Importance of Glutamine in Synaptic Vesicles Revealed by Functional Studies of SLC6A17 and Its Mutations Pathogenic for Intellectual Disability

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SUMMARY

Human mutations in the gene encoding the solute carrier (SLC) 6A17 caused intellectual disability (ID). The physiological role of SLC6A17 and pathogenesis of Slc6a17-based-ID were both unclear. Here we report learning deficits in SLC6A17 knockout and point mutants. Biochemistry, proteomics and electron microscopy (EM) support SLC6A17 protein localization in synaptic vesicles (SVs). Chemical analysis of SVs by liquid chromatography coupled to mass spectrometry (LC-MS) revealed glutamine (Gln) in SVs containing SLC6A17. Virally mediated overexpression of SLC6A17 increased Gln in SVs. Either genetic or virally mediated targeting of SLC6A17 reduced Gln in SVs. One ID mutation caused SLC6A17 mislocalization while the other caused defective Gln transport. Multidisciplinary approaches with 7 types of genetically modified mice have uncovered Gln as a new molecule in SVs, established the necessary and sufficient roles of SLC6A17 in Gln transport into SVs, and suggested SV Gln decrease as the key pathogenetic mechanism in human ID.

INTRODUCTION

With an approximate prevalence of 1%, intellectual disability (ID) in humans is a neurodevelopmental disorder with debilitating effects on patients (Maulik et al., 2011). Molecular genetic research into ID has identified multiple genes whose defects underly ID (de Ligt et al., 2012; Gilissen et al., 2014; Hamdan et al., 2014; Hu et al., 2019; Khan et al., 2016; Lelieveld et al., 2016; Rauch et al., 2012; Ropers, 2010; Vissers et al., 2016). Functional studies of products encoded by these genes are essential for our mechanistic understanding of ID pathogenesis.

Mutations causing ID were found in the gene encoding SLC6A17 (Iqbal et al., 2015; Waltl, 2015). SLC6A17, also known as Rxt1, NTT4, XT1, or B^0AT3 (el Mestikawy et al., 1994; Liu et al., 1993), was discovered 29 years ago (el Mestikawy *et al.*, 1994; Liu *et al.*, 1993). It is predominantly expressed in the nervous system (el Mestikawy *et al.*, 1994; Fischer et al., 1999; Hagglund et al., 2013; Jursky and Nelson, 1999; Kachidian, 1999; Luque et al., 1996; Masson et al., 1995; Masson et al., 1999). SLC6A17 protein was localized on SVs (Fischer *et al.*, 1999; Kachidian, 1999; Masson *et al.*, 1999). Those facts, together with its membership in the SLC6 or the neurotransmitter transporter (NTT) family, suggest that SLC6A17 could be a vesicular transporter for a neurotransmitter(s).

Early efforts failed to identify substrates transported by SLC6A17 (Broer, 2006; el Mestikawy *et al.*, 1994; Liu *et al.*, 1993). A later study using pheochromocytoma (PC)12 cells and SLC6A17-tranfected Chinese hamster ovary (CHO) cells found that SLC6A17 could transport 4 amino acids (AA): proline (Pro), glycine (Gly), leucine

(Leu) and alanine (Ala) (Parra et al., 2008). Another study using human embryonic kidney (HEK) cells reported transport of 9 AAs: Leu, methionine (Met), Pro, cysteine (Cys), Ala, glutamine (Gln), serine (Ser), histidine (His) and Gly (Zaia and Reimer, 2009). There are three differences between these two studies: that the second study reported 5 more amino acids (Met, Cys, Gln, Ser and His) as substrates of SLC6A17, that the second study used modified SLC6A17 to facilitate its membrane localization (Zaia and Reimer, 2009), and that one reported Na^+ dependence (Zaia and Reimer, 2009) whereas the other reported H^+ dependence (Parra *et al.*, 2008). However, none of these AAs have been found in the SVs. It remains unknown which of the 4 or 9 AAs, if any, are present in the SVs in an SLC6A17 dependent manner *in vivo*.

Thus, how mutations in a single AA of SLC6A17 lead to ID is unknown, the substrate(s) physiologically transported by SLC6A17 is unknown and behavioral phenotypes of animals lacking SLC6A17 or carrying any mutation pathogenic in human patients have not been characterized.

We have now generated mouse mutants either lacking the Slc6a17 gene or mimicking a point mutation found in ID patients. They had similar phenotypes including deficient learning and memory, indicating that the human mutation was a loss of function (LOF) mutation. These are the first animal models of ID caused by Slc6a17 mutations. While two in vitro studies with cell lines suggested that SLC6A17 could transport up to 9 neutral AAs, we could only find that Gln was present in the SVs. From multiple gain of function (GOF) and LOF experiments, we have obtained evidence that SLC6A17 is not only sufficient for Gln presence in the SVs *in vivo*, but

it is also physiologically necessary for Gln presence in the SVs. Of the two known human mutations pathogenic for ID (Iqbal *et al.*, 2015), we provide evidence that one caused the SLC6A17 to be mislocalized subcellularly and the other, while still on SVs, was defective in Gln transport. Decreases of Gln in SVs caused by SLC6A17 mutations were not correlated with any decrease in glutamate (Glu) or gamma-aminobutyric acid (GABA), dissociating vesicular Gln from the Glu/GABA-Gln cycle between neurons and glia. In addition to dissecting the molecular mechanisms underlying ID caused by Slc6a17 mutations, we report for the first time that Gln is robustly and reproducibly detected in the SVs, which should stimulate further research into its potential roles in neurotransmission.

RESULTS

Pattern of Slc6a17 Expression

To examine the pattern of Slc6a17 expression, we designed a knock-in mouse line Slc6a17$^{-2A\text{-}CreERT2}$ (Figure 1A). These mice were generated by in frame fusion of a T2A sequence (Ahier and Jarriault, 2014; Daniels et al., 2014; Trichas et al., 2008) and CreERT2 (Feil et al., 1996; Gu et al., 1994; Indra et al., 1999; Sauer and Henderson, 1988) to the C terminus of Slc6a17 with its stop codon removed (Figure S1A). We crossed the Slc6a17$^{-2A\text{-}CreERT2}$ mice with the Ai14 reporter line which contained floxed stop-tdTomato (Madisen et al., 2010). Treatment with Tamoxifen removed the stop codon and thus allowed specific expression of tdTomato in Slc6a17 positive cells (Figure 1A, Figure S1A). Labeled cells were exclusively neuronal with

no glial expression detected.

Slc6a17 expression was found in the neocortex, the thalamus, the amygdala, the hippocampus, the pontine nuclei and the brainstem (Figures 1B to 1K, Figures S1B and S1C). Strong expression of Slc6a17 was detected in the dentate gyrus (DG) and the CA3 region of the hippocampus (Figure 1K), which is essential for spatial learning and memory. Little expression was observed in either CA1 or CA2 (Figure 1K). Slc6a17 was also expressed in the basolateral amygdala (BLA) (Figure 1J), a brain area essential for emotion and fear learning.

Behavioral Deficits of Slc6a17 Mutant Mice

To investigate the functional role of Slc6a17, we generated a knock-out (KO) mouse mutant line with exon 2 deleted from the Slc6a17 gene (Figure 2A, Figure S2A). These Slc6a17-KO mice were not significantly different from the wild type (WT, Slc6a17$^{+/+}$) mice in body weight (Figure S3A), basal activities (Figure S3B), or short-term memory (Figure S3C).

To investigate learning and memory of Slc6a17-KO mice , we first tested Slc6a17-KO mice on a classical associative learning model of contextual and cued fear (Hitti and Siegelbaum, 2014; Phillips and Ledoux, 1992) (Figure 2B). Both the WT and the heterozygous (Slc6a17$^{+/-}$) mice spent a significant amount of time freezing (Figure 2C) on day 2 in Context-a environment. However, homozygous (Slc6a17$^{-/-}$) mutants showed a dramatic decrease in freezing behavior compared to the other genotypes (Figure 2C). On day 3, both Slc6a17$^{+/+}$ and Slc6a17$^{+/-}$ mice showed

significantly increased freezing behavior (Figure 2C), whereas Slc6a17$^{-/-}$ mutants showed significant memory impairment in response to the tone (Figure 2C). These results indicate that the Slc6a17-KO mice were severely deficient in contextual and cued fear memory.

We then tested hippocampus-dependent spatial learning and memory by evaluating the performance of mice in the Morris water maze (Morris, 1984; Morris et al., 1982) (Figure 2D). During training, control (Slc6a17$^{+/+}$ and Slc6a17$^{+/-}$) mice showed a gradually decreasing latency to find the platform in both visible and hidden sessions (Figure 2E), whereas Slc6a17$^{-/-}$ mutants exhibited significantly longer latency to find the platform (Figure 2E). Representative swimming traces of each genotype in probe trial are shown in Figure 2F. During the probe trial, control mice spent more time in the target quadrant (Figure 2F), whereas Slc6a17$^{-/-}$ mutants spent much less time searching the platform quadrant (Figure 2G and 2H). The frequency that control animals crossed through the platform area was above chance level (Figure 2G and 2H), whereas Slc6a17$^{-/-}$ mutants crossed the platform area much less than the other genotypes (Figure 2G and 2H).

Taken together, these data indicate that Slc6a17-KO mice are significantly impaired both in contextual and cued fear memory and in hippocampus-dependent spatial learning and memory.

Behavioral Deficits in Slc6a17^{P663R} Mutant Mice

We next generated a mouse line carrying a point mutation (P633R) found to be pathogenic in ID patients (Iqbal *et al.*, 2015). Three repeats of the hemagglutinin (HA) epitope (Kolodziej and Young, 1991) and three repeats of the FLAG tag were fused in-frame to the C terminus of the endogenous P633R mutant (Figures 3A and S2B). Homozygous (Slc6a17$^{P633R/P633R}$) mutant mice were not significantly different from the WT (Slc6a17$^{+/+}$) or heterzygous (Slc6a17$^{P633R/+}$) in body weight (Figure S4A), basal activities (Figure S4B) or novel objection recognition (Figure S4C).

We then investigated learning and memory in Slc6a17^{P633R} mice. In contextual and cued fear conditioning (Figure 3C), Slc6a17$^{P633R/P633R}$ mutants showed significantly less freezing behavior compared to Slc6a17$^{+/+}$ and heterozygous (Slc6a17$^{P633R/+}$) mice on day 2 in Context-a environment (Figure 3D). On day 3, Slc6a17$^{P633R/P633R}$ mutants showed a lower level of freezing behavior induced by cued tone compared to Slc6a17$^{+/+}$ and Slc6a17$^{P633R/+}$ mice (Figure 3D). These results indicate that Slc6a17^{P633R} mice are defective in contextual and cued fear memory.

