsbad, CA) and an Omniscript® Reverse Transcription kit (Qiagen Inc., Valencia, CA) or a ThermoScript™ Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) following the manufacturers' instructions. PCR using Taq polymerase and the primers denoted in Table 1 was performed on the cDNA. Bands of interest were extracted and sequenced by an in-house CORE facility. Sequence for a new Vamp1 splice variant and additional sequence for Vamp1as have been submitted to Genbank (accession no. EF653275 (Vamp1nv)) and accession no. EF653274 (Vamp1as)).

Total RNA from human medulla (Clontech, Mountain View, CA) was purchased to investigate the presence of the Vamp1nv splice variant in humans. This RNA from the tissue of interest was collected from 29 individuals between the ages of 18 and 64 experiencing sudden death. Human primers were designed, analogous to rat BFor and CRev, based upon the human genomic Vamp1 sequence to lay across the splice sites for Vamp1nv exon 4/5 and exon 5/6 (Table 1 and Figure 2A). Approximately 1μg of total RNA was DNAsed and reverse transcribed to cDNA. PCR was performed for 34 cycles at annealing temperatures of 55C, 58C and 61C with an elongation temperature of 72C for 1 minute. These were run on a 2% agarose gel for visualization. The 108bp band of interest was gel purified and analyzed by restriction digest and direct sequencing.

2.3 Real-Time RT-PCR

Real-time RT-PCR was performed using the ABI Taqman assay with primer/probe sets designed for Vamp1-conserved, Vamp1 (wild-type), Vamp1as and Vamp1b. Vamp1-conserved represents a primer/probe set to the 5′ conserved region found in wild-type Vamp1 and all variants. Vamp1 uses primers that specifically amplify the wild-type Vamp1 transcript exclusive of other known variants. In addition to these known variants, the new variant identified in this study, Vamp1nv, was studied with a probe specific for the unique splice site we identified. The reference gene for all real-time RT-PCR experiments was hypoxanthine guanine phosphoribosyl transferase (HPRT). HPRT was justified based upon our earlier SAGE and microarray experiments which showed robust and similar expression levels in all three subdivisions. We rejected other common reference genes such as B2M, GAPDH and β-actin as they showed wide ranges of expression among cochlear nucleus subdivisions by microarray and SAGE.

Taqman® Gene Expression Assays were purchased from Applied Biosystems (ABI, Foster City, CA) for Vamp1-conserved (ABI#: Rn00565308_m1) and HPRT (ABI#: Rn01527838_g1). Custom Taqman® Gene Expression Assays were designed and manufactured by Applied Biosystems for Vamp1 (wild-type), Vamp1as, Vamp1b and Vamp1nv (see Table 2). Real-time RT-PCR was performed on the iCyler iQ Multicolor Real-Time Detection System (Bio Rad Laboratories, Hercules, CA). All reactions contained 10μl 2× Taqman® Universal PCR Master Mix (ABI, Foster City, CA), 1μl of 20× Taqman® Gene Expression Assay (ABI, Foster City, CA), and 9μl cDNA in RNase-free water. Thermal cycling conditions were: 50°C hold for 2 min, 95°C hold for 10 min, followed by two-step PCR for 45 cycles of 95°C for 15 sec and 60°C for 1min. Negative controls were run for each sample and all reactions were run in triplicate. PCR was run out to 45 cycles to check for contaminants but the cycle threshold was identified in standard fashion as the point at which probe fluorescence crossed baseline. Each of these experiments was performed three times and the relative expression levels for each gene taken as the average of these runs. Relative expression levels were calculated by comparing the threshold cycle (Ct) values of HPRT and the Vamp variant of interest in each subdivision (Friedland et al., 2007).

3. RESULTS

3.1 Confirmation of Vamp1 Expression in the Cochlear Nucleus

The expression of Vamp1 within the cochlear nucleus was initially suggested by the isolation of a SAGE tag in prior experiments (Friedland et al., 2006). The sequence representing the SAGE tag, however, was not present in either wild-type Vamp1 (Genbank accession no. M24104) or two reported variants (i.e., Vamp1as (Genbank accession no. AF498262) and Vamp1b (Genbank accession no. U74621). In order to confirm that this SAGE tag represented Vamp1 expression, 40 cycles of RT-PCR was performed on pooled mRNA extracted from each subdivision of the cochlear nucleus of 20 rats (Figure 1A). Primers for this experiment

were designed to span exons 2 through 5 of wild-type Vamp1 (Figure 1B). An amplicon of expected size (i.e., 471bp) was identified in each cochlear nucleus subdivision (Figure 1C). The amplicon was gel extracted and confirmed by sequencing as being wild-type Vamp1.

