Received: 11 September 2023 | Revised: 5 May 2024 | Accepted: 23 May 2024

DOI: 10.1111/apha.14186

ACTA PHYSIOLOGICA

RESEARCH PAPER

ATP6V1A is required for synaptic rearrangements and plasticity in murine hippocampal neurons

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Funding information
#NEXTGENERATIONEU (NGEU) and funded by the Ministry of University and Research (MUR), National Recovery and Resilience Plan (NRRP), project MNESYS (PE0000006)—A multiscale inte- grated approach to the study of the nervous system in health and disease (DN. 1553 11.10.2022).; The Italian Ministry of Health (Ricerca Finalizzata Giovani Ricercatori GR-2019-12 370 176); IRCCS Ospedale Policlinico San Martino (5x1000 and Ricerca Corrente); Tuscany Region Call for Health 2018 (grant DECODE-EE); Fondazione Cassa di Risparmio di Firenze (Human Brain Optical Mapping Project); University of Genoa (Grant D.R. 3404, Heavy Equipment)

Abstract

Aim: Understanding the physiological role of ATP6V1A, a component of the cytosolic V_1 domain of the proton pump vacuolar ATPase, in regulating neuronal development and function.

Methods: Modeling loss of function of *Atp6v1a* in primary murine hippocampal neurons and studying neuronal morphology and function by immunoimaging, electrophysiological recordings and electron microscopy.

Results: *Atp6v1a* depletion affects neurite elongation, stabilization, and function of excitatory synapses and prevents synaptic rearrangement upon induction of plasticity. These phenotypes are due to an overall decreased expression of the V_1 subunits, that leads to impairment of lysosomal pH-regulation and autophagy progression with accumulation of aberrant lysosomes at neuronal soma and of enlarged vacuoles at synaptic boutons.

Conclusions: These data suggest a physiological role of ATP6V1A in the surveillance of synaptic integrity and plasticity and highlight the pathophysiological

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significance of ATP6V1A loss in the alteration of synaptic function that is associated with neurodevelopmental and neurodegenerative diseases. The data further support the pivotal involvement of lysosomal function and autophagy flux in maintaining proper synaptic connectivity and adaptive neuronal properties.

KEYWORDS

ATP6V1A, autophagy, LTP, lysosome, neurodevelopment, synapse, v-ATPase

1 | INTRODUCTION

Vesicular or vacuolar-type adenosine triphosphatase (v-ATPase) is a highly conserved multimeric complex that drives the transport of hydrogen ions upon ATP hydrolysis. In mammals, the complex is composed of the V_0 membrane domain, which consists of 7 distinct subunits and forms the hydrogen pore, and the V_1 cytosolic domain, which consists of 8 distinct subunits that bind and hydrolyze ATP.[1] The association/dissociation of V_0 and V_1 is reversible and regulates the proton pumping activity. Its activity is modified by several signals, including nutrients, hydrogen ion concentration, growth factors and kinases.[2–4] v-ATPase is embedded in various cellular membranes and the various subunits are typically expressed in a tissue-specific manner, resulting in different degrees of regulated acidification.[5,6] At lysosomal compartments, v-ATPase mediates the luminal acidification necessary for catabolic processes, such as endocytic degradation and autophagy. Autophagy is particularly relevant for neuronal cells, due to their post-mitotic nature, polarized morphology and high protein load, which sustain synaptic activity and plasticity.[7] In neurons, v-ATPase is additionally expressed by synaptic vesicles (SVs), where it creates the proton gradient that drives the loading of neurotransmitters.[8,9] Mutations in genes encoding the different subunits of v-ATPase and their regulators have been recently described in patients with a spectrum of neurodevelopmental disorders often associated with mental retardation and seizures.[10–18] In addition, loss of expression of ATP6V1A has been reported for late onset Alzheimer disease (AD).[19–21]