We then tested Slc6a17^{P633R} mice in the Morris Water Maze for hippocampal spatial learning and memory (Figure 3E) (Morris, 1984; Morris *et al.*, 1982). During training, Slc6a17$^{+/+}$ and Slc6a17$^{P633R/+}$ mice had a similar pattern in their learning curves with gradual decreases in the latency to find the platform in two sessions (Figure 3F). Similar to the Slc6a17-KO mice, Slc6a17$^{P633R/P633R}$ mutants required significantly longer time to find the platform in both visible and hidden sessions (Figure 3F). Representative swimming traces of each genotype in probe trials are shown in Figure 3G. During the probe trial, Slc6a17$^{+/+}$ and Slc6a17$^{P633R/+}$ mice spent

more time in the target quadrant (Figure 3H) and the more crossings through the platform area than Slc6a17$^{P633R/P633R}$ mutants (Figure 3I).

Taken together, these data indicate that Slc6a17^{P633R} mice are also significantly impaired both in contextual and cued fear memory and in hippocampus-dependent long-term spatial learning. Because all the phenotypes of Slc6a17^{P633R} are similar to Slc6a7-KO, the point mutation P633 is a loss of function (LOF) mutation.

Vesicular Localization of SLC6A17

Previous studies with anti-SLC6A17 antibodies provided evidence that SLC6A17 was localized in SVs (Fischer *et al.*, 1999; Kachidian, 1999; Masson *et al.*, 1999). We have now used multiple approaches to analyze the subcellular localization of SLC6A17.

Slc6a17$^{-HA-2A-iCre}$ mice were generated with the C terminus of the endogenous SLC6A17 protein fused in frame with three repeats of the HA epitope (Figures 4A and S1D). We used differential centrifugation to purify SVs from the brains of Slc6a17$^{-HA-2A-iCre}$ mice (Evans, 2015; Huttner et al., 1983). As shown in Figure 4B, SLC6A17-HA was enriched in LP2 fraction, similar to the other SV proteins such as synaptotagmin 1 (Syt1) (Geppert et al., 1994), vesicular ATPase (V-ATPase) (Cidon and Sihra, 1989), and synaptobrevin 2 (Syb2) (Link et al., 1992; Schiavo et al., 1992), but different from postsynaptic proteins such as postsynaptic density 95 (PSD95) (Cho et al., 1992; Woods and Bryant, 1991). Sucrose gradient centrifugation of the LP2 fraction showed copurification of SLC6A17-HA with SV proteins including

synaptophysin (Syp) (Jahn et al., 1985; Leube et al., 1987; Wiedenmann and Franke, 1985), Syt1, and VGluT1 (Bellocchio et al., 2000; Takamori et al., 2000), but not with proteins localized to the postsynaptic membrane (PSD95) (Woods and Bryant, 1991; Cho, Hunt and Kennedy, 1992) or (SNAP23) (Ravichandran et al., 1996; Suh et al., 2010), the endoplasmic reticulum (ER) (ERp72), the endosome (EEA1) (Mu et al., 1995), the proteosome (PSMC6) or the trans-Golgi network (synaptaxin 6, STX6) (Figure 4C).

We used the anti-HA antibody to immunoisolate SLC6A17-HA protein from Slc6a17$^{\text{-HA-2A-iCre}}$ mice (Slc6a17$^{\text{HA/+}}$), with WT (SLC6A17$^{+/+}$) mice as a control (Figure 4D and S5A). After examining markers of subcellular organelles, the fraction containing SLC6A17-HA was found to be associated with SV markers such as Syp, Syt1, Syb2, VATPase and vesicular neurotransmitter transporters including VGluT1, VGluT2, and VGAT (Figures 4D and S5A), but not with markers of axons or dendrites (GLUT4 or transferrin receptor), ER (ERp72), the lysosome (LAMP2, LC3b or cathepsin D), the cytoplasmic membrane (SNAP23, PSD95 and GluN1), the Golgi apparatus (GM130 and Goglin-97), mitochondria (VADC), the active zone (ERC1b/2), the endosome (EEA1) and the proteosome (PSMC6) (Figures 4D and S5A). In addition, immunostaining of HA-tagged SLC6A17 in Slc6a17$^{\text{HA/+}}$ mouse showed puncta co-localized with the anti-Syp positive immunoreactivity (Figures S5B and S5C).

We also used genetically assisted EM to confirm SLC6A17 localization. APEX2 is derived from ascorbate peroxidase (APEX) and can be used to genetically label

proteins for EM inspection (Lam et al., 2015; Martell et al., 2017). We fused APEX2 in-frame to the C terminus of SLC6A17 (Figure 4E). SLC6A17-APEX2 fusion protein was expressed in the adult mouse brain by adeno-associated virus (AAV)-PHP.eb mediated transduction (Chan et al., 2017; Deverman et al., 2016). SLC6A17-APEX2 was indeed localized on the SVs (Figures 4F and S6A, S6C, S6E, S6G). Quantitative analysis showed distinct populations of SVs distinguished by electron density (Figures 4G and S6B, S6D, S6F, S6H).

Our results from biochemistry and genetically assisted EM analysis support the SV localization of SLC6A17.

Functional Significance of the SV Localization of SLC6A17

We used a monoclonal anti-Syp antibody to immunoisolate SVs from brains of Slc6a17$^{+/+}$ or Slc6a17$^{-/-}$ mice (Boyken et al., 2013; Gronborg et al., 2010; Jahn *et al.*, 1985). Quantitative proteomic analysis showed that only SLC6A17 was dramatically reduced in SVs immunopurified from Slc6a17-KO mice, whereas other transporters such as VGluT1, VGluT2, VGluT3, VGAT, VMAT2, SV2A, SV2B, SV2C, ZnT3 and VAT-1 were not different between Slc6a17$^{-/-}$ and Slc6a17$^{+/+}$ (Figure S7). Thus, Slc6a17 gene knockout indeed specifically decreased SLC6A17 protein, but none of the other transporters.

To investigate the functional significance of the subcellular localization of SLC6A17, we examined the subcellular localization of SLC6A17^{P633R} protein in the mouse brain. SLC6A17^{P633R} mutation was found in human ID patients (Iqbal et al.,

2015) and transfection of a cDNA encoding this mutant into cultured primary neurons from mice found that its distribution was different from that of the WT SLC6A17 (Iqbal et al., 2015), but the disturbance on SV localization for the transfected mutant protein was not unambiguous and the effect of the mutation on the endogenous protein was unknown.

With the endogenous SLC6A17^{P633R} protein tagged by the HA and FLAG epitopes (Figures 3A and 3B), we found that SLC6A17^{P633R}-HA signal was primarily localized to the soma with no overlap detected with the anti-Syp antibody staining (Figure 3B). We also performed differential centrifugation and sucrose gradient purification. HA and FLAG signals were presented in LP2 but did not co-purified with SVs in sucrose gradients, indicated that the SLC6A17^{P633R} protein was not present in SVs (Figure S8A).

Presence of Gln in Immunoisolated SVs

Neurotransmitters such as glutamate (Glu) and gamma-aminobutyric acid (GABA) have been reliably found in SVs immunoisolated from the mammalian brain (Bradberry et al., 2022; Burger et al., 1991; Burger et al., 1989; Chantranupong et al., 2020; Martineau et al., 2013). Previous experiments suggest that SLC6A17 could transport Ala, Gly, Leu and Pro into PC12 and CHO cells (Parra et al, 2008), or Ala, Gly, Leu, Pro, Cys, Gln, Gly, His and Ser into HEK cells (Zaia and Reimer, 2009). However, none of the reported substrates of SLC6A17 has been reliably detected in SVs.

To analyze the contents of SVs, we used the anti-Syp antibody to immunoisolate SVs from the mouse brain (Burger *et al.*, 1991; Burger *et al.*, 1989; Martineau *et al.*, 2013), before their contents were subjected to chemical analysis by liquid chromatography coupled to high resolution mass spectrometry (LC-MS) (Figures 5A and 5B, and Figures S9B to S9G). IgG was used as a control for the anti-Syp antibody in SV immunoisolation. The specificity of the anti-Syp antibody mediated SVs immunoisolation was confirmed (Figure S9A, with a total of 16 markers for SVs, lysosomes, endosomes, the Golgi apparatus, mitochodria, the ER and pre- or post-synaptic membrane).

The ratio of a molecule immunoisolated with the anti-Syp antibody to that with IgG was calculated and analyzed (Figures 5C and 5D). Glu, GABA and ACh were reliably detected from SVs immunoisolated with the anti-Syp antibody (Figures 5C to 5G, and S9B, S9D and S9F). Monoamine neurotransmitters such as 5-hydroxytryptamine (5-HT) (Figure S9C), dopamine (DA) (Figure S9G) and histamine (Figure S9E) could also be detected but not further analyzed for this paper. Hereafter, we focused our analysis on Glu, GABA and ACh as the positive controls and the nine previously reported AAs as candidate substrates of SLC6A17 *in vivo*.

Surprisingly, of the 9 AAs reported previously using cultured cells, 8 (Ala, Gly, Leu, Pro, Cys, Gly, His and Ser) were not found to be significantly enriched in SVs (Figures 5C and 5D). Only Gln was reproducibly found to be enriched in the anti-Syp antibody immunoisolated SVs (Figures 5C, 5D and 5H).

There could be several reasons why molecules transported into cultured cells

were not detected in SVs of the mouse brain, including, e.g., relative abundance in subsets of SVs specific for different transporters, or the redundancy of multiple transporters for a single molecule among the 9 candidates. However, the positive finding of Gln in the SVs is definitive.

Enrichment of Gln in SLC6A17 Containing SVs from the Mouse Brain

The above results with SVs purified by the anti-Syp antibody revealed Gln presence in SVs, but did not show association of Gln with specific transporter on SV, because Syp is a universal marker of SVs (Jahn, Schiebler, Quimet and Greengard, 1985; Wiedenmann and Franke, 1985; Leube et al., 1987; Südhof et al., 1987). To determine the potential relationship of SLC6A17 with Gln in the SVs, we used magnetic beads coated with the anti-HA antibody to immunoisolate SLC6A17-positive SVs from Slc6a17$^{-HA-2A-iCre}$ mice (Figures 4A and S1D).

The monoclonal anti-HA antibody could enrich SVs containing SLC6A17 from the brains of Slc6a17$^{-HA-2A-iCre}$ mice but not from wt mice, as confirmed by the analysis of 23 markers (Figures 4D and S5A). SVs thus isolated (Figure 6A) were subject to chemical analysis with LC-MS (Figure 6B). Glu, GABA and ACh were detected in SVs containing SLC6A17-HA (Figures 6C to 6G), consistent with the immunoblot results that these SVs contained VGluT1, VGluT2 and VGAT (Figure 4D). Significant enrichment of Gln was detected in SVs containing SLC6A17-HA (Figures 6C, 6D and 6H). By contrast, the other 8 AAs had not been found to be enriched in SVs containing SLC6A17-HA (Figures 6C, 6D and 6I).

