Alternative Splicing Controls Neuronal Expression of v-ATPase Subunit α1 and Sorting to Nerve Terminals*

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Vacuolar proton ATPase accumulates protons inside various intracellular organelles such as synaptic vesicles; its membrane domain V0 could also be involved in membrane fusion. These different functions could require vacuolar proton ATPases possessing different V0 subunit isoforms. In vertebrates, four genes encode isoforms α1–α4, and α1 variants are also generated by alternative splicing. We identified a novel α1 splice variant α1-IV and showed that the two α1 variants containing exon C are specifically expressed in neurons. Single neurons coexpress α2, α1-I, and α1-IV, and these subunit isoforms are targeted to different membrane compartments. Recombinant α2 was accumulated in the trans-Golgi network, and α1-I was concentrated in axonal varicosities, whereas α1-IV was sorted to both distal dendrites and axons. Our results indicate that alternative splicing of exon N controls differential sorting of α1 variants to nerve terminals or distal dendrites, whereas exon C regulates their neuronal expression.

Vacuolar H⁺-ATPases (v-ATPases) translocate protons across the membrane of various organelles (lysosomes, endosomes, trans-Golgi cisternae, secretory granules, etc.) (1, 2). Acidification of these organelles is required for many cellular processes (maturation and processing of proteins, receptor-mediated endocytosis, coupled intracellular transport of small molecules, etc.). v-ATPases are large multimeric enzymes made up of several different subunits. These subunits are organized in two domains: a cytoplasmic domain V1 that hydrolyzes ATP and a membrane domain V0 involved in proton translocation. V1 is composed of eight different subunits (A–H). V0 contains four to five transmembrane domains located in its C-terminal half and a large N-terminal sector in the cytoplasm (3). Several isoforms of the α subunit have been identified. In yeast, the two α isoforms (Vph1p and Stv1p) are associated to vacuolar and Golgi/endosomal membranes, respectively (4, 5). In mice, four different isoforms (α1–α4), encoded by different genes with tissue-specific expression, have been described (6–8). Except α4, which is specifically expressed in kidney (6), these isoforms are expressed to various extents in all tissues (7, 8). Three variants of α1 are generated by alternative mRNA splicing, with α1-I specifically expressed in brain, whereas α1-II and -III mRNAs, which lack the C-terminal exon C, were found in all other tested tissues (7, 9). The diversity of the α subunit isoforms is not only important for tissue specificity and targeting to different membrane compartments; it could also result in the generation of v-ATPases with different functional properties (10, 11).

In neurons, the V1 and V0 domains are transported independently in the axon and assemble once arrived in nerve terminals (12). v-ATPase is present both in the presynaptic plasma membrane (13) and in the membrane of synaptic vesicles (14, 15). The large electrochemical H⁺ gradient generated by this enzyme, pH 5.2–5.5 inside the synaptic vesicles (16, 17), is used by specific vesicular transporters to accumulate the neurotransmitter. V0 could also participate in the constitution of a fusion pore involved in membrane fusion (18) and neurotransmitter release (19, 20). Recently, in Drosophila nerve terminals, the V0 subunit α1 was shown to be required for evoked synaptic vesicle exocytotic fusion, independently of proton translocation (20). This could suggest the involvement of V0 domains with different subunit isoforms in these two processes.

In the present work, we studied the expression of different v-ATPase subunit α isoforms in rat neurons. We found that identified neurons coexpress isoforms α1 and α2 of subunit α. A new α1 splice variant was identified, α1-IV, that contains both exons N and exon C. Neurons express the two α1 splice variants that contain exon C, α1-I and α1-IV, whereas α1-II and α1-III are expressed by glial cells and in all tissues tested. The three different α subunits expressed by neurons, α1-I, α1-IV, and α2, are sorted to different neuronal membrane compartments. Only α1-I was specifically concentrated in nerve terminals.