3.2 Identification of Vamp1 Splice Variants

While wild-type Vamp1 was confirmed as being present in the cochlear nucleus, it was not confirmed that the SAGE tag represented this transcript. Thus, we designed primers to amplify Vamp1 from the 5′ conserved region to a downstream primer based upon the SAGE tag sequence (Figure 2A). If an amplicon was generated, and sequencing confirmed homology to Vamp1 as well as the inclusion of the tag sequence, it would validate the SAGE findings and also add 3′ sequence to existing rat Vamp1 Genbank entries. Using a reverse transcribed whole rat brain library, amplicons of 2530 base-pairs, 960 base-pairs and 724 base-pairs were produced (Figure 2B, lane 1). The 2530bp amplicon was extracted and sequenced and showed homology to wild-type Vamp1 with inclusion of the SAGE tag at the 3′ end. The amplicon was 100% homologous to a more recent report of full length Vamp1 (Genbank accession no. BC092206) which contained the SAGE tag. Sequencing of the 960bp band showed homology to Vamp1as (Figure 2B, lane1). Using a further downstream primer located 3′ of the SAGE tag produced a longer 1109bp amplicon of Vamp1as (Figure 2B, lane 3). This was also sequence confirmed and added to the existing sequence reported for this variant (Genbank accession no. EF653274). This downstream primer failed to amplify Vamp1 or other variants and we feel it may reflect an alternate poly-adenylation site for Vamp1as.

The smaller 724 base-pair amplicon from the above experiment showed 5′ homology to wild-type Vamp1 but had a unique downstream sequence (Figure 2B, lane 1). This sequence was 100% homologous to the genome for rat Vamp1 but showed a different splice pattern beyond exon 4. The splice pattern is presented in figure 2A and further characterization is presented below. We termed this novel variant Vamp1nv (Genbank accession no. EF653275).

The remaining known splice variant, Vamp1b, was expressed in the commercially available rat brain library. This variant ends before the SAGE tag and did not show up with the original primer set. A primer set specific for Vamp1b showed a single band of appropriate size with PCR (Figure 2, lane 2). This was confirmed as Vamp1b by sequencing.

3.3 Expression Patterns of Vamp1nv

Using the novel Vamp1nv sequence, a PCR primer set was designed bridging the novel splice sites so as to be specific for Vamp1nv (Figure 2A). This primer set, termed BFor and CRev, was used in libraries of whole rat brain, cerebellum, cerebral cortex, thalamus and medulla (Figure 2C). After 45 cycles of PCR, Vamp1nv was amplified from whole rat brain and medulla only. There was no expression in cerebellum, cerebral cortex or thalamus.

The expression in the medulla suggested that Vamp1nv may be present in the cochlear nucleus. The same primer set was applied to mRNA extracted from each subdivision of the cochlear nucleus. An identical amplicon of 110bp was identified in the AVCN, PVCN and DCN also after 45 cycles of PCR (Figure 2D).

3.4 Sequence of Vamp1nv (new variant)

Gel extraction and sequencing of the Vamp1nv amplicon showed a unique splice pattern and nucleotide sequence as compared to previously reported rat Vamps (online supplemental data). The Vamp1nv transcript has identical splice sites for exons 1–4 but bypasses the start of exon 5 seen in wild-type Vamp1. Rather, Vamp1nv splices in a 77bp sequence in the mid-portion of wild-type exon 5 and then adds a 397bp sequence from the terminal end of wild-type exon 5. The 77bp sequence is unique to Vamp1nv and to wild-type Vamp1 but is not present in

either Vamp1b or Vamp1as. The 397bp tail is in both wild-type Vamp1 and Vamp1as but Vamp1b terminates upstream of this region.

The identified splice regions for Vamp1nv were flanked by canonical GT- and -AG donor and acceptor dinucleotides (see online supplemental data). Further strengthening the validity of the splice sites is a pyrimidine rich sequence upstream of the AG acceptor sites terminating in a cytosine just before the acceptor (Abril et al., 2005). In addition, there are guanine and adenine residues flanking the GT donor site (Abril et al., 2005). The acceptor AG preceding the novel exon is present in the human genome and the downstream acceptor to the terminal exon is present in the mouse genome. The donor GT following the novel exon was found only in the rat genome and aligns with an AT dinucleotide in humans. These sequence data, the amplification of Vamp1nv by RT-PCR in native tissue and brain libraries, and the amplification of Vamp1nv by qRT-PCR with a probe specific for the splice site provide strong evidence for the validity of this transcript.

3.5 Predicted Protein Sequence of Vamp1nv

The alternate splice sites seen for Vamp1nv, Vamp1b, and Vamp1as result in unique amino acid sequences in the c-terminus of the Vamp1 protein (Figure 3). These sites are immediately c-terminal of the hydrophobic region that anchors the protein in the synaptic vesicle membrane. Thus, the unique termini fall in the intra-vesicular portion of the synaptic vesicle. For Vamp1, Vamp1as and Vamp1b this intra-vesicular tail is short and contains 3 to 5 amino acids. In contrast, the predicted protein sequence for Vamp1nv produces a 17 amino acid tail c-terminal of the trans-membrane region.

The carboxy termini of all the Vamp1 variants contain hydrophilic and polar residues which contrast with the immediately upstream trans-vesicular membrane region which is strongly hydrophobic. Five of the terminal 7 amino acids for Vamp1nv are hydrophobic after a 10 amino acid stretch that is largely hydrophilic. Thus, Vamp1nv may form an intra-vesicular loop with the carboxy-terminus anchored into the synaptic membrane.