The atomic structure of the mammalian brain v-ATPase has been recently resolved, showing that the ATP6AP1 and ATP6AP2 subunits act as assembly factors for the V_0 domain.[22] Specific functions of the neuronal v-ATPase subunits have been investigated. However, due to the complexity of the supramolecular complex, a complete picture of their roles in the neuronal system is far from being elucidated. For instance, both the membrane-associated ATP6V0A1 subunit and the V_0 assembly factor ATP6AP2 have been shown to play a role in brain development in terms of synaptic integrity and connectivity.[10,17] Moreover, experimental evidence supports a complex and not fully elucidated role of the different members of the v-ATPase at presynaptic sites. The V_0 domain subunits V_0a and V_0c interact with the SNARE complex and modulate neurosecretion,[23,24] whereby V_1/V_0 disassembly represents a precondition for SV fusion, thus defining availability of SV for release.[25] Assembled V_1/V_0 was also identified on clathrin-coated endocytosed vesicles, where clathrin blocks v-ATPase activity and prevents acidification before uncoating.[26]

In accordance with the above-mentioned essential roles of v-ATPase, alterations in genes encoding v-ATPase subunits or their regulators were found to perturb brain development and synaptic function in animal models and cause human disease.[10–18]

v-ATPase dysfunctions were also described in aging and neurodegeneration.[27] In particular, deficits in *ATP6V1A* – encoding for the ubiquitously expressed subunit A of the V_1 complex – have been associated with both early-onset epileptic encephalopathy (EOEE)[14,16] and late onset AD.[19–21] These findings reveal that early neurodevelopmental and neurodegenerative disorders may share common pathways such as lysosomal and autophagic failures as consequence of neuronal v-ATPase dysfunction.

To investigate the role of v-ATPase in neuronal development and connectivity, we developed an in vitro neuronal model of*Atp6v1a* loss. We demonstrated that Atp6v1a depletion results in overall decrease of V_1 subunits, affects synapse stabilization and prevents long-term potentiation (LTP) at excitatory hippocampal synapses. These phenotypes were due to impairment of neuronal pH homeostasis and of autophagy with the accumulation of aberrant lysosomes at neuronal cell bodies and enlarged cisternae at synapses.

2 | RESULTS

2.1 | Loss of *Atp6v1a* impairs neuronal lysosomal acidification

We modeled neuronal *Atp6v1a* loss by silencing its expression in cultured primary rat neurons using RNAi-mediated knockdown. A small hairpin (sh)-RNA, targeting the coding sequence of the rat *Atp6v1a* mRNA (*Atp6v1a* KD), was generated and cloned into lentiviral vector for neuronal infection. Neurons were transduced at 10 days in vitro

(DIV) and analyzed at 17 DIV. Western blot analysis revealed a significant reduction of Atp6v1a protein in *Atp6v1a* KD with respect to control neurons transduced with the scrambled sh-RNA virus (Figure 1A). By evaluating the expression of other v-ATPase subunits, we observed a parallel reduction of different members of the V_1 domain – namely Atp6v1h, Atp6v1b2 and Atp6v1c1- with no change in the V_0 subunit Atp6v0a1 (Figure 1A).

Loss of expression and activity of v-ATPase subunits in neurons is predicted to primarily impair intracellular pH homeostasis.[28,29] We therefore analyzed the consequences of Atp6v1a depletion on intraorganellar acidification, using the acidic dye Lysotracker (LTR). Staining with LTR was significantly lower in *Atp6v1a* silenced neurons, and the decrease in the LTR intensity was phenocopied in neurons treated with the v-ATPase blocker bafilomycin (Figure 1B,C). To verify the specificity of the phenotype and exclude off-target effects, we designed an ATP6V1A expressing construct that is resistant to silencing (r-ATP6V1A). Its efficacy was tested by western blot and immunocytochemistry (Figure S1). Re-expression of r-ATP6V1A in *Atp6v1a* KD neurons restored LTR intensity to control levels while no significant impact was observed in control scamble-sh-RNA transduced cells (Figure 1D).

These data demonstrate that the observed LTR phenotype is specifically associated to Atp6v1a loss and caused by inhibition of v-ATPase activity due to overall decrease of V1 subunits.

2.2 | The acidic shift in *Atp6v1a* KD neurons primarily involves lysosomes

To determine if the LTR decrease was cell autonomous and mainly affected lysosomal structures, we transfected neurons with *Atp6v1a* sh-RNA alone or together with the red fluorescent protein (RFP)-tagged lysosomal marker LAMP1 (LAMP1-RFP). LTR signal was heavily reduced in transfected cells (Figure 2A) and significantly lower in the LAMP1-positive compartments of both *Atp6v1a* KD transfected and bafilomycin-treated neurons (Figure 2B,C). These data suggest that the effect of *Atp6v1a* KD is cell autonomous and the basic shift at neuronal soma primarily involves lysosomes.