EXPERIMENTAL PROCEDURES

Rat Hippocampal Neuron Culture and Transfection—Primary cultures of hippocampal neurons were performed essentially as described by Goslin et al. (21). Hippocampi of 18-day-old rat embryos were removed and mechanically dissociated after a mild trypsin digestion. The cells were plated at medium density (17 or 34,103 cells/cm2 for immunofluorescence or transfection, respectively) onto poly-L-lysine-coated glass coverslips and allowed to settle for 2 h. They were then transferred onto a glial cell monolayer in the neurobasal medium supplemented with B27 (Invitrogen). Proliferation of non-neuronal cells was prevented by the addition, 1 day later, of cytosine arabinoside (final concentration 1 mm). Most experiments were performed on cells kept for 14 days in vitro. The neurons were transfected at 7 days in vitro with the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Isolation of cDNA Clones—RNA was extracted by TRIzol (Invitrogen) from various rat tissues (brain, kidney, liver, or adrenal medulla) or rat cells (hippocampal neurons or astroglial cells) in primary culture. It was used to synthesize first strand cDNAs using oligo(dT) priming and Moloney murine leukemia virus RNAse H reverse transcriptase (Finnzymes). Then clones encoding the α1, α2, and α3 isoforms of rat v-ATPase subunit α, and the four α1 splice variants (α1-I, α1-II, α1-III, and α1-IV) were generated by PCR. Specific primers allowing the amplification of the entire coding sequence were designed based on the mouse α1, α2, and α3 isoform sequences and on partial sequences of rat isoforms.
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Isolation and Sequencing of cDNAs Encoding Isoforms of Rat v-ATPase Subunit a—Clones encoding the a1, a2, and a3 isoforms of rat v-ATPase subunit a and the four a1 splice variants (a1-I, a1-II, a1-III, and a1-IV) were generated by PCR. Primers were designed based on the sequences of the mouse a1, a2, and a3 isoforms (7, 8) and of the rat a1 isoform (22) and on partial sequences of the corresponding rat genes. All of these clones were sequenced. The a1 isoform sequences we obtained (GenBank™ accession numbers DQ286421 to DQ286424) were identical with the published one (22) with only two base differences (a conservative Thr1086 to Gly change and Gly40 instead of Ala, leading to replacement of Glu29 by Gly).

In mice, three alternatively spliced variants of the a1 isoform have been cloned (7). From rat brain, we cloned the a1-I variant (DQ286421), which contains the exon C (a 18-bp insertion in between exons 17 and 18 of the a1 cDNA). The a1-II (DQ286423) and a1-III (DQ286424) variants, cloned from rat kidney, do not contain the exon C and differ according to a 21-bp insertion in a1-II (exon N between exons 4 and 5). We looked for the forth possible splice variant of a1, a1-IV, containing both exon N and exon C. Starting from cDNAs of rat hippocampal neurons in primary culture, we isolated two clones encoding a1-IV (DQ286422), of 86 a1 clones. The a2 (DQ286425) and a3 (DQ286426) isoforms were cloned from brain and liver, respectively.

Brain Expression of Subunit a Isoforms—The expression of the a1, a2, a3, and a4 isoforms and subunit c of v-ATPase in brain, kidney, liver, and adrenal medulla was studied by semi-quantitative RT-PCR (Fig. 1). Expression by hippocampal neurons and glial cells in culture were also compared. As expected (6), the kidney-specific a4 isoform was strongly expressed in kidney and not detected elsewhere. Expression of a3 was detected in all tissues tested, higher in liver, in agreement with Northern blot data from mice (7, 8). Neither glial cells nor hippocampal neurons in culture express detectable amounts of a3. The isoform a2 is ubiquitously expressed in all of the tissues and cells tested. Expression of a1 was detected in all tissues. It was much more highly expressed in neurons than in glial cells where it was hardly detectable. Neurons therefore express the a1 and a2 subunit a isoforms, but none of these isoforms is neuron-specific. Subunit c expression was similar in all samples except in glial cells, which showed only a faint signal. Because subunit c is encoded by a single gene in vertebrates (2), its expression likely reflects overall expression of the V0 domain of v-ATPase.