The tail of rat Vamp1nv also contains two predicted serine kinase recognition sites. The serine in sequence VIVN**S**GTED and the middle serine in sequence DRSC**S**VCFG have a high phosphorylation potential (i.e., 0.892 and 0.893 out of 1.0, respectively) based upon analysis through the online NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/). Similarly, analysis through PPSP (http://bioinformatics.lcd-ustc.org/PPSP/) confirms these two serines as being strong kinase targets. The strongest predicted kinase for the former site is casein kinase II (CasKII). For the latter there are multiple predicted kinases including ribosomal S6 kinase (RSK) and serum- and glucocorticoid-induced kinase (SGK). Other potential kinases for the latter include Ca^{++}/calmodulin dependent kinase II (CaMKII) and protein kinase B (PKB) based upon recognition homology to RXX(S/T)X. There are no predicted phosphorylation sites in the intra-vesicular carboxy-terminus of any of the other rat Vamp1 splice variants.

The predicted protein sequences and phosphorylation sites for Vamp1nv in human and mouse are shown aligned to rat in figure 5E. The human retains the initial serine but the sequence is truncated by a stop codon prior to the second potential phosphorylation site. Casein kinase II phosphorylation requires an acidic amino acid located 3 amino acids to the carboxy-terminal side of the phosphorylated serine (Marchiori et al., 1988). As this protein is truncated at this site it may not be a functional CasKII site. NetPhos weakly predicts this serine to be phosphorylated.

The mouse retains neither rat phosphorylation site but multiple serines in this sequence are predicted to be kinase targets by NetPhos 2.0 and PPSP. The first and second serines in the mouse variant (e.g., AAQ**S**V**S**GA) are strongly predicted to be targets of CaMKII and/or SGK.

These serines correspond in location to the region of the rat intra-vesicular sequence also predicted to be targeted by these kinases. A stop codon was not found in the mouse sequence and this carboxy-terminus extends beyond the rat sequence and contains additional potential phosphorylation sites.

3.6 Real-time RT-PCR on Vamp1 and Variants in Cochlear Nucleus

The original impetus for investigating Vamp1 was the finding of differential expression in the cochlear nucleus by SAGE. Thus, we employed real-time RT-PCR to characterize the relative levels of expression of each variant among the cochlear nucleus subdivisions. Primer-probe sets were designed specifically for each splice variant with the probe overlying the unique splice sites. We were not able to consistently and cleanly amplify Vamp1as but were able to obtain valid data for Vamp1 (wild-type), Vamp1b and Vamp1nv. In addition, we used a primer-probe set proximal to the splice sites that would amplify all species of Vamp1 (i.e., Vamp1 conserved). The amplification of Vamp1nv by a specific primer-probe set further supports the validity of this splice variant and confirms its expression in the medulla (i.e. cochlear nucleus).

Real-time RT-PCR results for the 3 cochlear nucleus subdivisions is graphically depicted in Figure 4. Fold-difference was normalized to the subdivision with lowest levels of expression of the probed transcript. For Vamp1nv the lowest levels were in the PVCN while for all other variants the lowest level was in the DCN. The Vamp1 conserved primer set, which shows expression of all Vamp1 species', demonstrated statistically significant higher levels of Vamp1 in the AVCN and PVCN than in the DCN ($p \leq 0.05$). The wild-type Vamp1 showed a nearly identical expression pattern but the ratio did not reach statistical significance ($p=0.07$ for AVCN and $p=0.08$ for PVCN). Vamp1b and Vamp1nv showed no significant differential expression among regions.

The primer-probe sets were not assessed for relative efficiency and comparison of the levels of the transcripts to each other within a subdivision are not precise. However, a consistent pattern emerged within the real-time experiments in which Vamp1b and Vamp1nv crossed the baseline threshold 5–7 cycles later than Vamp1 wild-type (Figure 4). This suggests an abundance of Vamp1 wild-type relative to the other forms. This is further supported by the finding that Vamp1 (conserved), which shows all Vamp1 variants pooled together, has a nearly identical pattern of expression to Vamp1 wild-type. That is, Vamp1b and Vamp1nv do not significantly affect the overall pattern of Vamp1 expression suggesting they contribute little to the total transcript pool.

3.7 Vamp1nv in the Human Medulla

The rat Vamp1nv splice sites identified in this study were not found in the NCBI human genome VAMP1 sequence. This may be specific to the individual from whom the sequence was obtained, or potentially reflect the methodology and tissue of origin used for sequencing. Therefore, we purchased commercially available medulla RNA pooled from many individuals to assess whether an analogous splice variant may be expressed in humans. RT-PCR, restriction digest, and direct sequencing support the presence of the unique exon 5 we initially identified in the rat (Figure 5A-C). There is strong sequence homology of this region among rat, human and mouse (Figure 5D).

4. DISCUSSION

Fusion of the synaptic vesicle to the cell membrane during exocytosis is regulated by the SNARE complex of proteins. This complex includes Vamp (a.k.a. synaptobrevin) of which two isoforms have been reported: Vamp1 and Vamp2 (Elferink et al., 1989, Trimble et al., 1990). Vamp2 is globally distributed throughout the brain and appears essential for life as

knockout mice do not survive beyond birth (Schoch et al., 2001). Vamp1 shows more localized expression, although co-expression with Vamp2 in the same cell populations has been reported (Rossetto et al., 1996, Raptis et al., 2005). Vamp1 appears to have distinct functions based upon its more localized distribution and its inability to substitute for Vamp2 in knockout animals (Schoch et al., 2001, Raptis et al., 2005, Li et al., 1996). Vamp1 is most abundant in motor nuclei of cranial and spinal nerves and is under independent regulation from Vamp2 in these regions (Trimble et al., 1990, Jacobsson et al., 1998). Vamp1 has been reported in the dorsal cochlear nucleus which is consistent with our findings (Trimble et al., 1990).