2.3 | *Atp6v1a*-silencing in neurons induces blockade of autophagy and lysosome ultra-structural alterations

v-ATPase-mediated acidification is necessary for lysosomal function and autophagic progression. To analyze the effects of *Atp6v1a* silencing on neuronal autophagy, we evaluated the expression of autophagic markers and revealed an accumulation of LC3 and p62 in the soma of neurons transduced with *Atp6v1a* KD with respect to that with scramble controls (Figure 3A,B). When analyzed by Western blot, we confirmed an increased expression of the autophagic markers Lc3 and p62 and revealed a net accumulation of Lc3-II, the lipidated form of Lc3 (Figure 3C). The phenotype was phenocopied by *Atp6v1a*-sh-RNA transfection and bafilomycin treatment (Figure S2). These data support the pivotal role of ATP6V1A expression and v-ATPase activity in sustaining autophagy progression in neurons.

When analyzed at the ultrastructural level, *Atp6v1a*-silenced neurons displayed increased density and size of multilamellar bodies (MLBs) in the soma, reminiscent of terminal non-degradative lysosomes (Figure 3D). Taken together these findings demonstrate that knockdown of the *Atp6v1a* subunit results in a prominent lysosomal alteration associated with impaired autophagy.

2.4 | *Atp6v1a* silencing impairs dendritic outgrowth and synaptogenesis due to selective loss of presynaptic sites

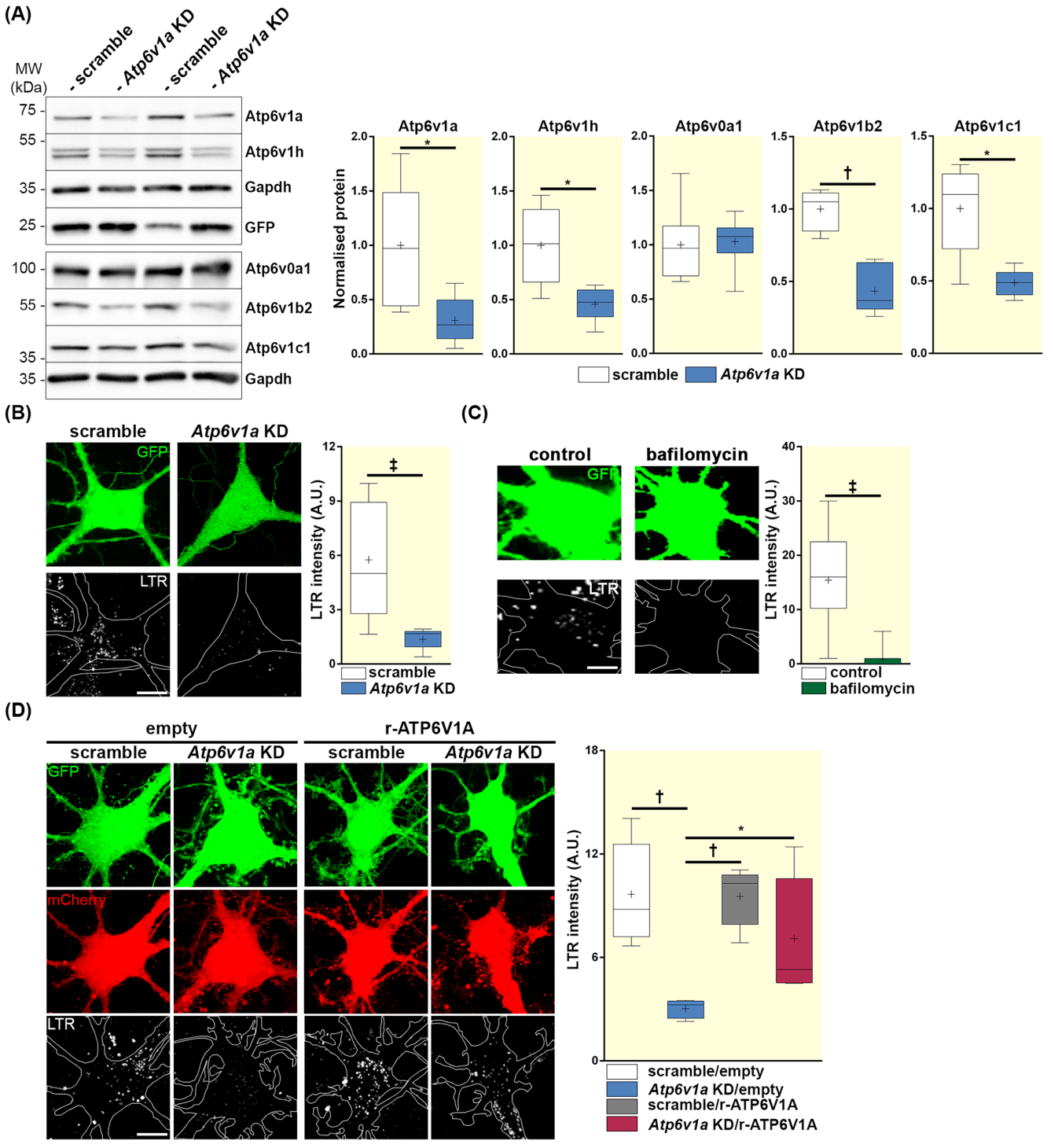
To examine the impact of the Atp6v1a loss on neuronal morphology and function, we quantified neurite arborization and synaptic connections in *Atp6v1a*-silenced neurons. Sparse transfected neurons, showed a significant decrease in neurite complexity, quantified by Sholl analysis (Figure 4A). The phenotype is accompanied by a significant loss of excitatory synaptic contacts, identified by the presynaptic marker v-Glut1 and the postsynaptic marker Homer 1. When analyzed separately, a significant decrease was observed only for the presynaptic transporter, suggesting a primary role of Atp6v1a at presynaptic sites (Figure 4B). Such decrease was phenocopied treating neurons with bafilomycin, implying that loss v-ATPase activity results in defective neuronal development and synaptic connectivity (Figure S3).