Four a1 variants can be generated by alternative splicing of two small exons in a1 mRNAs. They differ according to the insertion of either exon C (a1-I), exon N (a1-II), neither (a1-III), or both (a1-IV). The expression of the a1 splice variants was studied by PCR (Fig. 2). In a first series of experiments (Fig. 2A), we used a combination of PCR primers that allows the specific amplification of each a1 splice variant, as dem-
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onstrated using the four corresponding plasmids. The exon C containing variants, a1-I and a1-IV, are expressed in brain and neurons and to some extent in the adrenal medulla. The a1-II and a1-III splice variants are expressed in all tissues tested, but brain expression is much lower than that of kidney or liver. Glial cells express a1-II and a1-III. Neurons express a1-I and a1-IV, the small amplification of the a1-II and -III variants from the neuronal sample being most probably due to some contaminating glial cells (see also Fig. 4).

In a second series of experiments (Fig. 2B), we compared the expression of the different a1 splice variants. PCRs were performed using two pairs of primers (primers 33-34 and 42-2) that bind on each side of the site of insertion of exon N or exon C (respectively). They allowed, as shown using plasmids encoding a1-I and a1-II (Fig. 2B), the amplification of fragments of slightly different sizes according to the presence or absence of the corresponding exon. With primers 33–34, DNA fragments of 176 and 155 bp were amplified containing or devoid of exon N, respectively. From kidney, liver, and adrenal medulla, the two fragments were similarly amplified. From brain, glial cells, and neurons, the two bands are detected, but the 155-bp one is much more intense. With primers 42-2, plasmids containing or without exon C led to amplification of DNA fragments of 175 and 157 bp, respectively. A single 157-bp band was visualized with kidney, liver, and glial cells, showing that they express mostly the a1-II and -III variants that do not contain exon C. From brain and neurons in culture, only the 175-bp fragment was detected that corresponds to the exon C containing the a1-I and -IV variants. From adrenal medulla, the two fragments were amplified. All together, data from Fig. 2B show that a1-I expression is much higher than that of a1-IV in neurons and in the brain; glial cells mainly express a1-III and to a lower extent a1-II, whereas the expression of these two variants is similar in kidney and liver; finally, the four variants are expressed in adrenal medulla.

Three Isoforms of the a Subunit Are Coexpressed in a Single Neuron—Coexpression of the a1-I, a1-IV, and a2 isoforms in hippocampal neurons in culture could reflect the existence of several subpopulations of neurons, each expressing a different subunit a. Alternatively, each individual neuron could coexpress the three isoforms. To address this question, we performed single-cell RT-PCRs. Cytoplasm was harvested from individual identified neurons and patch-clamped in the whole cell configuration by aspiration through the patch pipette. This was done either on pyramidal neurons in brain slices (in layer V of the visual cortex) or on hippocampal neurons in primary culture.
reverse transcription, cDNAs encoding subunits a1, a2, and c were amplified simultaneously in a first PCR. Further specific amplifications of each subunit or splice variant were obtained in second parallel PCRs (PCR2). Fig. 3 (A and B) presents results from a typical hippocampal neuron after 14 days in culture. The neuron was patch-clamped in the whole cell configuration, its resting membrane potential was measured (−65 mV), and its response to a square depolarizing pulse was recorded (evoked action potentials are shown in Fig. 3A). By RT-PCR on an aliquot of its cytoplasm, this neuron was shown to express subunit c, the a1 and a2 isoforms (Fig. 3B). The a1 amplicon comprises the site of exon N insertion. Two DNA fragments were amplified with a1 primers, whereas the smaller and abundant one corresponds to a splice variant without exon N, whereas the larger one contains exon N. Coexpression of two a1 splice variants containing or not containing exon N was directly demonstrated in two nested parallel PCRs using the same forward primer and reverse primers that bind either in exon N (+N) or only when exon N is absent (−N). These variants most likely correspond to a1-I and a1-IV (see later). The specificity of each amplification product has been verified by digestion with appropriate endonucleases that generated restriction digest products of the expected size (Fig. 3B, right panel).