This study identified expression of Vamp1 and several splice variants in the cochlear nucleus subdivisions of the rat. The novel splice variant Vamp1nv showed uniform expression throughout the nucleus. In contrast, Vamp1wt and Vamp1b showed higher levels of transcript in ventral subdivisions than in the dorsal nucleus. Thus, the ratio of Vamp1nv to wild-type or Vamp1b differs between the DCN and the VCN. This ratio may affect the physiology of synaptic vesicle release in specific cell types in the cochlear nucleus.

Differences in function between wild-type and variant forms of Vamp1 would be solely dependent on the c-terminal intravesicular domain. One role of the intravesicular tail may be in protein sorting and trafficking (Mandic et al., 1997). Isenmann and colleagues found that the alternate c-terminus of VAMP1B led to trafficking to the mitochondria rather than the plasma membrane as seen with VAMP1A (Isenmann et al., 1998). The human VAMP1B has a c-terminus most akin to rat Vamp1as although 1b and 1as both have a short sequence of positively charged and hydrophilic residues. Vamp1nv differs by having a longer tail than that normally seen with trafficking signals, such as those to the endoplasmic reticulum, and may therefore localize to a different cellular location (Berglund et al., 1999). That the most distal c-terminal region contains hydrophobic amino acids, similar to wild-type Vamp1, may indicate trafficking to the synaptic vesicle. A specific antibody would be necessary to localize Vamp1nv within the sub-cellular domain.

Assuming localization to the synaptic vesicle, the unique c-terminus of Vamp1nv may alternatively function to modulate the kinetics of the SNARE complex similar to that found for other Vamp proteins (Siddiqui et al., 2007). For example, the transmembrane α-helical domain of Vamp2 inserts at a specific angle into the vesicle lipid bilayer which may regulate rates of vesicle fusion (Bowen and Brunger, 2006). The angle of insertion appears to be affected by the juxta-membrane protein sequence (Bowen and Brunger, 2006). Vamp1 is similar to Vamp2 in the transmembrane region and may similarly be influenced by surrounding amino acid sequence. By regulating the expression of alternatively spliced Vamp1, the cell may be capable of modulating vesicle fusion and thus synaptic firing rates.

A unique feature of the predicted rat Vamp1nv sequence is the appearance of two kinase recognition sites in the intra-vesicular domain. Phosphorylation plays a role in regulating the activity of many SNARE complex associated proteins (Pyle et al., 2000, Risinger and Bennett, 1999, Snyder et al., 2006). The identified c-terminal phosphorylation sites in Vamp1nv may affect Vamp1 insertion into the plasma membrane or alter the tension on the SNARE complex promoting or inhibiting fusion. Regulation of Vamp1 and Vamp2 by CaMKII and CasKII has been postulated after identification of several kinase recognition sites in the cytoplasmic domain (Nielander et al., 1995). Both these kinases are abundant in synaptic terminals and CaMKII, in particular, plays an important role in neural plasticity and regulation of neuronal activity (Thiagarajan et al., 2002). The identification of c-terminal kinase recognition sites in Vamp1nv may indicate another means of potentiation of neuronal activity in response to afferent input.

The potential role of such a mechanism in humans is tempered by the early truncation of the variant protein and the weak prediction of the intra-vesicular serine as being a kinase target. The mouse, however, has strongly predicted serine phosphorylation sites with a region similar in location and kinase-recognition as that seen in the rat. Thus, phosphorylation of Vamp1nv in rodents may represent a regulatory mechanism affecting synaptic activity. This may be important for studies using rodent models of central auditory activity.

A wide variety of neuronal discharge patterns have been observed in the cochlear nucleus (Stabler et al., 1996, Rhode and Smith, 1986, Blackburn and Sachs, 1989). Some of these patterns remain unexplained electrophysiologically and may involve underlying molecular and cellular mechanisms. For example, the notch in the primary-like with notch response is unlikely to be simply a function of cell refractoriness (Blackburn and Sachs, 1989). Similarly, transiently adapting choppers rapidly alter their initial discharge patterns by an unexplained mechanism (Blackburn and Sachs, 1989). Although highly speculative, a mechanism such as phosphorylation of SNARE proteins may rapidly influence synaptic vesicle release and recycling and effect such observations. Alternatively, varying the ratio of synaptic vesicle protein variants, each with differing docking and release properties, may also effectuate changes in discharge patterns.