Single cell re-expression of r-ATP6V1A, in neuronal network silenced by viral transduction, efficiently restored the density of v-Glut1 positive puncta to the control level (Figure 4C).

These data demonstrate a specific impairment of neurite development and excitatory synaptic contact upon loss of V_1 subunits and v-ATPase dysfunction.

2.5 | Loss of Atp6v1a decreases the frequency of miniature excitatory postsynaptic currents and results in cisternae accumulation at presynaptic sites

To directly measure glutamatergic neurotransmission, we performed whole-cell voltage-clamp recordings of



miniature excitatory postsynaptic currents (mEPSCs). We recorded voltage (−70 mV) clamped neurons in the presence of TTX to measure spontaneous miniature currents (Figure 5A). We found that the mEPSC frequency recorded in *Atp6v1a* KD neurons was significantly lower compared to that in controls (scramble: 1.423 Hz ± 0.175; *Atp6v1a* KD: 0.487 Hz ± 0.070; Figure 5B), without significant changes in mEPSC amplitude, charge, rise and decay kinetics (Figure 5C,D). At the ultrastructural level, *Atp6v1a* KD synapses are preserved in term of synaptic area, active zone (AZ) length and total SV density. However, they present accumulation of enlarged cisternae suggesting impaired endosomal clearance at presynaptic sites (Figure 6).

Altogether, these findings suggest a specific loss of active excitatory contacts in *Atp6v1a* silenced neurons with a presynaptic deficit but preserved postsynaptic response to glutamate exocytosis.