In each experiment, several neuronal samples (12–18 samples) were harvested and processed in parallel. The results of an experiment on hippocampal neurons after 14 days in vitro are shown (Fig. 3C). Because the amount of cytoplasm that is aspirated from each neuron is difficult to estimate, expression of subunit c was taken as an internal control. Subunit c mRNAs are expected to be rather abundant in neurons; they are frequently found in expressed sequence tag data banks, V0 contains several copies of subunit c, which is encoded by a single gene in vertebrates (2). Only neuronal samples that gave positive results for c were considered (98 c+/116 harvested neurons, from seven different experiments). Considering all of the experiments, expression of the a1 isoform was detected in 80% of neurons (78 a1+/98 c+ neurons, seven experiments). Two series of experiments were performed, with primers that hybridize on both sides of the alternative splicing sites; in the first series (five experiments), we amplified 500-bp DNA fragments that contained an exon C insertion site; in the second series (two experiments), we amplified 200-bp DNA fragments containing the exon N site. Detection of the isoform a1 expression was more efficient in the 200-bp series (31 a1+/33 neurons, 94%) than in the 500-bp series (47 a1+/65 neurons, 72%). This is probably due to an increase of PCR efficacy rather than a sequence effect because a similar difference was found for subunit c expression (see below). Nested specific PCRs were performed to identify the a1 splice variants that are expressed. In the first series, all of the a1 positive neurons expressed exon C-containing variants (47 +C/47 a1 +), and none expressed variants without exon C. Therefore, no neuronal expression of the a1-II and a1-III variants was detected. In the second series, a1 amplification led, in some cases, to the detection of two fragments, suggesting coexpression within the same neurons of two splice variants of a1 (not shown). This was directly tested using nested reverse primers (Fig. 3C, two upper lanes). All of the neurons that express the a1 isoform expressed variants without exon N (31 -N/31 a1 +), whereas ∼40% of them (12 +N/31 neurons) were shown to also express variants that contain exon N. These variants correspond most probably to a1-I and a1-IV, respectively.

Expression of the a2 isoform was detected in approximately half of the neurons (Fig. 3C). Considering the two series of experiments, a2 isoform expression was detected in 26 neurons of 57 neurons (46%, four different experiments). As also observed for subunit c and a1 expression (see above), detection of a2 expression was more efficient in the 200-bp series (20 a2+/33 neurons, 61% from two experiments) than in the 500-bp one (6 a2+/24 neurons, 25%, two experiments).

It has been reported that, at a low mRNA copy number, PCR detection leads to a stochastic all-or-none amplification with either positive samples or apparently negative ones (23). It was of importance to know whether, in our single-cell RT-PCR experiments, negative detection...
reflected the failure of amplification of some low abundance mRNAs or whether it was related to neuronal heterogeneity. This was tested on very low amounts of total brain RNA, which contain all of the mRNAs of interest. In the representative experiment shown (Fig. 3D), six identical aliquots of brain RNA (1 pg of total RNA) were reverse transcribed and, after duplication, amplified as was done for neuron samples. One can estimate that 1 pg of total brain RNA (of which 2–5% would be mRNA) corresponds to 20–100 molecules of a medium abundance mRNA species (representing 1/1000 to 1/500 of the cell mRNAs, with an average 2-kb size). Subunit c mRNAs were successfully amplified in the 12 replicates. But the a1 and a2 subunit mRNAs, which are expected to be less abundant (see above), were not detected in all of the samples. Subunit a1-I was detected in nine replicates, whereas a1-IV was amplified only once. A similar all-or-none amplification was observed for a2 mRNAs. We have observed that the percentage of amplified samples depends on the number of cDNA molecules used in the first PCR amplification step. Because the efficacy of reverse transcription is not expected to be very high at low RNA copy number (10–20%; Ref. 23), all-or-none amplification was observed for ~2–20 cDNA molecules, in accordance with data from Tsuzuki et al. (23). These control experiments illustrate that, in our single-cell RT-PCRs, all-or-none detection of certain isoforms does not necessarily reflect a neuronal heterogeneity. The percentage of positive neurons seemed to be related to the abundance of the mRNA species concerned. For example, when we performed single-cell RT-PCRs on neurons after 2 or 14 days in culture, the percentage of positive neurons for the expression of subunit c or a1 doubled (two different experiments not shown) when we observed a 4–5-fold overall increase in the expression of these subunits by semi-quantitative RT-PCR (Fig. 4A). The fact that similar results were obtained from two different neuronal populations (hippocampal neurons in culture or cortical neurons in situ) and that neurons that were shown to express a2 or a1-IV were also positive, because the more abundant a1-I renders very unlikely the existence of neuronal subpopulations that do not express one of these subunit a isoforms.