This study identified a novel Vamp1 splice variant in the rat cochlear nucleus: Vamp1nv. Although not abundantly expressed, this variant may provide a means for modulating auditory neuronal activity by kinase signaling pathways. Such modulation may play a role in normal sound processing but may be more pronounced in disorders of auditory perception associated with hearing loss. For example, tinnitus or poor word discrimination may reflect compensatory changes in synaptic activity along central auditory pathways in response to reduced afferent input. Additional studies may better localize the expression of Vamp1 and its splice variants and identify changes in such expression in response to peripheral deafferentation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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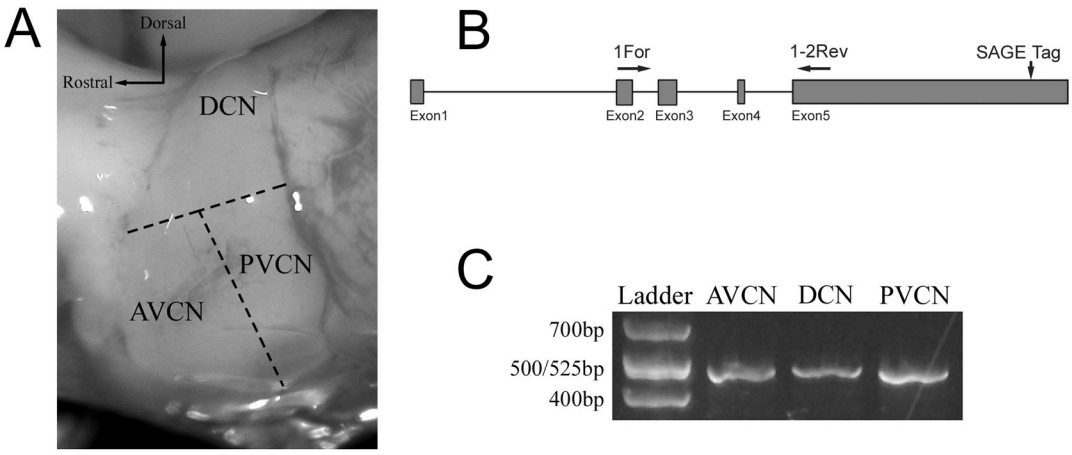

Figure 1.

Confirmation of expression of Vamp1 in the cochlear nucleus. (A) Rat cochlear nucleus subdivisions were dissected and total RNA extracted. (B) Primers were designed to identify wild-type Vamp1 with an expected amplicon size of 471bp. (C) RT-PCR and gel electrophoresis demonstrated Vamp1 expression in each subdivision of the cochlear nucleus.

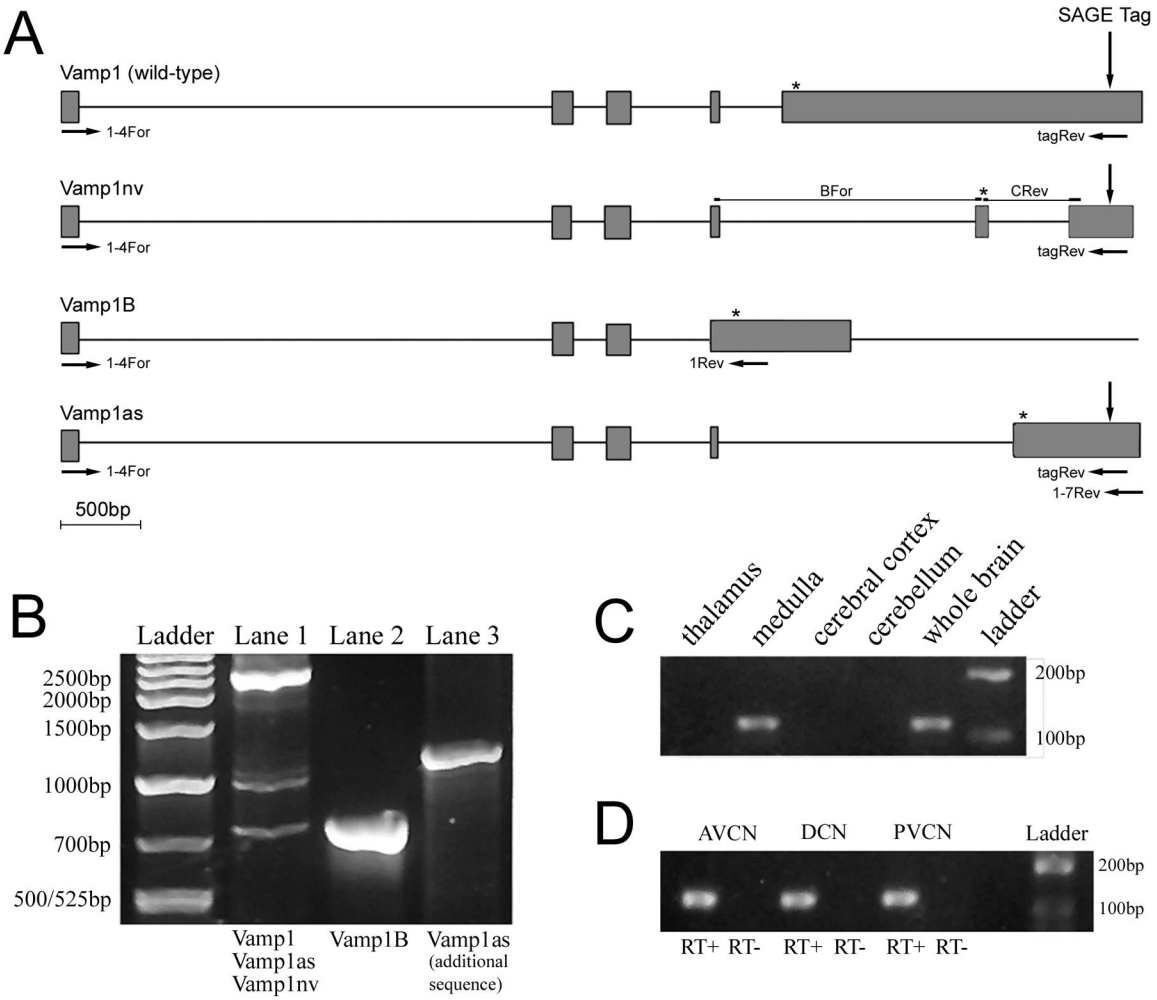

Figure 2.