Thus, only Gln has been reproducibly found to be enriched in SLC6A17 containing SVs *in vivo*.

Sufficiency of SLC6A17 for Gln Localization in SVs

To further investigate whether SLC6A17 could increase Gln in SVs, we overexpressed SLC6A17 in the mouse brain. AAV-PHP.eb was used to express hSLC6A17-HA in the mouse brain (OE-hSLC6A17-HA) (Chan *et al.*, 2017; Deverman *et al.*, 2016) (Figure 7A). The overexpressed hSLC6A17-HA protein was localized in SVs, as analyzed by immunoblot (Figure S9A).

We then analyzed the chemical contents of immunoisolated SVs by LC-MS. The ratio of a molecule purified from OE-hSLC6A17-HA mice by the anti-HA antibody vs that purified from WT mice by the same antibody was calculated and analyzed (Figure 7B). SVs from mice overexpressing hSLC6A17-HA contained significantly higher levels of Glu and GABA (Figures 7B to 7G). Gln was dramatically increased (Figures 7B and 7F), to the extent that Gln was higher than GABA in SVs overexpressing hSLC6A17-HA (Figures 7B, 7C, 7H and 7M). Quantification of the enriched molecules in SVs containing hSLC6A17-HA showed Gln to be approximately 2 times that of GABA (Figures 7H and 7M). In addition, His was moderately increased in SVs overexpressing hSLC6A17-HA (Figures 7B, S10C and S10D). These results from our virally introduced overexpression experiments support that SLC6A17 is sufficient for Gln transport into SVs *in vivo*.

Functional Significance of SLC6A17 for Gln Presence in the SVs

SLC6A17^{G162R} is another pathogenic mutation of human ID (Iqbal *et al.*, 2015), with a point mutation in the 3rd transmembrane domain of SLC6A17. To investigate whether SLC6A17^{G162R} affected Gln transportation, we used AAV-PHP.eb to overexpress either hSLC6A17^{G162R}-HA or Syp-HA in mouse brains. Overexpressed hSLC6A17^{G162R}-HA was still localized on SVs (Figures S8B and S10A). Overexpressed Syp-HA was previously reported to be localized on SVs (Chantranupong *et al.*, 2020), which we confirmed (Figure S10A).

LC-MS analysis of contents of SVs immunoisolated by the anti-HA antibody from OE-Syp-HA mice, when compared with immunoprecipitates by the anti-HA antibody from the brains of WT mice, would indicate SV enriched molecules. As expected, Glu, GABA and ACh were all found to be enriched in these SVs (Figures 7J, 7K, and 7L), whereas Ser was not enriched (Figure 7N).

The contents of SVs immunoisolated from OE-hSLC6A17^{G162R}-HA mice, when compared with that from OE-Syp-HA and WT mice, would indicate molecules enriched in SVs containing hSLC6A17^{G162R} mutation, above the general contents of all SVs. Levels of Glu and GABA in SVs containing hSLC6A17^{G162R}-HA were higher than those in WT mice (Figure S10B), but not significantly different with those from OE-Syp-HA mice (Figures 7J and 7K). These results indicate that the subset of SVs containing hSLC6A17^{G162R}-HA did not enrich Glu or GABA beyond the levels of Glu and GABA in the general population of SVs. The level of Gln in SVs containing hSLC6A17^{G162R}-HA were much lower than that in from OE-hSLC6A17^{G162R}-HA

mice (Figure 7M), indicating that hSLC6A17^{G162R} was defective in transporting Gln into SVs *in vivo*. The level of Gln in SVs containing hSLC6A17^{G162R}-HA was higher than that in WT mice, but not different from that in OE-Syp-HA mice (Figure 7M). These results indicate that Gln was present in SVs, but not enriched in SVs containing SLC6A17^{G162R}-HA over the general populations of SVs expressing Syp-HA. The levels of Gln and the other 8 AAs in SVs containing hSLC6A17^{G162R}-HA were not significantly higher than those in WT mice (Figures 7N, S10B and S10D).

We compared the contents of SVs immunoisolated from OE-hSLC6A17-HA mice with those from either OE-Syp-HA mice or OE-SLC6A17^{G162R}-HA mice. The levels of GABA and ACh were not different among SVs from mice expressing SLC6A17-HA, Syp-HA, or SLC6A17^{G162R}-HA (Figures 7H, 7I, 7K and 7L), indicating that SVs containing SLC6A17-HA were similar to the general populations of SVs. A moderate enrichment of Glu in SVs overexpressing SLC6A17-HA, as compared to those overexpressing SLC6A17^{G162R}-HA, was observed (Figures 7H and 7J). Gln was increased most dramatically in SVs overexpressing SLC6A17-HA, as compared to those overexpressing Syp-HA (Figures 7I and 7M) or SLC6A17^{G162R}-HA (Figures 7H and 7M). The levels of Ser (Figures 7I and 7N), Pro, Gly, Met, Leu, Ala, Cys were not significantly increased in SVs overexpressing SLC6A17-HA, as compared to those overexpressing Syp-HA (Figure 7I) or SLC6A17^{G162R}-HA (Figure 7H).

Taken together, our data provide *in vivo* evidence that the pathogenic mutation SLC6A17^{G162R} could not transport Gln into SVs in the mouse brain, supporting the

functional significance of Gln in the SVs.

Requirement of SLC6A17 for Gln Transport into the SV in Slc6a17 Knockout Mice

We have generated SLC6A17-KO mice (Figure 2A) and found them to be defective in learning and memory (Figures 2B to 2H). We then investigated Gln level in these mice. The anti-Syp antibody was used to immunoisolate SVs from the brains of SLC6A17$^{+/+}$, SLC6A17$^{+/-}$ and SLC6A17$^{-/-}$ mice (Figures 8A-8L).

LC-MS analysis of SVs revealed that Gln was the only molecule found to be decreased in SVs from Slc6a17$^{-/-}$ mice, as compared with Slc6a17$^{+/+}$ or Slc6a17$^{+/-}$ mice (Figure 8H). Quantitative analysis showed that the levels of classic transmitters such as Glu (Figure 8A), GABA (Figure 8B) and ACh (Figure 8C) were not significantly different among the SVs immunoisolated from the Slc6a17$^{+/+}$, the Slc6a17$^{+/-}$ or the Slc6a17$^{-/-}$ mice. The levels of the other 8 AAs were also not significantly different among SVs immunoisolated from all 3 genotypes (Figures 8D, 8E, 8F, 8G, 8I, 8J, 8K and 8L). These results further support that Gln was transported into the SVs by SLC6A17 *in vivo*.

Physiological Requirement of SLC6A17 for Gln Presence in the SVs in Adult Mice

To investigate whether the endogenous SLC6A17 is required in adulthood for Gln presence in the SVs, we used a viral mediated CRISPR-based approach to remove the

Slc6a17 gene from adult mice.

Briefly, Slc6a17$^{-HA-2AiCre}$ mice generated earlier (Figure S1D) were crossed with LSL-Cas9 transgene-carrying mice (Platt et al., 2014) and injected with an AAV-PHP.eB virus to express Syp-HA and guide RNAs (gRNA) targeting Slc6a17 (AAV-PHP.eb-hSyn-DIO-Syp-HA-U6-sgRNAs) (Figures 9A and S11A). A tandemly arrayed tRNA-gRNA structure was used to maximize the cleavage possibility *in vivo* (Port and Bullock, 2016; Xie et al., 2015) (Figure S10A). This strategy caused the excision of the Slc6a17 gene and simultaneous expression of Syp-HA to tag SVs specifically in Slc6a17-expressing cells of adult mice (Figure S11A). Slc6a17-HA was significantly reduced both in the homogenates of total brains and in immunoisolated SVs from AAV-sgRNA/Slc6a17^{iCre}/Cas9^{+} mice (Figures 9B and S10B).

Importantly, when the SV contents from the brains of knockout (AAV-sgRNA/Slc6a17^{iCre}/Cas9^{+}) and control (AAV-sgRNA/Slc6a17^{iCre}/Cas9^{-}) mice were compared after LC-MS analysis, Gln was the only vesicular content significantly decreased in SVs from the knockout mice (Figures 9C, 9D and 9H).

To our surprise, GABA and Glu were moderately increased (Figures 9C, 9D, 9E and 9F). The level of ACh, and those of the other 8 AAs (Ala, Gly, Leu, Pro, Cys, Gly, His and Ser) were not significantly changed in the SVs from adult Slc6a17 knockout mice (Figures 9C, 9D, 9G and 9I). The increased levels of Glu and GABA in SVs of virally mediated knock-out mice were different from the absence of Glu and GABA increases in the straightforward Slc6a17 KO (Figures 8A, 8B), making the significance of the observed increases of Glu and GABA in the virally mediated

Slc6a17 knockout unclear.

These results indicate that SLC6A17 is physiologically necessary for Gln in SVs, but not for the other 8 AAs.

Functional Significance of Gln in SVs Supported by Analysis of Mice Carrying a Mutation Mimicking a Human ID Mutation

To further address the question whether Gln transport into the SVs was functionally important, we examined the SVs from Slc6a17^{P633R} mutant mice (Figures 10A to 10L). This mutation mimicked one of the human ID mutations (Iqbal et al., 2015), and its behavioral phenotypes have been found by us (Figure 3). We have shown that the SLC6A17^{P633R} protein was not localized in the SVs (Figures 3B and S8A).

LC-MS analysis of contents from SVs showed a significant decrease of Gln level in Slc6a17$^{P633R/P633R}$, as compared to Slc6a17$^{+/+}$ and Slc6a17$^{P633R/+}$ mice (Figure 10H). The difference in Gln between Slc6a17$^{+/+}$ and Slc6a17$^{P633R/+}$ was not statistically significant (Figure 10H). The levels of Glu (Figure 10A), GABA (Figure 10B) and ACh (Figure 10C) in SVs were not significantly different among Slc6a17$^{+/+}$, Slc6a17$^{P633R/+}$ and Slc6a17$^{P633R/P633R}$ mice. The levels of the other 8 AAs in the SVs were also not significantly different among Slc6a17$^{+/+}$, Slc6a17$^{P633R/+}$ and Slc6a17$^{P633R/P633R}$ mice (Figures 10D, 10E, 10F, 10G, 10I, 10J, 10K and 10L).

Thus, mislocalization of SLC6A17^{P633R} outside the SVs correlates with the inability of Gln enrichment in SVs (Figure 10), as well as behavioral impairment (Figures 3 D, F, H and I), establishing the significance of SLC6A17 localization on

the SVs.