FIGURE 1 Loss of *Atp6v1a* impairs the expression of V1 subunits and acidification of intracellular organelles. (A) Representative Western blot images showing v-ATPase protein levels in rat cortical neuronal transduced with control scramble sh-RNA (scramble) or *Atp6v1a* sh-RNA (*Atp6v1a* KD) pGFP-C-shLenti vectors. Gapdh and turbo-GFP (GFP) are shown as reporters of equal loading and transduction levels. Densitometric quantification is shown on the right. Data are from $n = 6$ independent preparations. *$p < 0.05$, †$p < 0.01$ Mann–Whitney's *U*-test. (B) Representative images of rat hippocampal neurons transduced with scrambled or *Atp6v1a* KD sh-RNAs pGFP-C-shLenti vectors (GFP) and incubated with LysoTracker (LTR, 50 nM, 30 min) at 17 DIV. Quantitative analysis of LTR fluorescence intensity is shown on the right. Data are from 8 to 9 coverslips from 2 independent preparations. ‡$p < 0.001$, unpaired Student's *t*-test. Scale bar 10 μm. (C) Representative images of rat hippocampal neurons transfected with pCAGIG-IRES-enhanced green fluorescent protein (GFP) under control conditions or treated with bafilomycin (100 nM, 3 h) and incubated with LTR as above. Quantitative analysis of LTR fluorescence intensity is shown on the right. Data are from 32 cells for each experimental condition from 3 independent preparations. ‡$p < 0.001$, Mann–Whitney's *U*-test. Scale bar, 5 μm. (D) Representative images of rat hippocampal neurons transduced with scramble or *Atp6v1a* KD sh-RNAs pGFP-C-shLenti vectors (GFP), successively transfected with sh-resistant ATP6V1A in pLVX-IRES-mCherry (r-ATP6V1A) or pLVX-IRES-mCherry empty vector (empty). At 17 DIV neurons were incubated with LTR as above. Quantitative analysis of LTR fluorescence intensity is shown on the right. Data are from 5 coverslips from 2 independent preparations. *$p < 0.05$; †$p < 0.01$, versus *Atp6v1a* KD/empty two-way ANOVA Dunnett's multiple comparison test. Scale bar, 10 μm.

2.6 | *Atp6v1a* silencing impairs autophagy-dependent synaptic plasticity

Compelling evidence demonstrated that lysosomal trafficking and autophagy regulate synaptic activity and plasticity.[30–37] To investigate this aspect, we challenged *Atp6v1a*-silenced neurons with an established chemical long-term potentiation (cLTP) protocol (Figure S4A)[33] and analyzed the excitatory synapses rearrangement. We observed a boost in neuronal autophagy upon LTP induction, as demonstrated by increased Lc3-II and decreased p62 signals, accompanied by the previously reported increase in excitatory synaptic contacts[33] (Figure S4B–F). In contrast, by challenging with the cLTP protocol either *Atp6v1a*-silenced neurons (Figure 7A,B) or bafilomycin-treated neurons (Figure 7C,D), we found that the increase in excitatory synapses was virtually abolished in both experimental groups. These findings demonstrate that neuronal v-ATPase is fundamental for synapse stabilization and plasticity, and loss of V_1 subunit results in defective functional connectivity and plasticity.

3 | DISCUSSION

v-ATPase is a ubiquitously expressed multi-subunit proton pump that regulates several essential functions in cells, ranging from nutrient sensing to catabolism. In neurons, it additionally regulates SV loading and neurotransmission. In recent years, several mutations in genes coding for the various v-ATPase subunits have been described in patients with neurodevelopmental disorders, often associated with epilepsy and intellectual disability. In particular, mutations in *ATP6V1A*, coding for the V_1A member of the cytosolic V_1 complex, have been associated with epileptic encephalopathies and a broad spectrum of clinical severity.[14,16,38–40] Altered ATP6V1A expression has been recently identified in Alzheimer's patient brains and proposed to represent a key factor for neuronal impairment and neurodegeneration.[20] In this scenario, clarifying the morphological and functional consequences of neuronal ATP6V1A loss is fundamental to unravel disease pathophysiology and design novel treatments.

By selectively silencing *Atp6v1a* subunit in rat hippocampal neurons, we observed impairment of lysosomal acidification and of autophagy progression, with an accumulation of aberrant lysosomes and MLB at neuronal soma. This phenotype was accompanied by impaired neuronal arborization and a prominent loss of excitatory synapses. This defect was due to a selective pruning of presynaptic contacts as demonstrated morphologically, by selective decrease of the glutamatergic presynaptic marker and, functionally, by a significant decrease in mEPSCs frequency. At the ultrastructural level, synaptic terminals showed accumulation of enlarged vacuoles with preserved AZ length and SV density within the boutons. The vacuole accumulation may represent a compensatory mechanism that allows the maintenance of the SV pool and their quantal size, as revealed by the preserved amplitude of mEPSCs. A similar, selective loss of presynaptic contacts has been recently described in human neurons lacking ATP6V1A accompanied by impaired neuronal activity.[20] Overall, our results support the evidence for v-ATPase-dependent lysosomal function and autophagy progression that is required for the maintenance of functionally active presynaptic contacts. In neurons lacking Atp6v1a, not only neuronal development and formation of excitatory synaptic connections were defective but also the surviving excitatory synapses did not respond to an LTP protocol that triggered synaptic plasticity in healthy neurons. This defective synaptic plasticity was associated with the lack of autophagy progression, as revealed by the prominent autophagy stimulation upon LTP induction. These data support the emerging and complex role