Identification of Vamp1 splice variants. (A) Primers were designed to span from exon 1 through the 3′ variable region of each splice variant. A specific primer set was also developed for Vamp1nv that spanned introns (i.e., BFor and CRev). The approximate locations of the stop codons for each variant are noted (*). The start codon for all variants is at the splice juncture of exon 1 and exon 2. (B) RT-PCR and gel electrophoresis of RNA extracted from whole brain was used to identify the Vamp1 variants. Bands were extracted and sent for sequencing which identified the 724bp amplicon representing a novel variant. Additional sequence for Vamp1as was also found. (C) Individual brain libraries were probed for the expression of Vamp1nv using a specific primer set. The expected 110bp amplicon was found in medulla but not in any other brain region after 40 cycles of PCR. (D) Expression of Vamp1nv was identified in all cochlear nucleus subdivisions. This was non-quantitative and represents 40 cycles of RT-PCR.

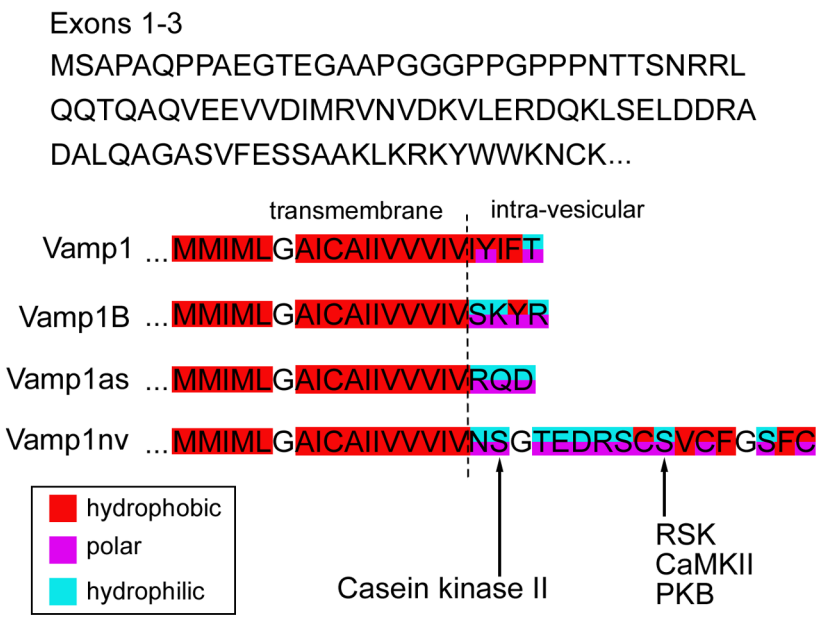

Figure 3.

Predicted protein sequence of Vamp1 variants. The protein sequence is conserved through the hydrophobic transmembrane region encoded by exon 4. The end of the exon 4 coding region is indicated by the dashed line. The variable intra-vesicular c-terminus is similar in all variants by containing polar and hydrophilic residues. Vamp1nv is unique in the length of the intra-vesicular tail and by the presence of two serine residues predicted to be targets for several kinases. CasKII and CaMKII are abundant in synaptic terminals and may provide a means of modulating Vamp1nv activity though phosphorylation.