DISCUSSION

We have taken multiple approaches to analyze 7 kinds of genetically modified mice (4 modified in germlines and 3 in adult brains) and our results indicating that SLC6A17 is necessary and sufficient for Gln presence in SVs in vivo.

We have discovered the presence of Gln in the SVs and shown that it is functionally important because two mutations pathogenic for human ID patients both resulted in reduction of Gln in the SVs. We discuss these findings in basic neurobiology with implications in human ID pathology.

The Discovery of Gln in SVs and Its Functional Significance

With behavioral studies in two types of Slc6a17 mutant mice, we have shown that one ID pathogenic Slc6a17 mutation (Slc6a17^{P633R}) causes defective learning and memory (Figure 3), similar to the phenotype of Slc6a17 knockout mice (Figure 2). With biochemical purifications and genetically assisted EM, we have confirmed SLC6A17 localization on SVs (Figure 4).

We demonstrate the functional significance of the SV localization of SLC6A17 by showing that the human ID mutation Slc6a17^{P633R} causes mislocalization of SLC6A17 outside the SVs (Figures 3B and S6A). Gln transport into the SVs was decreased in Slc6a17^{P633R} mutant mice (Figure 10I).

With multiple rounds of biochemical purification of SVs and chemical analysis of

SV contents of genetically modified mice, we have found that Gln is present in SVs containing SLC6A17 and that SLC6A17 is sufficient for Gln presence in SVs (Figures 5, 6 and 7). With two lines of germline mutant mice and virally mediated gene edited mice, we demonstrate that Gln is the only molecule reliably and reproducibly reduced in SVs when Slc6a17 gene is deleted (Figures 8 and 9).

We have also characterized mice carrying a human ID mutation $SLC6A17^{G162R}$ and found it could not transport Gln into SVs (Figure 7), further supporting the importance of Gln in SVs. Thus, two mutations pathogenic in human ID are both LOF mutations: $SLC6A17^{P633R}$ with mislocalization outside the SVs and the $SLC6A17^{G162R}$ with defective Gln transport into the SVs.

The fact that all four kinds of SLC6A17 LOF mutations (two KOs, two point mutations) have led to reduction of Gln in SVs strongly support the functional significance of Gln in SVs.

Functional Mechanisms of Gln in SVs

There are three possible functions for Gln in SVs: serving as a neurotransmitter, serving as a carbon source and serving in a function so far unknown.

The first possibility is most attractive. SLC6A17 is a member of the SLC6 family, which is also known as the NTT family because many members of this family are transporters for neurotransmitters (Blakely and Edwards, 2012; Broer, 2006; Kristensen et al., 2011; Rudnick et al., 2014): SLC6A1 for GABA (Guastella et al., 1992; Clark et al., 1992; Borden et al., 1992; Lopez-Corcuera et al. 1992), SLC6A2

for noradrenaline (Pachoczyk, Blakely and Amara, 1991), SLC6A3 for DA (Giros et al., 1991; Kilty et al., 1991; Shimada et al., 1991), SLC6A4 for 5-HT or serotonin (Blakely et al., 1991; Hoffman et al., 1991), SLC6A5 and SLC6A9 for Gly (Guastella et al., 1992; Smith et al. 1992; Liu et al., 1992). Most of these are cytoplasmic transporters for transporting neurotransmitters from the synaptic cleft into the presynaptic cytoplasm.

The presence of the SLC6A17 protein on the vesicular membrane further supports the possibility that it is a neurotransmitter transporter, because most of the vesicular transporters with known substrates transport neurotransmitters. If this is true for SLC6A17, then Gln would be a good candidate for a new neurotransmitter. However, this exciting possibility remains to be investigated. For Gln to be a transmitter, in addition to its presence in the SVs, more criteria should be satisfied: 1) it should be released from the nerve terminals upon electric stimulation, 2) it should be active on postsynaptic membrane, 3) it should be up-taken by a cytoplasmic transporter back into the presynaptic terminal or be removed otherwise (such as degraded enzymatically), and 4) a pharmacological blocker inhibiting its activity should interfere with chemical transmission in a physiologically significant context. Because Gln is abundant both intracellularly and extracellularly, we expect that demonstration of all the above would take some time, and likely by many laboratories.

There is a Glu/Gln cycle in which Glu released from neurons is taken up by glia and transformed into Gln, followed by Gln release from glia and taken up by neurons

and transformed into Glu (Bak et al., 2006; Benjamin and Quastel, 1972; Ottersen et al., 1992; van den Berg and Garfinkel, 1971). Gln uptake across the neuronal cytoplasmic membrane relies on system A transporters (Mackenzie et al., 2003; Reimer et al., 2000; Varoqui et al., 2000; Weiss et al., 2003), while Gln uptake across the glia cytoplasmic membrane uses system L (Deitmer et al., 2003; Kanai et al., 1998), system N transporters (Broer and Brookes, 2001; Chaudhry et al., 2002; Chaudhry et al., 1999) and system ASC (Broer et al., 2000). It is unlikely that SLC6A17 and the Gln it transports could be part of the Glu/Gln cycle, because: 1) the Glu/Gln cycle uses cytoplasmic transporters for Gln, not vesicular transporters for Gln; 2) Gln is transformed into Glu in the cytoplasm of neurons before being transported in the SVs as Glu; 3) in all of our experiments presented here, when SLC6A17 was genetically disrupted, Gln was consistently decreased, but Glu was either unchanged but not concomitantly increased, which was against the expectation of the Glu/Gln cycle. The only result in which Glu was increased when SLC6A17 was decreased came from an experiment with virally mediated genetic knockdown of SLC6A17 (Figure 9E), but GABA was also increased in that experiment (Figure 9F). When the same experiment was carried with a germline genetic knockout of SLC6A17, Glu was not changed when SLC6A17 was decreased (Figure 9A).

The possibility of Gln as a carbon source is based on the fact that it is one of the major carbon sources (Hensley et al., 2013; Reitzer et al., 1979). It would be very curious if a carbon source should be stored in the SVs. It would suggest an entirely new function for Gln in the nerve terminal which has never been suspected before and

may require considerable efforts before one can fully understand.

Multiple other functions for Gln have been proposed (i.e., Curi et al., 2005; Wischmeyer, 2006).

Other Substrates for SLC6A17

Of the nine AAs (Ala, Gly, Leu, Pro, Cys, Gln, Gly, His and Ser) previously reported to be in vitro substrates for SLC6A17 transport in cell lines (Parra *et al.*, 2008; Zaia and Reimer, 2009), we have reliably and reproducibly found evidence supporting only one (Gln) as present *in vivo* in all 5 types of experiments conducted with SV purifications: SVs immunoisolated from the WT brains by anti-Syp (Figure 5), SVs immunoisolated from the brains of mice with the HA tag fused in frame to the endogenous SLC6A17 protein (Figure 6), SVs immunoisolated from mice with virally introduced overexpression of Syp-HA, SVs immunoisolated from mice with virally introduced overexpression of SLC6A17^{G162R}-HA, and SVs immunoisolated from mice with virally introduced overexpression of SLC6A17-HA (Figure 7). While we did observe a moderate increase of His in one set of experiments (OE SLC6A17-HA vs WT), we did not find evidence for its changes in other experiments.

Because multiple transporters can transport AAs, and the relative contribution of each transporter varies and the relative abundance of each small molecules and their detectability varies, we can not rule out the possibility that SLC6A17 can contribute to the transport of other AAs into the SVs. However, our results can establish that Gln is transported *in vivo*.

In summary, integration of genetics, biochemistry, behavioral analysis, mass spectrometry and electron microscopy has resulted in the discovery of Gln as a novel chemical in SVs, provided *in vivo* evidence that SLC6A17 is necessary and sufficient for Gln in SVs, generated animal models for, and suggested pathogenetic mechanism of, human ID. However, while we have answered the question how Slc6a17 mutations may cause ID in animals, our research has also raised more questions. This natural process of questions begetting answers begetting questions is intrinsic to scientific inquiries and is a source of endless fun in conducting research.

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SUPPLEMENTAL INFORMATION

Supplemental information includes MATERIALS AND METHODs and 11 figures.

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FIGURE LEGENDS

Figure 1. Slc6a17 Expression in the Mouse Brain. (A) A schematic diagram illustrating the strategy for the generation of Slc6a17$^{-2A\text{-}CreERT2}$ mice. Crossing of Slc6a17$^{-2A\text{-}CreERT2}$ mice with Ai14 (LSL-tdTomato) mice allowed specific labeling of Slc6a17-expressing neuron after tamoxifen injection. More details in Figure S1A. (B-I) Representative coronal sections of Slc6a17$^{-2A\text{-}CreERT2}$::Ai14 mice. Numbers above images indicate the anteroposterior position of the section from Bregma in millimeters (mm), based on(Paxinos and Franklin, 2019). Scale bars=1 mm. (J) Slc6a17-positive neurons in the basolateral amygdaloid nucleus (BLA) and the central amygdaloid nucleus (CeA). Scale bars=100 μm. (K) Hippocampal expression of Slc6a17. Slc6a17-positive neurons are densely distributed in the granule cell layer of the dentate gyrus (GrDG), and CA3, with little expression in the CA1 and the CA2. Scale bars=200 μm.

Abbreviations: 2Cb, lobule 2 of the cerebellar vermis; 3Cb, lobule 3 of the cerebellar vermis; 4/5Cb, lobule 4 and 5 of the cerebellar vermis; AON, accessory olfactory nucleus; AM, anteromedial thalamic nucleus; AV, anteroventral thalamic nucleus; BLA, basolateral amygdaloid nucleus; Ent, entorhinal cortex; CB, cerebellum; Cg1/2, cingulate ccortex; CIC, central nucleus of the inferior colliculus; Ctx, cortex; Hip, hippocampus; LO, lateral orbital cortex; LS, lateral septal nucleus; M1, primary motor cortex; M2, secondary motor cortex; OB, main olfactory bulb; Pir, piriform cortex; Pn, pontine nuclei; Rt, reticular thalamic nucleus; S1, primary somatosensory cortex; S2, secondary somatosensory cortex; SCN, suprachiasmatic nucleus; SFO, subfornical

organ; SuG, superficial gray layer of superior colliculus; Sim, simple lobule; Th, thalamus; V1, primary visual cortex; V2, secondary visual cortex; VPM, ventral posteromedial nucleus.