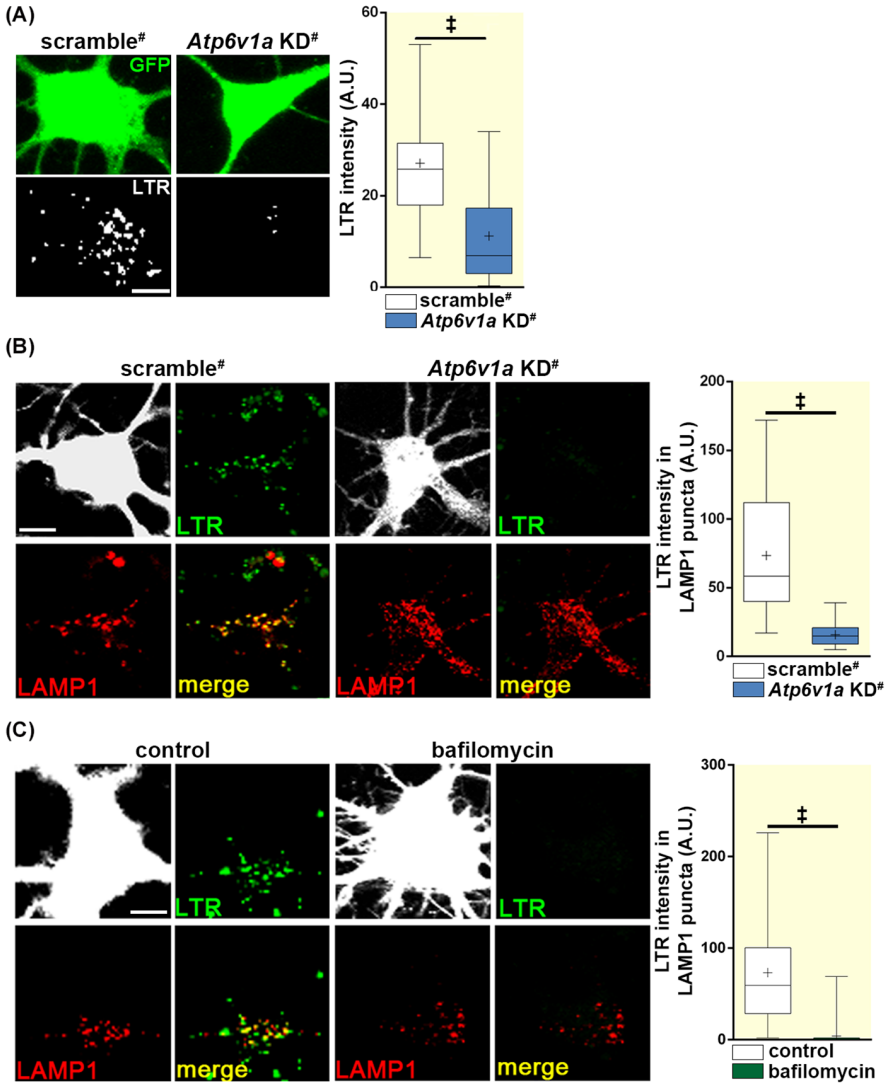


FIGURE 2 The acidic shift in *Atp6v1a* KD neurons primarily involves lysosomes. (A) Representative images of rat hippocampal neurons transfected (#) with scramble or *Atp6v1a* KD sh-RNAs pGFP-C-shLenti vector (GFP) and incubated with LTR (50 nM, 30 min). Quantitative analysis of LTR fluorescence intensity is shown on the right. Data are from 26 scramble and 33 *Atp6v1a* KD neurons from 3 independent preparations. ǂ$p < 0.001$, unpaired Student's *t*-test with Welch's correction. (B) Representative images of rat hippocampal neurons co-transfected with LAMP1-RFP (LAMP1) and either control scramble sh-RNA (scramble) or *Atp6v1a* sh-RNA (*Atp6v1a* KD) pGFP-C-shLenti vector (GFP) and incubated with LTR as above. Quantitative analysis of LTR fluorescence intensity in LAMP1-positive puncta is shown on the right. Data are from 74 scramble and 75 *Atp6v1a* KD neurons from 3 independent preparations. ǂ$p < 0.001$, Mann–Whitney's *U*-test. (C) Representative images of rat hippocampal neurons co-transfected with GFP plasmid (GFP) and LAMP1-RFP (LAMP1) under either control conditions or treated with bafilomycin (100 nM, 3 h). Quantitative analysis of LTR fluorescence intensity in LAMP1-positive puncta is shown on the right. Data are from 66 neurons for each experimental condition obtained from 3 independent preparations. ǂ$p < 0.001$, Mann–Whitney's *U*-test. Scale bars, 10 μm.

of the autophagy process in different forms of synaptic plasticity acting at both presynaptic and postsynaptic compartments.[31,32,36,41,42]

Our findings emphasize the central role of even a single v-ATPase subunit in the regulation of presynaptic homeostasis, synaptic rearrangements and plasticity. The loss of expression of Atp6v1a is paralleled by reduction of its main partners in the cytosolic V_1 domain, whereas no similar decrease is detected for Atp6v0a1, a member of the membrane embedded V_0 domain. These findings are consistent with the reported independent biosynthetic origin of V_0 and V_1 subunits[43] and demonstrate an overall decrease of the cytosolic enzymatic V_1 subunit in *Atp6v1a*-silenced neurons. The closely similar phenotypes which were observed by molecular silencing of *Atp6v1a* and pharmacological v-ATPase inhibition by bafilomycin,