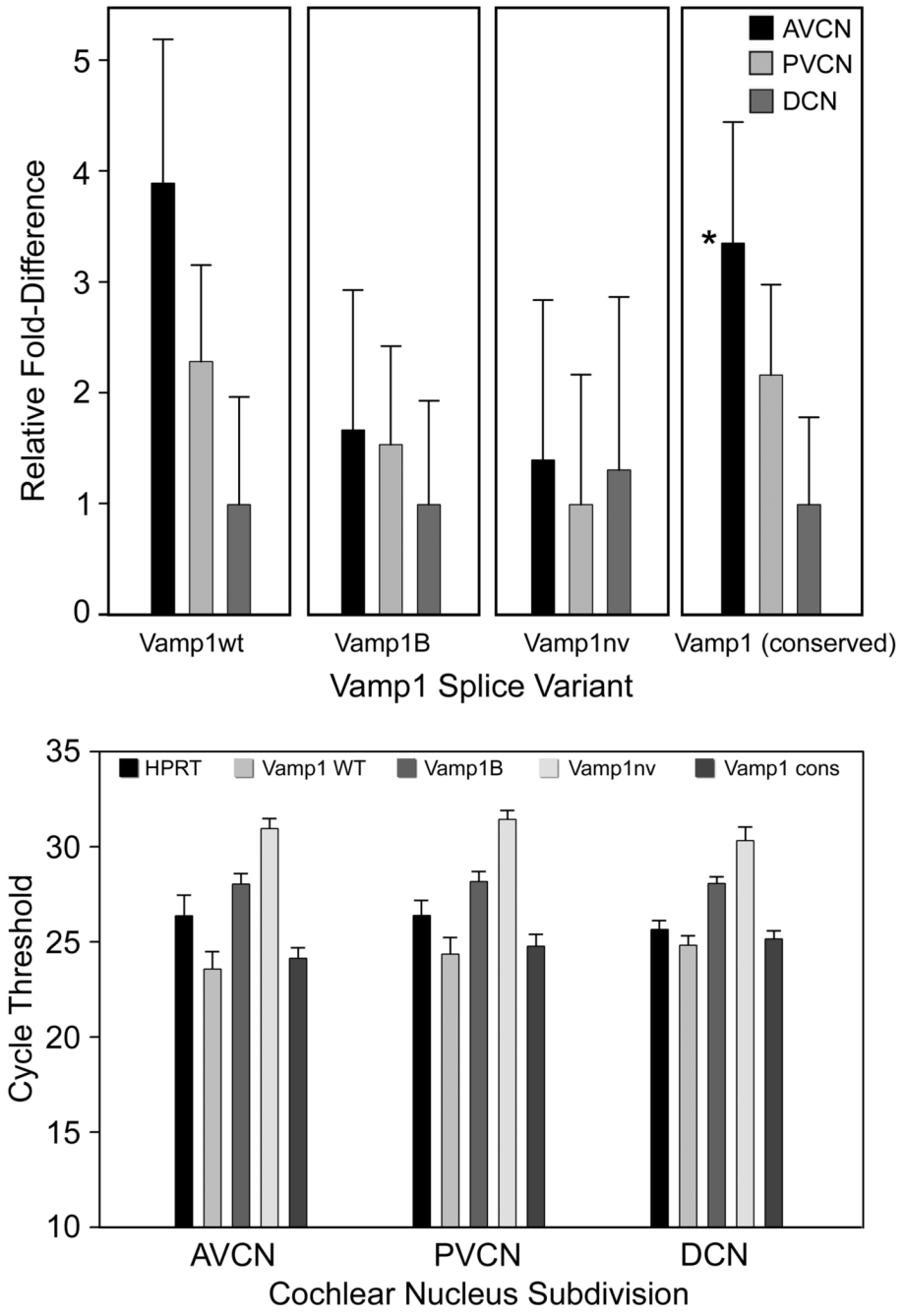

Figure 4.

Upper panel: real-time RT-PCR results comparing relative Vamp1 variant levels among subdivisions of the cochlear nucleus (±SEM). Vamp1 (conserved) used a primer-probe set proximal to the splice sites and represents the expression of all Vamp1 species regardless of splice variant. Expression of all Vamp1 variants is higher in the AVCN which may reflect the predominance of large endbulb synapses. Vamp1nv is not differentially expressed between subdivisions but represents a higher proportion of total Vamp1 in the DCN than in ventral divisions. Lower panel: raw cycle thresholds of real-time experiments comparing the HPRT reference and Vamp1 variants in each cochlear nucleus subdivision (±SEM). Probes were

designed to lie on the unique splice sites and are thus specific for Vamp1wt, Vamp1B and Vamp1nv.