Figure 2. Slc6a17-KO Mice Exhibited Impaired Memory Formation. (A) A schematic diagram illustrating the knock-out strategy for generating Slc6a17-KO mice by CRISPR/Cas9. Exon 2 encoding the first 99 amino acids of Slc6a17 was deleted. More details in Figure S2A. (B) A diagram of experiments for contextual and cued fear conditioning (n=16, 23, 18 for Slc6a17$^{+/+}$, Slc6a17$^{+/-}$ and Slc6a17$^{-/-}$, respectively). Detailed description in Experimental Procedures. (C) Slc6a17$^{-/-}$ mice exhibited a significant decrease of freezing behavior in both cued and contextual fear conditioning (Day 2 context a, p=0.0062 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, p<0.0001 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$; Day 3 tone, p=0.0093 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, p=0.0012 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$). (D) A diagram of experiments for modified Morris water maze task (n=15, 18, 16 for Slc6a17$^{+/+}$, Slc6a17$^{+/-}$ and Slc6a17$^{-/-}$, respectively). Detailed description in Experimental Procedures. (E) Latency to find the hidden platform during the training session. Slc6a17$^{-/-}$ mice differred significantly from Slc6a17$^{+/+}$ and Slc6a17$^{+/-}$ mice (Two-way ANOVA; main effect of training day: F (4.681, 210.7) = 46.55, p<0.0001; main effect of genotype: F (2, 45) = 21.53, p<0.0001; main effect of training day x genotype: F (12, 270)=3.762, p< 0.0001; Tukey's multiple comparisons test: day 2, p=0.0004 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, p=0.0002 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$; day 5, p= 0.0002 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$,

p= 0.008 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$; day 6, p=0.0002 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, p=0.0027 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$; day 7, p<0.0001 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, p=0.0005 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$). (F) Representative swimming traces of each genotype. (G) Percentage of time spent in the target quadrant was significantly different (p=0.0004 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, p< 0.0001 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$). (H) Number of crossings through the platform area was significantly different (p=0.0194 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, p=0.0054 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$). Here and hereafter, data in the figures are presented as the mean ± SEM, with * indicating p< 0.05, ** indicating p< 0.01, *** indicating p< 0.001, and **** indicating p< 0.0001.

Figure 3. Deficient Memory in Slc6a17^{P633R} Mice. (A) A diagram illustrating the knock-in strategy for Slc6a17 with a pathogenic point mutation (Slc6a17^{P633R}). It was tagged with 3 repeats of the HA epitope and 3 repeats of the FLAG epitope. More details in Figure S2B. (B) Immunocytochemistry of Slc6a17^{P633R} mouse brains with the anti-HA antibody showed that the subcellular localization of SLC6A17^{P633R}-HA did not co-localized with synaptophysin. It was present in the cytoplasm. Representative view of CA3 was shown. Scale bar=50 μm. (C) Experiments for contextual and cued fear conditioning (n=17, 18, 14 for Slc6a17$^{+/+}$, Slc6a17$^{P633R/+}$, Slc6a17$^{P633R/P633R}$, respectively). Detailed description in Experimental Procedures. (D) Slc6a17^{P633R} homozygous mutants exhibited significant decrease of freezing behavior in both cued and contextual fear conditioning (Day 2 context A, p< 0.0001 for

Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p< 0.0001 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$; Day 3 tone, p= 0.0017 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p= 0.0009 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$). (E) Cartoon figure of experiments for Morris water maze task (n=12, 15, 9 for Slc6a17$^{+/+}$, Slc6a17$^{P633R/+}$ and Slc6a17$^{P633R/P633R}$, respectively). Detailed description in Experimental Procedures. (F) Latency to find the hidden platform during the training session. Slc6a17$^{P633R/P633R}$ mice showed significantly different learning curve from Slc6a17$^{+/+}$ and Slc6a17$^{P633R/+}$ mice (Two-way ANOVA; main effect of training day: F (4.381, 144.6) = 20.02, p<0.0001; main effect of genotype: F (2, 33) = 22.65, p<0.0001; main effect of training day x genotype: F (12, 198) = 2.389, p=0.0067; Tukey's multiple comparisons test: day 2, p= 0.0150 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p= 0.0078 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$; day 3, p= 0.0403 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p= 0.0388 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$; day 5, p= 0.0023 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p= 0.0243 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$; day 6, p= 0.0199 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/+}$,p= 0.0010 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p= 0.0077 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$; day 7, p= 0.0074 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p= 0.0129 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$). (G) Representative swimming traces of each genotype. (H) Percentage of time spent in the target quadrant was significantly different (p= 0.0031 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p= 0.0325 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$). (I) Number of crossings through the platform area was significantly different (p= 0.0003 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p= 0.0123 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$).

Data in the figures are presented as the mean ± SEM, with * indicating $p < 0.05$, ** indicating $p < 0.01$, *** indicating $p < 0.001$, and **** indicating $p < 0.0001$.

Figure 4. Biochemical and Genetically Assisted EM Validation of the Vesicular Localization of SLC6A17. (A) A schematic diagram illustrating the knock-in strategy for generating Slc6a17$^{-HA-2A-iCre}$ mice. More details in Figure S1D. (B) With fractions of whole brains by differential centrifugation, SLC6A17-HA was co-immunoisolated with synaptic markers including Syp, Syt1, Syb2 and V-ATPase, but not with PSD95. (C) Further purification of the LP2 fraction by sucrose gradient showed that SLC6A17-HA was co-immunoisolated with Syp, Syt1 and vGluT1, but not PSD95, ERp72, EEA1, SNAP23, PSMC6 or STX6. (D) Immunoisolation of SLC6A17-HA containing vesicles by the anti-HA antibody coated on magnetic beads. SLC6A17-HA fraction was positive for Syp, Syt1, Syb2, v-ATPase, vGluT1, vGluT2, and vGAT, but negative for GluT4 and transferin receptor. (E) A schematic diagram illustrating the APEX2-based labeling strategy with AAV-PHP.eb virus mediated hSLC6A17-APEX2 overexpression *in vivo*. SLC6A17 was fused in-frame to 3 repeats of the HA tag, a V5 tag, and APEX2. (F) Representative EM image of SVs labeled by hSLC6A17-APEX2. Red arrow pointing to APEX2 labeled SVs. White arrow pointing to unlabeled SVs. (G) Darkness distribution of DAB positive and DAB negative SVs in sections of Slc6a17-APEX2 mouse brains.

Figure 5. Presence of Glutamine in SVs from the Mouse Brain. (A) A schematic

diagram illustrating the procedure to immunoisolate SVs with the anti-Syp antibody for LC-MS analysis of the contents in the SVs. (B) Representative MS results showing Gln signals from SVs immunoisolated by the anti-Syp antibody (anti-Syp) vs the control sample immunoisolated with IgG. (C) Volcano plot of chemical contents in the SVs isolated by anti-Syp vs IgG. The y axis shows *p* values in $\log_{10}$ and the x axis shows the $\log_2$ of the ratio of the level of a molecule immunoisolated by anti-Syp vs IgG. Classical neurotransmitters Glu, GABA and ACh, as well as previously reported substrates of SLC6A17 are listed. (D) Ratios of the level of a chemical immunoisolated by anti-Syp vs IgG (transformed into $\log_2$). (E-I) Chemicals were quantified to mole per μg antibody (n=12 for each group): Glu (E, $p<0.0001$ for anti-Syp vs. IgG); GABA (F, $p<0.0001$ for anti-Syp vs. IgG); ACh (G, $p<0.0001$ for anti-Syp vs. IgG); Gln (H, $p<0.0001$ for anti-Syp vs. IgG); Ser (I, $p=0.7553$ for anti-Syp vs. IgG).

Figure 6. Gln Enrichment in SVs Containing SLC6A17. (A) A schematic diagram illustrating the procedure to isolate SLC6A17-containing SVs for chemical analysis of SV contents. (B) Representative result showing MS Gln signals in SVs immunoisolated by the anti-HA beads in SLC6A17$^{HA/+}$ mice vs those from Slc6a17$^{+/+}$ mice. (C) Volcano plot of chemical contents in the SVs immunoisolated by anti-HA from Slc6a17$^{HA/+}$ mice vs Slc6a17$^{+/+}$ mice. The y axis shows *p* values in $\log_{10}$ and the x axis shows the $\log_2$ of the ratio of the level of a molecule immunoisolated by anti-HA from Slc6a17$^{HA/+}$ mice vs that from Slc6a17$^{+/+}$ mice. Classical

neurotransmitters such as Glu, GABA and ACh as well as the previously reported substrates of SLC6A17 are listed. (D) Ratios of the level of a chemical immunoisolated by anti-HA from Slc6a17$^{HA/+}$ mice vs that from Slc6a17$^{+/+}$ mice (transformed into $\log_2$). (E-I) Contents of SLC6A17-containing SVs were quantified to mole per 10 μl anti-HA beads (n=12, for each group): Glu (E, $p<0.0001$ for Slc6a17$^{HA/+}$ vs. Slc6a17$^{+/+}$); GABA (F, $p<0.0001$ for Slc6a17$^{HA/+}$ vs. Slc6a17$^{+/+}$); ACh (G, $p<0.0001$ for Slc6a17$^{HA/+}$ vs. Slc6a17$^{+/+}$); Gln (H, $p< 0.0001$ for Slc6a17$^{HA/+}$ vs. Slc6a17$^{+/+}$); Ser (I, $p= 0.7553$ for Slc6a17$^{HA/+}$ vs. Slc6a17$^{+/+}$).

Figure 7. Increased Gln Transport into SVs Containing SLC6A17 but not into SVs Containing SLC6A17^{G162R}. (A) A schematic diagram illustrating the strategy for the AAV-PHP.eb virus mediated *in vivo* overexpression of hSLC6A17-HA and hSLC6A17^{G162R}-HA. (B) Volcano plot of chemical contents in the SVs isolated by anti-HA from the OE-hSLC6A17-HA mice vs WT mice. The y axis shows *p* values in $\log_{10}$ and the x axis shows the $\log_2$ of the ratio of the level of a molecule immunoisolated by anti-HA beads from mice overexpressing hSLC6A17-HA vs that from WT mice. Classical neurotransmitters and previously reported substrates of SLC6A17 are listed.