Subunit a Isoform Expression and Neuritogenesis—The expression of subunit a isoforms a1 and a2 in hippocampal neurons after 2 or 14 days in primary culture were compared (Fig. 4A). During that period, neu-
rons extend neurites that make progressively a dense network with numerous synaptic contacts. Expression of the vesicular glutamate transporter (VGlut1), a specific protein of synaptic vesicles, was markedly increased (\(8\)-fold, determination from two independent experiments, normalized at constant \(\beta\)-actin expression) when comparing neurons from the same primary cultures at 2 and 14 days in vitro (Fig. 4A). Subunit c expression increased (\(5\)-fold) during that period, as did isoform a1 expression. In contrast, expression of isoform a2 remained unchanged (two independent experiments; Fig. 4A). We then compared the expression of the a1 splice variants in the same conditions (Fig. 4B). Expressions of a1-I and, to a lesser extent, a1-IV were increased at 14 days in vitro. Plasmids encoding the different a1 splice variants (2 pg of DNA) were amplified in parallel to illustrate that detection of a1-I expression required much less amplification (PCR cycles) than detection of a1-IV. Trace amounts of a1-II and a1-III in both 2- and 14-day in vitro samples were hardly detected, even after overamplification (compare plasmid amplifications). Therefore a1 and a2 isoforms expressions are differently regulated. Expression of a2 appears independent of neurites extension and synapse formation, whereas the a1-I and c mRNA levels are markedly increased.

The a1 Isoform Is Concentrated in Nerve Terminals in Hippocampal Neurons—The distribution of the a1 isoform in 14 days in vitro hippocampal neurons (Fig. 5) was studied using an antibody that binds to the 10 C-terminal amino acids of the subunit and therefore does not discriminate the different a1 variants. Immunolabeling of a1 led to rather large fluorescent spots that are aligned on neurites and the neuronal cell body (Fig. 5A). This punctate distribution colocalized with that of SV2 (Fig. 5B). SV2 is a specific marker of synaptic vesicles, regardless of the neurotransmitters they contain and therefore of all nerve terminals. The a1 isoform and SV2 both appeared therefore concentrated within nerve terminals.

Differential Targeting of Recombinant Tagged a1 and a2 Isoforms—To compare the subcellular sorting of the a2 isoform and of the a1 isoform variants for which no specific antibodies are available, recombinant a1 and a2 isoforms with an N-terminal FLAG tag were expressed in hippocampal neurons in culture, and their targeting was visualized by confocal microscopy imaging (Figs. 6 – 8). Neurons expressing very high levels of exogenous proteins exhibited signs of toxicity and were not analyzed. All of the expressed tagged isoforms were visualized in the neuron soma (Fig. 6) and the proximal part of dendrites. Dendrites were
stained with anti-MAP-2 antibodies that specifically label dendritic microtubules. Punctate staining of recombinant a1-IV was visualized in dendrites over a much longer distance than was observed for a1-I- and a2-transfected neurons (Fig. 6). It has been possible to detect recombinant a1-I and a1-IV in the initial portion of axons (not shown), in contrast to a2. In the vicinity of a1-I-transfected neurons, aligned stained spots, frequently apposed onto dendrites, were always observed (Fig. 6, arrowheads). Such stained spots correspond to axonal varicosities (see Fig. 8). They were never detected when neurons express recombinant a2 (Fig. 6) and only occasionally for the recombinant a1-IV variant.

In the cell soma when observed at a higher magnification (Fig. 7), the recombinant a1-I and a1-IV variants appeared evenly distributed, giving a punctate staining with no colocalization with TGN-38, a protein located inside trans-Golgi cisternae. In contrast, the a2 isoform was concentrated in the trans-Golgi network where it matched the TGN-38 distribution (Fig. 7). A similar codistribution of TGN-38 and the endogenous a2 isoform has been observed in epididymis cells (24). The recombinant a2 isoform appeared therefore restricted to cell soma organelles and has never been detected in distal parts of dendrites or axons or in nerve terminals (not shown).