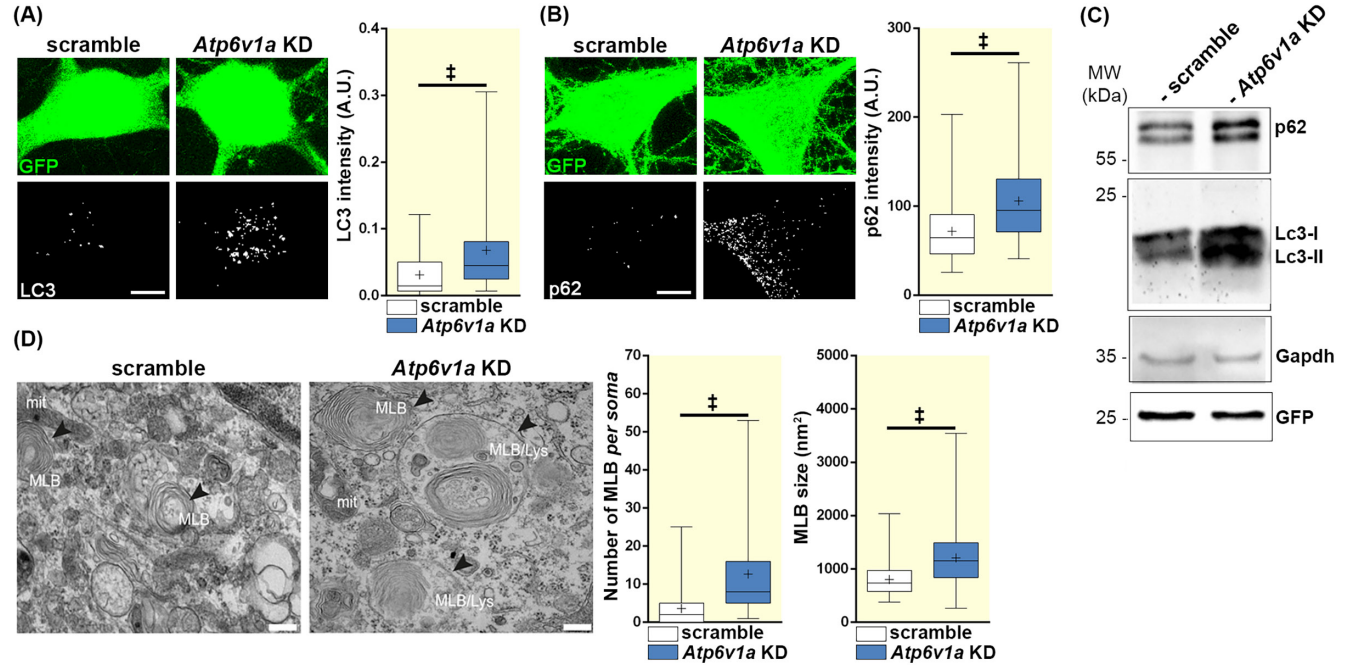


FIGURE 3 *Atp6v1a*-silencing in neurons induces blockade of autophagy and lysosomal structural alterations. (A) Representative images of rat hippocampal neurons transduced with scramble or *Atp6v1a* KD sh-RNAs pGFP-C-shLenti vector (GFP) and successively transfected with LC3-RFP (LC3). Quantitative analysis of LC3-RFP fluorescence intensity is shown on the right. Data are from 39 scramble and 51 *Atp6v1a* KD neurons from 2 independent preparations. ǂ$p < 0.001$, Mann–Whitney's *U*-test. (B) Representative images of rat hippocampal neurons transduced with scramble or *Atp6v1a* KD sh-RNAs pGFP-C-shLenti vector (GFP) and immunostained with anti-p62 antibody. Quantitative analysis of p62 fluorescence intensity is shown on the right. Data are from 50 scramble and 60 *Atp6v1a* KD neurons from 2 independent preparations. ǂ$p < 0.001$, Mann–Whitney's *U*-test. Scale bar, 5 μm. (C) Protein levels of p62, Lc3I and Lc3-II detected by Western blot in transduced neurons. Gapdh and GFP are shown as reporters for equal loading and infection levels. (D) Representative TEM micrographs of neurons transduced as above. Arrowheads indicate multilamellar bodies (MLBs) and enlarged lysosomes containing MLB (MLBs/Lys). Mitochondria (mit) are also shown. Scale bar, 200 nm. Box plots on the left represent numbers and size of MLBs in neuronal soma. All parameters were obtained from 48 cells per each condition from 3 independent preparations. ǂ$p < 0.001$, Wilcoxon's and Mann–Whitney's *U*-tests for MLB number and size, respectively.

further demonstrate the pilot role of V_1 expression for the physiological regulation of v-ATPase activity that propells both lysosomal acidification and autophagy flux.