(C-G) Contents of OE-hSLC6A17-containing SV are quantified to mole per 10 μl HA beads (n=10, for each group): Glu (C, $p<0.0001$ for OE-hSLC6A17-HA vs. WT); GABA (D, $p<0.0001$ for OE-hSLC6A17-HA vs. WT); ACh (E, $p<0.0001$ for OE-hSLC6A17-HA vs. WT); Gln (F, $p<0.0001$ for OE-hSLC6A17-HA vs. WT); (G,

p=0.1655 for OE-hSLC6A17-HA vs. WT). (H) Volcano plot comparing the chemical contents of SVs containing OE-hSLC6A17-HA with those containing OE-hSLC6A17^{G162R}-HA. Classical neurotransmitters and 9 putative substrates of SLC6A17 are listed. Glu is the only classical transmitter significantly increased. Gln is the only substrates significantly increased in hSLC6A17-HA containing SVs vs hSLC6A17^{G162R}-HA containing SVs. (I) Volcano plot comparing the chemical contents of SVs containing OE-hSLC6A17-HA with those containing OE-Syp-HA. Classical neurotransmitters and 9 putative substrates of SLC6A17 are listed. Gln is the only substrates significantly increased in SLC6A17 containing SVs vs Syp-HA containing SVs. (J-N) Contents of SVs containing SCL6A17-HA, SVs containing OE-SLC6A17^{G162R}-HA, SVs containing Syp-HA immunosilated by anti-HA immunoisolation with that from WT mouse brains were quantified to mole per 10 μl HA beads (n=9, 9, 6, 6, for OE-hSLC6A17-HA, OE-hSLC6A17^{G162R}-HA, OE-Syp-HA, and WT, respectively): Glu (J, *p*<0.0001 for OE-hSLC6A17-HA vs. OE-hSLC6A17^{G162R}-HA; *p*<0.0001 for OE-hSLC6A17-HA vs. WT; *p*<0.0001 for OE-hSLC6A17^{G162R}-HA vs. WT; *p*= 0.0009 for OE-Syp-HA vs. WT; *p*= 0.1551 for OE-hSLC6A17-HA vs. OE-Syp-HA; *p*= 0.0618 for OE-hSLC6A17^{G162R}-HA vs. OE-Syp-HA); GABA (K, *p*= 0.0002 for OE-hSLC6A17-HA vs. WT; *p*= 0.0002 for OE-hSLC6A17^{G162R}-HA vs. WT; *p*= 0.0039 for OE-Syp-HA vs. WT; *p*= 0.9885 for OE-hSLC6A17-HA vs. OE-hSLC6A17^{G162R}-HA; *p*= 0.9000 for OE-hSLC6A17-HA vs. OE-Syp-HA; *p*= 0.5840 for OE-hSLC6A17^{G162R}-HA vs. OE-Syp-HA); ACh (L *p*= 0.0019 for OE-hSLC6A17-HA vs. WT; *p*= 0.0006 for OE-hSLC6A17^{G162R}-HA vs.

WT; *p*= 0.0467 for OE-Syp-HA vs. WT; *p*= 0.1054 for OE-hSLC6A17-HA vs. OE-hSLC6A17^{G162R}-HA; *p*= 0.7563 for OE-hSLC6A17-HA vs. OE-Syp-HA; *p*= 0.8702 for OE-hSLC6A17^{G162R}-HA vs. OE-Syp-HA); Gln (M, *p*= 0.001 for OE-hSLC6A17-HA vs. OE-hSLC6A17^{G162R}-HA; *p*= 0.0018 for OE-hSLC6A17-HA vs. OE-Syp-HA; *p*= 0.0003 for OE-hSLC6A17-HA vs. WT; P<0.0001 for OE-hSLC6A17^{G162R}-HA vs. WT; *p*= 0.0189 for OE-Syp-HA vs. WT; *p*= 0.2749 for OE-hSLC6A17^{G162R}-HA vs. OE-Syp-HA); Ser (N, *p*= 0.626 for OE-hSLC6A17-HA vs. OE-hSLC6A17^{G162R}-HA; *p*= 0.8551 for OE-hSLC6A17-HA vs. OE-Syp-HA; *p*= 0.9874 for OE-hSLC6A17-HA vs. OE-Syp-HA; P<0.0865 for OE-hSLC6A17^{G162R}-HA vs. WT; *p*= 0.1017 for OE-Syp-HA vs. WT).

Figure 8. Reduced Levels of Gln in the SVs of Slc6a17 KO Mice. Contents of SVs purified by the anti-Syp antibody from Slc6a17-KO mice were quantified to mole per 10 μg antibody (n=12 for Slc6a17$^{+/+}$, Slc6a17$^{+/-}$, Slc6a17$^{-/-}$): Glu (A, *p*=0.2749 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, *p*=0.2466 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$); GABA (B, *p*=0.665 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, *p*=0.2503 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$); ACh (C, *p*=0.9993 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, *p*=0.7186 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$); Pro (D, *p*=0.9986 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, *p*=0.8035 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$); Leu (E, *p*=0.826 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, *p*= 0.61 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$); Gly (F, *p*= 0.3211 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, *p*=0.442 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$); Ala (G, *p*=0.9092 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, *p*=0.1321 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$); Gln (H, *p*=0.002 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, *p*=0.0489 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$); Cys (I,

p=0.3753 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, p=0.5718 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$); Met (J, p=0.9735 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, p= 0.13 for +/- vs. Slc6a17$^{-/-}$); Ser (K, p=0.8678 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, p=0.7834 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$); His (L, p>0.9999 for Slc6a17$^{+/+}$ vs. Slc6a17$^{-/-}$, p=0.9922 for Slc6a17$^{+/-}$ vs. Slc6a17$^{-/-}$).

Figure 9. Physiological Requirement of SLC6A17 for Gln Transport into SVs in vivo. (A) A schematic diagram illustrating the strategy of Cas9-mediated cleavage of Slc6a17 specifically in Slc6a17 positive neurons, and simultaneous labeling of all SVs in these neurons by Syp-HA. (B) Immunoblot results showing SLC6A17 protein was significantly reduced in targeted neurons, while Syp-HA was efficiently tagged onto the SVs of these neurons. (C) Volcano plot of contents of SVs from AAV-sgRNA /Slc6a17^{iCre}/Cas9^{+} targeted neurons compared to contents of SVs from control (AAV-sgRNA /Slc6a17^{iCre}/Cas9^{-}) neurons. Glu, GABA, ACh and the 9 previously reported substrates of SLC6A17 are listed. (D) Ratios of the level of a molecule from the SVs of AAV-sgRNA /Slc6a17^{iCre}/Cas9^{+} neurons vs the level of the same molecule from SVs of AAV-sgRNA /Slc6a17^{iCre}/Cas9^{-} neurons shown as fold change (log2 transformed). GABA level was significantly increased (p=0.0034 for AAV-sgRNA /Slc6a17^{iCre}/Cas9^{+} vs. AAV-sgRNA /Slc6a17^{iCre}/Cas9^{-}). Gln level was significantly decreased (p< 0.0001 for AAV-sgRNA /Slc6a17^{iCre}/Cas9^{+} vs. AAV-sgRNA /Slc6a17^{iCre}/Cas9^{-}). (E-H) Contents of SVs from Slc6a17 containing neurons were quantified to mole per 10 μl HA beads (n=18, 27 for Slc6a17^{iCre}/Cas9^{-} and Slc6a17^{iCre}/Cas9^{+}, respectively): Glu (E, p= 0.0005 for AAV-sgRNA

/Slc6a17^{iCre}/Cas9^{+} vs. AAV-sgRNA /Slc6a17^{iCre}/Cas9^{-}); GABA (F, p=0.0032 for AAV-sgRNA /Slc6a17^{iCre}/Cas9^{+} vs. AAV-sgRNA /Slc6a17^{iCre}/Cas9^{-}); Gln (G, p<0.0001 for AAV-sgRNA /Slc6a17^{iCre}/Cas9^{+} vs. AAV-sgRNA /Slc6a17^{iCre}/Cas9^{-}); Ser (H, p=0.0979 for AAV-sgRNA /Slc6a17^{iCre}/Cas9^{+} vs. AAV-sgRNA /Slc6a17^{iCre}/Cas9^{-}).

Figure 10. Reduced Levels of Gln in the SVs of Slc6a17^{P633R} Mice. Contents of SVs purified by the anti-Syp antibody from Slc6a17^{P633R} mice were quantified to mole per 10 μg antibody (n=12 for all genotype): Glu (A, p=0.9804 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p=0.9982 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$); GABA (B, p=0.4432 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$ p=0.3898 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$); ACh (C, p=0.8175 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p=0.9804 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$); Pro (D, p=0.9674 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p=0.9989 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$); Leu (E, p=0.7116 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p=0.6696 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$); Gly (F, p=0.7376 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p=0.9971 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$); Ala (G, p=0.9871 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p=0.986 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$); Gln (H, p=0.0029 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p=0.0117 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$); Cys (I, p=0.8143 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p=0.5475 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$); Met (J, p=0.9942 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p=0.995 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$); Ser (K, p=

0.9955 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p=0.8355 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$); His (L, p=0.6177 for Slc6a17$^{+/+}$ vs. Slc6a17$^{P633R/P633R}$, p=0.4316 for Slc6a17$^{P633R/+}$ vs. Slc6a17$^{P633R/P633R}$).