Outside the cell soma, the a1-I and a1-IV variants exhibited different subcellular distributions. Tagged a1-I was found concentrated in 2–3-μm intense spots, where it colocalized with endogenous SV2 (Fig. 8). This staining pattern very much resembled that of endogenous a1 subunit (Fig. 5), showing that recombinant a1-I is accumulated in nerve terminals. Axonal staining of recombinant a1-I was restricted to these nerve terminal varicosities as shown by colabeling with anti-neurofilament antibodies (not shown). In contrast to a1-I, overexpressed recombinant a1-IV was observed both in distal parts of dendrites and axons where it does not strictly colocalize with SV2 (Fig. 8). For some transfected neurons, a1-IV was also accumulated in SV2-stained axonal varicosities. These differences in the recombinant a1-I and a1-IV distributions suggest that insertion of the peptide encoded by exon N (peptide N) influences neuritic sorting of a1 variants. To evaluate the relative importance of insertion of the peptide encoded by exon C (peptide C) in targeting of the a1 subunits, neurons were also transfected with tagged a1-II and a1-III, two splice variants that are normally not expressed in neurons (see above). Recombinant a1-II showed the same distribution as a1-IV, whereas expression of tagged a1-III led to a staining pattern that is identical to that of a1-I (Fig. 8). These results show that targeting of the a1 variants to different neuritic domains is influenced by insertion of peptide N, in contrast to that of peptide C.

These results show that the three variants of the v-ATPase subunit a that are expressed in neurons, a1-I, a1-IV, and a2, are sorted to different membrane compartments, even under conditions of overexpression.
The a2 isoform is located in the cell soma and abundant in the trans-Golgi network. Nerve terminals are enriched in the a1-I isoform, whereas the a1-IV splice variant appears more evenly distributed.

**DISCUSSION**

In mice as in other vertebrates, four different genes code for the a1–a4 isoforms of subunit a (6–8). Three a1 variants generated by alternative mRNA splicing of two small exons (exons N and C) have been described in mice (7). They differ by the presence or absence of 7- and 6-amino acid-long insertions near the amino (peptide N) and C (peptide C) termini of the subunit, respectively. In the present work, we have detected in rat brain a fourth a1 splice variant, a1-IV, that contains both the N and C exons. The two variants that lack the C insertion, a1-II and a1-III, are expressed in non-neuronal tissues (kidney, liver, and adrenal medulla); their small expression in brain is most probably due to the glial cell contribution because a1-II and a1-III are expressed by astroglial cells in culture, and not by neurons. The two variants that possess the C insert, a1-I and a1-IV, are both specifically expressed in neurons and possibly by neurosecretory cells as suggested by the expression of both a1-I and a1-IV by adrenal medulla (Fig. 2) and PC12 cells.3 The insertion of peptide C in the cytoplasmic loop between transmembrane domains VI and VII (according to the transmembrane topography proposed by Leng et al. (3)) generates a PEST motif (9). PEST sequences have been proposed to serve as conditional proteolytic signals (25), and therefore insertion of peptide C could result in a1 variants with a shorter half-life. Peptide C is in a portion of subunit a that contains numerous conserved amino acids that were shown, by mutagenesis of the yeast subunit a orthologue, to be essential for v-ATPase activity (5, 26). A functional comparison of neuronal v-ATPases possessing different subunit a isoforms or a1 variants may reveal significant differences. Yeast v-ATPases that contain either the Vph1 or Stv1 subunit a isoforms exhibit different functional properties, concerning either the coupling of proton-transmembrane domain V0 or the ability of membrane fusion (11).

Efficient sorting mechanisms are required to address the correct amount of v-ATPase to the proper membrane compartments. The membrane V0 domain appears to contain the necessary targeting information (1). It can be synthesized, assembled, and sorted independently of the catalytic V1 sector (27, 28). In neurons, V0 is transported to the nerve terminals by the fast anterograde axonal flow, independently of V1 (12). The two yeast subunit a isoforms, Vph1 and Stv1, are addressed to vacuolar and Golgi/endoosomal membranes, respectively (4, 5), and their subcellular targeting is controlled by their cytosolic N-terminal domains (5). Torpedo electromotoneurons synthesize at least two different subunit a isoforms, the a1 isoform being specifically sorted to nerve endings where it is present both in synaptic vesicles and in the presynaptic membrane (13). Targeting of V0 appears therefore to be controlled by the subunit a isoform it contains.