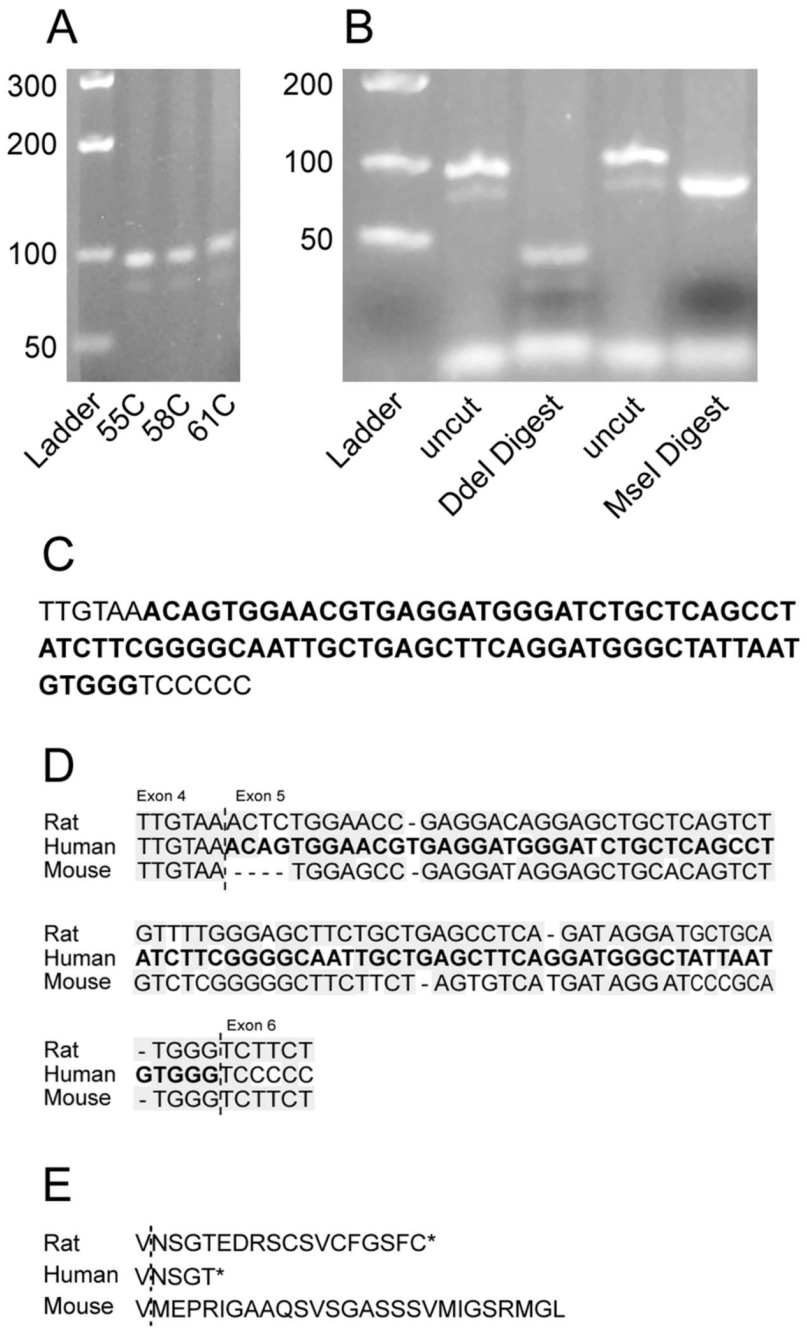

Figure 5.

RT-PCR primer probes lying at the 3′ and 5′ unique splice sites for VAMP!nv were applied to human medulla total RNA. **A**: Using several different annealing temperatures (i.e. stringency), the expected 108bp amplicon was consistently identified. **B**: Restriction digest with DdeI and MseI identified the predicted large fragments: 45bp (DdeI) and 82bp (MseI). The smaller fragments are lost in the dye-front. **C**: Direct sequencing of the amplicon produced a sequence containing the unique VAMP1nv exon 5 (bold) and the flanking sequences from exon 4 and exon 6. **D**: Alignment of the human VAMP1nv sequence with the same region in rat and mouse. Sequence homologies to the rat variant are shaded. **E:** Predicted protein sequences for

Vamp1nv in rat, human and mouse. The carboxy-terminus is indicated by “*”; the mouse had no identified stop codon.

Table 1

Primers used in Vamp1 PCR experiments

GENE	NAME	PRIMER SEQUENCE	EXPECTED AMPLICON SIZE (bp)
Vamp1 (wild-type, full-length)	1-4For	5′-TGCCTGTCTCATTGCATTCTC	2530
	tagRev	5′-CGCCTTTGTTGAGAATACATG	
Vamp1 (wild-type specific)	1For	5′-ATTACAGCAAACCCAGGCAC	471
	1-2Rev	5′-TCTCTTCGGGCAGTGACTTT	
Vamp1as	1-4For	5′-TGCCTGTCTCATTGCATTCTC	960
	tagRev	5′-CGCCTTTGTTGAGAATACATG	
Vamp1as (extended length)	1-4For	5′-TGCCTGTCTCATTGCATTCTC	1109
	1-7Rev	5′-CACAGAGACTTTAATCTCCAGG	
Vamp1b	1-4For	5′-TGCCTGTCTCATTGCATTCTC	750
	1-Rev	5′-GGGCAAGTTACAGCACATCA	
Vamp1nv (full-length)	1-4For	5′-TGCCTGTCTCATTGCATTCTC	724
	tagRev	5′-CGCCTTTGTTGAGAATACATG	
Vamp1nv (specific)	BFor	5′-CGTGGTAGTAATTGTAAACTC	110
	CRev	5′-TGGGAGGGACAGAAGACCCATG	
Human VAMP1nv (specific)	BFor3	5′-TTGTAAACAGTGGAACCTGAGG	108
	CRev	?5′-AAAGGGGGACCCACATTAATAGC	

Forward and reverse primers used for the identification of Vamp1 variants in the brain and cochlear nucleus. The rat Vamp1nv (specific) primer set spanned introns and generated a 110bp sequence unique to this variant.

Table 2

Primer-Probe Sequences used in Vamp1 Splice Variant Taqman® Real-Time RT-PCR experiments

Vamp1	Forward Primer	Reverse Primer	Probe	Size
Vamp 1 WT	GATCATGCTGGGAGCTATCTGT	AGGGATGGCACATTCTCAAGTAAAA	ATCGTGGTAGTAATTGTAATCTA	79
Vamp 1 B	GATCATGCTGGGAGCTATCTGT	CAGTGGCCTCAGCGATACTTAC	CATCATCGTGGTAGTAATTGTAA	69
Vamp 1 nv	TGCCATCATCGTGGTAGTAATTGT	CAGAAGCTCCCAAAACAGACTGA	CCTCGGTTCCAGAGTTT	75
Vamp 1 as	CTATCTGTGCCATCATCGTGGTA	TGCTTCCTTCCAGCTCTTCAG	TCCTGCCTTACAATTAC	61

Primer-probe sets used for real-time RT-PCR experiments. These were used to identify the relative levels of each variant among the subdivisions. Primers were designed around the unique splice sites to be specific for each variant.