Figure 1

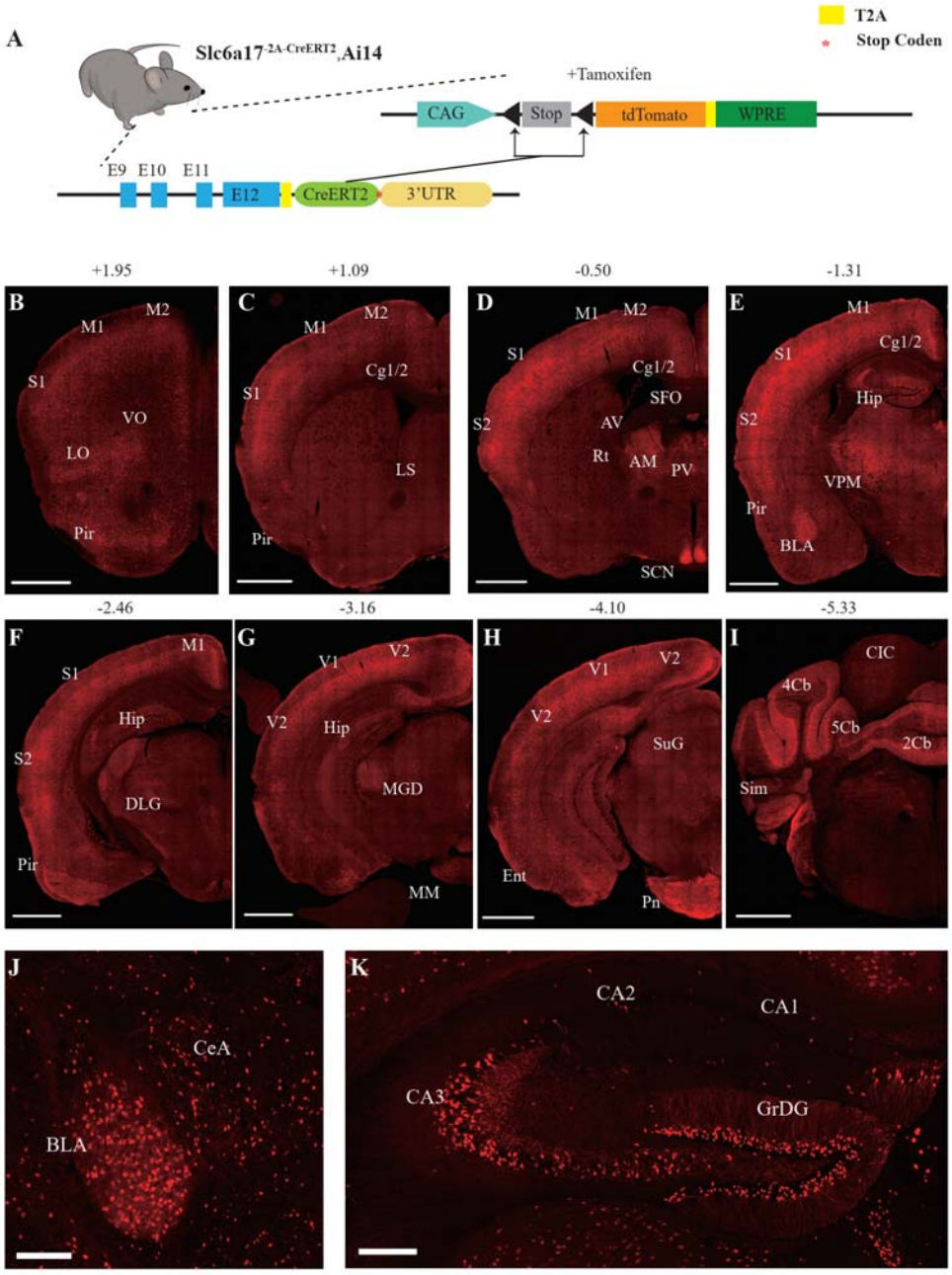

A
Slc6a17-2A-CreERT2,Ai14
T2A
Stop Coden
+Tamoxifen
CAG
Stop
tdTomato
WPRE
E9 E10 E11
E12
CreERT2
3'UTR
+1.95
+1.09
-0.50
-1.31
B
M1
M2
S1
VO
LO
Pir
C
M1
M2
Cg1/2
S1
LS
Pir
D
M1
M2
S1
Cg1/2
SFO
S2
AV
Rt
AM
PV
SCN
E
M1
Cg1/2
S1
Hip
S2
VPM
Pir
BLA
-2.46
-3.16
-4.10
-5.33
F
M1
S1
Hip
S2
DLG
Pir
G
V1
V2
V2
Hip
MGD
MM
H
V1
V2
V2
SuG
Ent
Pn
I
CIC
4Cb
5Cb
2Cb
Sim
J
CeA
BLA
K
CA2
CA1
CA3
GrDG

Figure 2

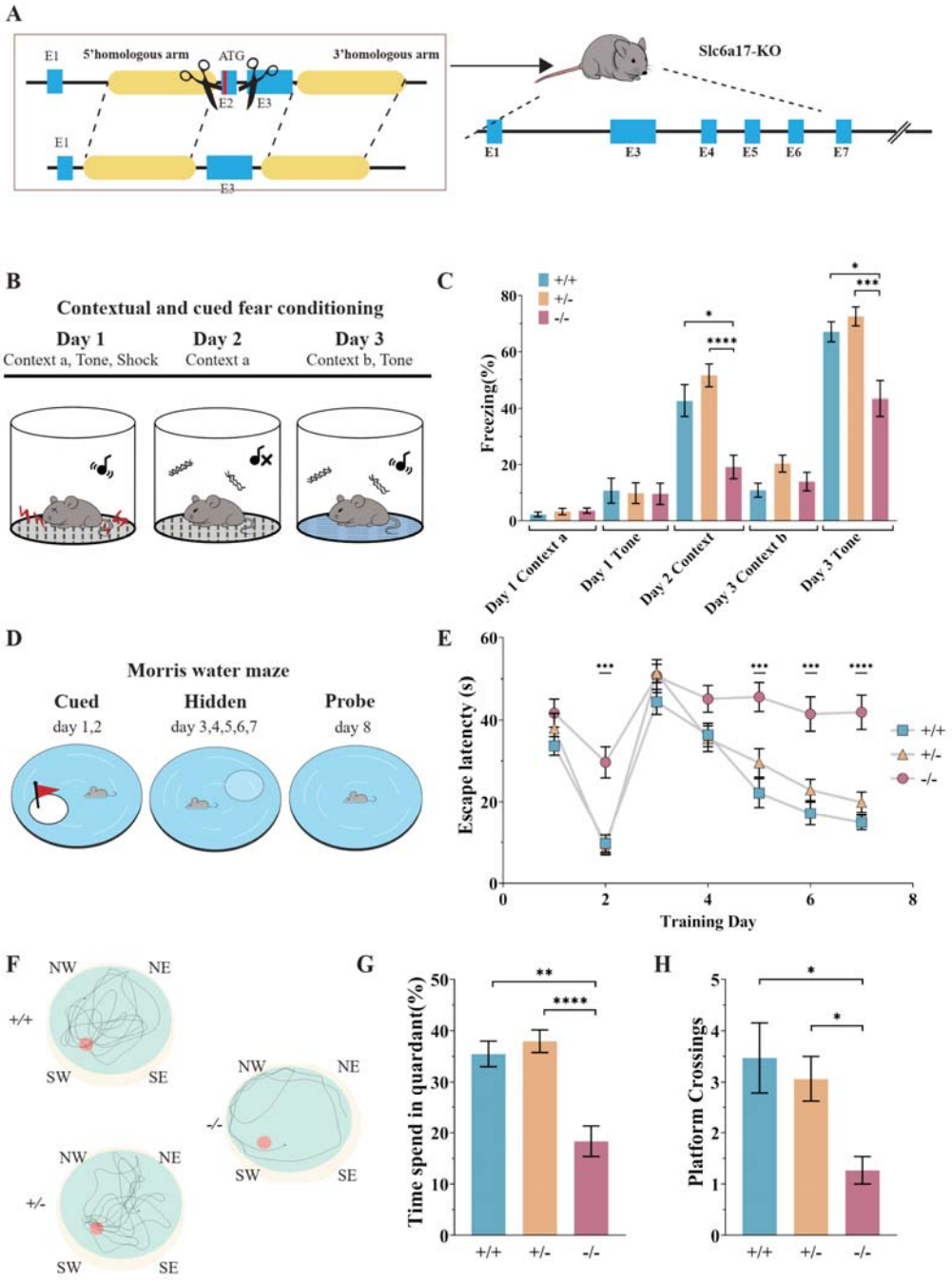

A
E1
5'homologous arm
ATG
3'homologous arm
E2
E3
Slc6a17-KO
E1
E3
E4
E5
E6
E7
B
Contextual and cued fear conditioning
Day 1
Context a, Tone, Shock
Day 2
Context a
Day 3
Context b, Tone
C
+/+
+/-
-/-
Freezing(%)
Day 1 Context a
Day 1 Tone
Day 2 Context
Day 3 Context b
Day 3 Tone
D
Morris water maze
Cued
day 1,2
Hidden
day 3,4,5,6,7
Probe
day 8
E
Escape latency (s)
Training Day
+/+
+/-
-/-
F
NW
NE
SW
SE
+/+
+/-
-/-
G
Time spend in quardant(%)
+/+
+/-
-/-
H
Platform Crossings
+/+
+/-
-/-

Figure 3

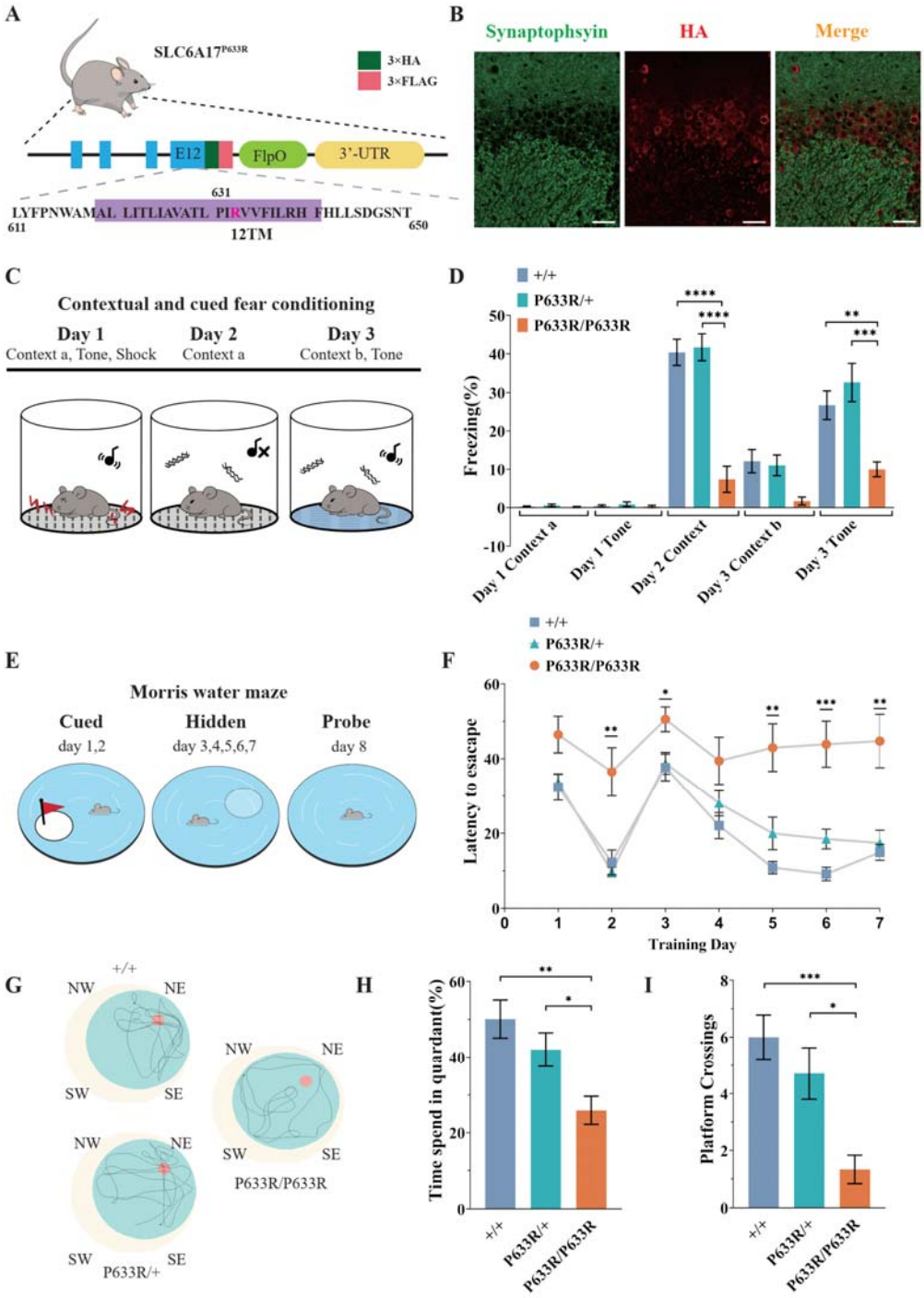

A
SLC6A17P633R
3×HA
3×FLAG
E12
FlpO
3'-UTR
631
LYFPNWAMAL LITLIAVATL PIRVVFILRH FHLLSDGSNT
611
650
12TM
B
Synaptophsyin
HA
Merge
C
Contextual and cued fear conditioning
Day 1
Context a, Tone, Shock
Day 2
Context a
Day 3
Context b, Tone
D
+/+
P633R/+
P633R/P633R
Freezing(%)
Day 1 Context a
Day 1 Tone
Day 2 Context
Day 3 Context b
Day 3 Tone
E
Morris water maze
Cued
day 1,2
Hidden
day 3,4,5,6,7
Probe
day 8
F
Latency to esacape
Training Day
G
NW
NE
SW
SE
P633R/+
P633R/P633R
H
Time spend in quardant(%)
I
Platform Crossings