In the present work, we showed that neurons, the majority at least and probably all of them, express three different subunit a isoforms, a1-I, a1-IV, and a2. To compare the subcellular sorting of the a2 isoform and of the a1 isoform variants for which no specific antibodies are available, recombinant a1 and a2 isoforms with an N-terminal FLAG tag were expressed in hippocampal neurons in culture. Obviously, sorting of overexpressed recombinant proteins should be interpreted with caution, especially for proteins with a low endogenous expression. However, comparison of the subcellular distributions of the tagged a1-I, a1-IV, and a2 isoforms revealed that each of them have unique localizations that therefore cannot simply be attributed to overexpression.

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3 S. Pœa-Guyon and N. Morel, unpublished results.

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The a2 isoform, which is expressed in all of the tissues tested (Refs. 7 and 8 and the present work), is present in cell soma organelles including the trans-Golgi network. Its expression is not modified after differentiation of the neuritic network and establishment of synaptic contacts. v-ATPases containing the a2 subunit are probably some sort of housekeeping v-ATPase common to all cells.

During neuritic extension and synaptogenesis, expression of a1-I and a1-IV increased, a large up-regulation that was associated with an increased expression of subunit c, indicating synthesis of larger amounts of v-ATPases. This was expected because a1-containing v-ATPases are concentrated within nerve endings (Ref. 13 and the present work). Recombinant a1-I was accumulated in synaptic vesicle enriched varicosities, whereas overexpressed a1-IV was detected both in distal dendrites and axons, where, in contrast to a1-I, it was not restricted to nerve terminals. Using an antibody that does not discriminate a1 variants, the endogenous a1 isoform was observed highly concentrated in nerve terminal varicosities. This shows that a1-I is the most abundant a isoform expressed in neurons, in accordance with PCR data. Endogenous a1-IV was probably not detected because it was present in too low amounts, but an absence of a1-IV mRNAs translation or the possibility that a1-IV could be trafficked like a1-I when not overexpressed cannot be excluded. Because a1-containing v-ATPase membrane domains were shown to be localized both in synaptic vesicles and in the presynaptic plasma membrane (13), one can speculate that a1-I could be synaptic vesicle-specific, whereas a1-IV could be targeted to neuritic plasma membranes.

Recombinant a1-II and a1-III, variants that are not endogenously expressed in neurons, exhibited staining patterns identical to those of a1-IV and a1-I, respectively. Therefore, alternative splicing of exon N modifies sorting to synaptic vesicles of the a1 subunit variants, in contrast to that of exon C. It is not known how insertion of the small 7-amino acid-long peptide encoded by exon N influences subunit a1 targeting. One obvious possibility would be that it regulates interactions of the N-terminal cytoplasmic domain of a1 with some important protein partners. Axonal versus dendritic targeting is likely to involve several splicing steps, including insertion into specific membrane carriers in the Golgi, preferential transport along axonal microtubules, fusion into the plasma membrane, and selective retrieval by endocytosis (29, 30). Different domains of the cargo could interact at each of these steps with various protein partners, as shown for VAMP-2 targeting to synaptic vesicles (31, 32). Recently, in Drosophila neurons, the a subunit a isoform was shown to interact with the t-SNAREs syntaxin-1 and SNAP-25 via its N-terminal domain (20), but alternative splicing of Drosophila a1 was not studied.

In the present work, neurons were shown to coexpress three different isoforms of subunit a, which are targeted to different membrane compartments. A new splice variant of the a1 isoform, a1-IV, has been identified. Peptide N appears to play a role in targeting subunit a1 variants to nerve terminals. The specific expression of the two variants that contain peptide C in neurons, and neurosecretory cells, suggests that these variants could be specially fitted for neurotransmitter storage and/or release during synaptic activity (13, 20).

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