Our findings reveal that neurons are particularly vulnerable to dysregulation of ATP6V1A, with loss of synaptic stability and plasticity as primary signs of neuronal dysfunction. They also highlight the central role of ATP6V1A in presynaptic homeostasis and activity-dependent rearrangement of synaptic connectivity. Our data further support the role of lysosomal integrity and autophagy flux for morphology and maintenance of connectivity in post-mitotic perennial cells such as neurons,[36,44] and explain the prevalence of brain dysfunction in ATP6V1A related disorders.[14,16] The early synaptic defects described here induced by autophagy-lysosomal dysregulation may trigger a pathophysiological cascade which can possibly result in neuronal shrinkage and neurodegeneration. This progression could underlie the early onset of neurodegeneration associated with the more severe expression of the *ATP6V1A*-related neurodevelopmental disorders[16] as well as the late onset AD associated with ATP6V1A loss.[20,21] Considering the broad spectrum of neurological pathologies related to dysregulation of ATP6V1A and the neuronal effects of its loss describe here in, identifying compounds that selectively act on ATP6V1A becomes a priority. Different ATP6V1A selective drugs are available and could establish a significant therapeutic basis. In particular, the immunosuppressive drug FK506 was reported to selectively bind ATP6V1A and exert neuroprotective effects.[45] The histone deacetylase inhibitor NCH-51 is a selective enhancer of ATP6V1A expression and normalizes neuronal impairment and neurodegeneration caused by ATP6V1A deficits.[20] In addition, the small molecule EN6 interacting with cysteine 277 of ATP6V1A increases v-ATPase activity, lysosomal acidification, and autophagy.[46] On the other hand, Kim et al, observed decreased levels of amyotrophic lateral sclerosis associated protein ataxin-2 upon constitutive downregulation of *ATP6V1A* and lowered ataxin-2 levels in cellular models and in the brains of adult mice after administration of the FDA approved ATP6V1A blocker etidronate.[47] These data, although at odds with previous findings showing pathogenicity