Figure 4

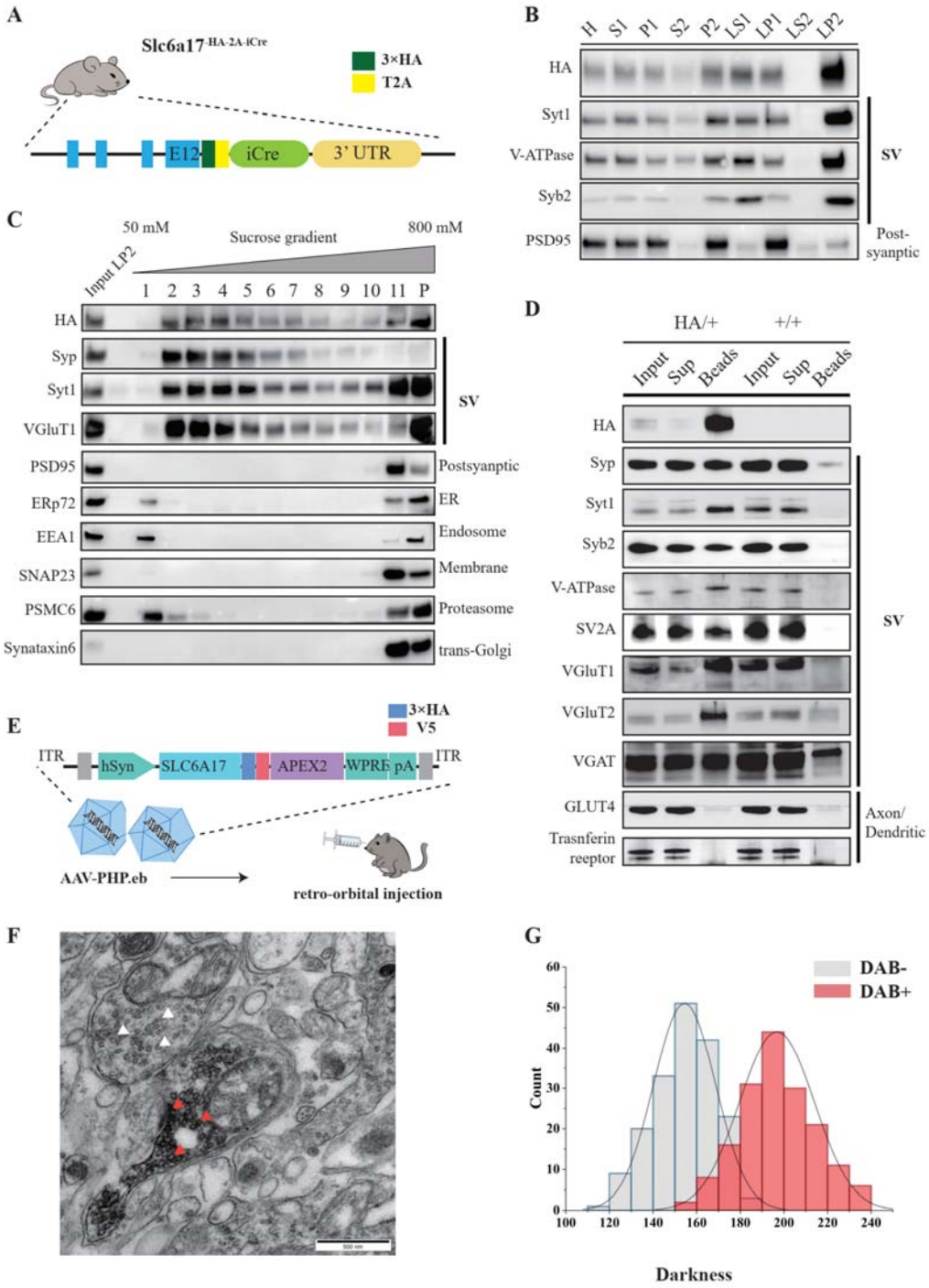

A
Slc6a17-HA-2A-iCre
3×HA
T2A
E12
iCre
3' UTR
B
H1 S1 P1 S2 P2 LS1 LP1 LS2 LP2
HA
Syt1
V-ATPase
Syb2
PSD95
SV
Post-syanptic
C
50 mM
Sucrose gradient
800 mM
Input LP2
1 2 3 4 5 6 7 8 9 10 11 P
HA
Syp
Syt1
VGluT1
SV
PSD95
Postsyanptic
ERp72
ER
EEA1
Endosome
SNAP23
Membrane
PSMC6
Proteasome
Synataxin6
trans-Golgi
D
HA/+
+/+
Input Sup Beads Input Sup Beads
HA
Syp
Syt1
Syb2
V-ATPase
SV2A
VGluT1
VGluT2
VGAT
SV
GLUT4
Trasnferin reeptor
Axon/ Dendritic
E
3×HA
V5
ITR
hSyn
SLC6A17
APEX2
WPRE
pA
ITR
AAV-PHP.eb
retro-orbital injection
F
G
DAB-
DAB+
Count
Darkness

Figure 5

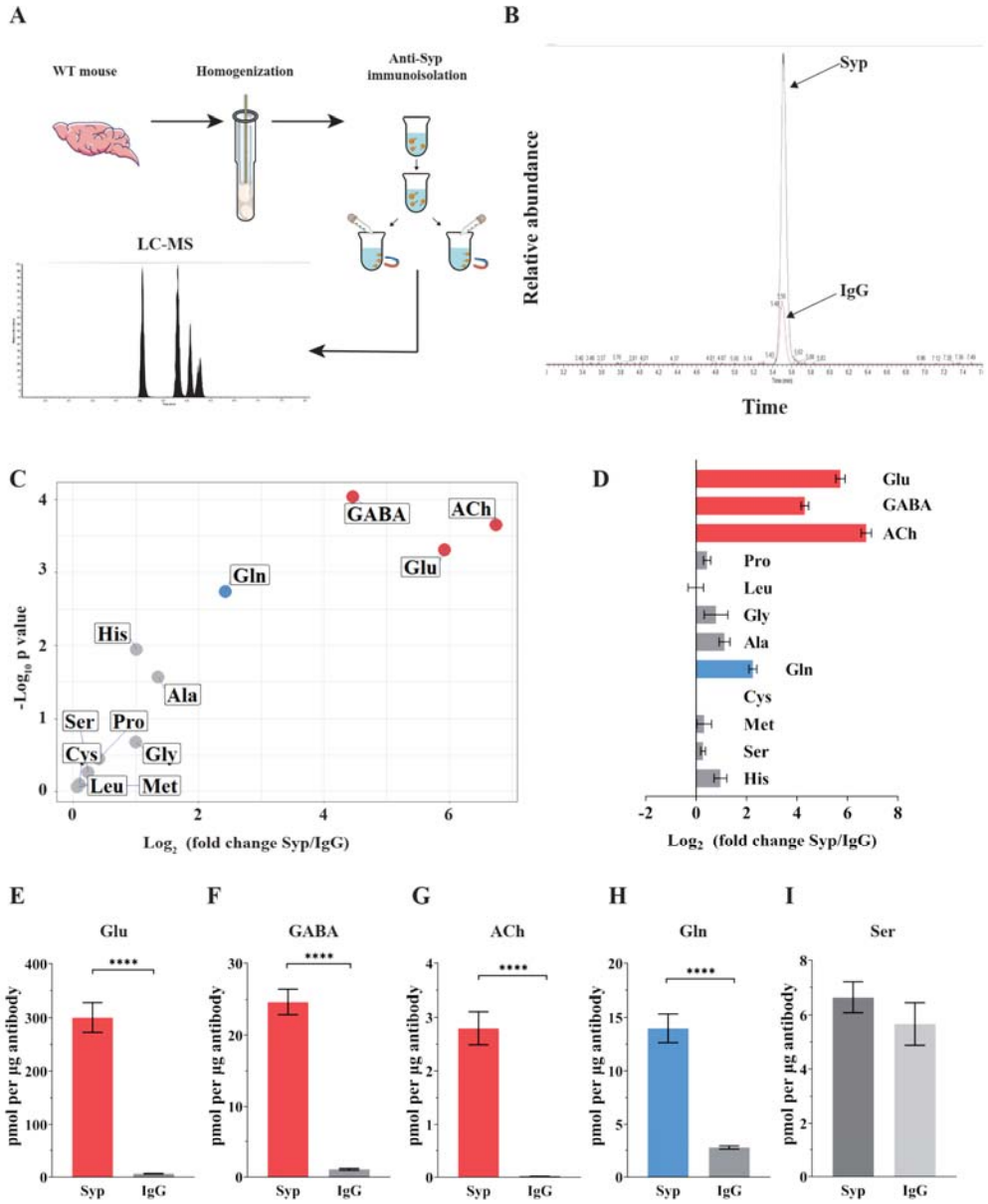

A
WT mouse
Homogenization
Anti-Syp immunoisolation
LC-MS
B
Syp
IgG
Relative abundance
Time
C
GABA
ACh
Glu
Gln
His
Ala
Ser
Pro
Cys
Gly
Leu
Met
-Log10 p value
Log2 (fold change Syp/IgG)
D
Glu
GABA
ACh
Pro
Leu
Gly
Ala
Gln
Cys
Met
Ser
His
Log2 (fold change Syp/IgG)
E
Glu
F
GABA
G
ACh
H
Gln
I
Ser
pmol per µg antibody
****
Syp
IgG

Figure 6

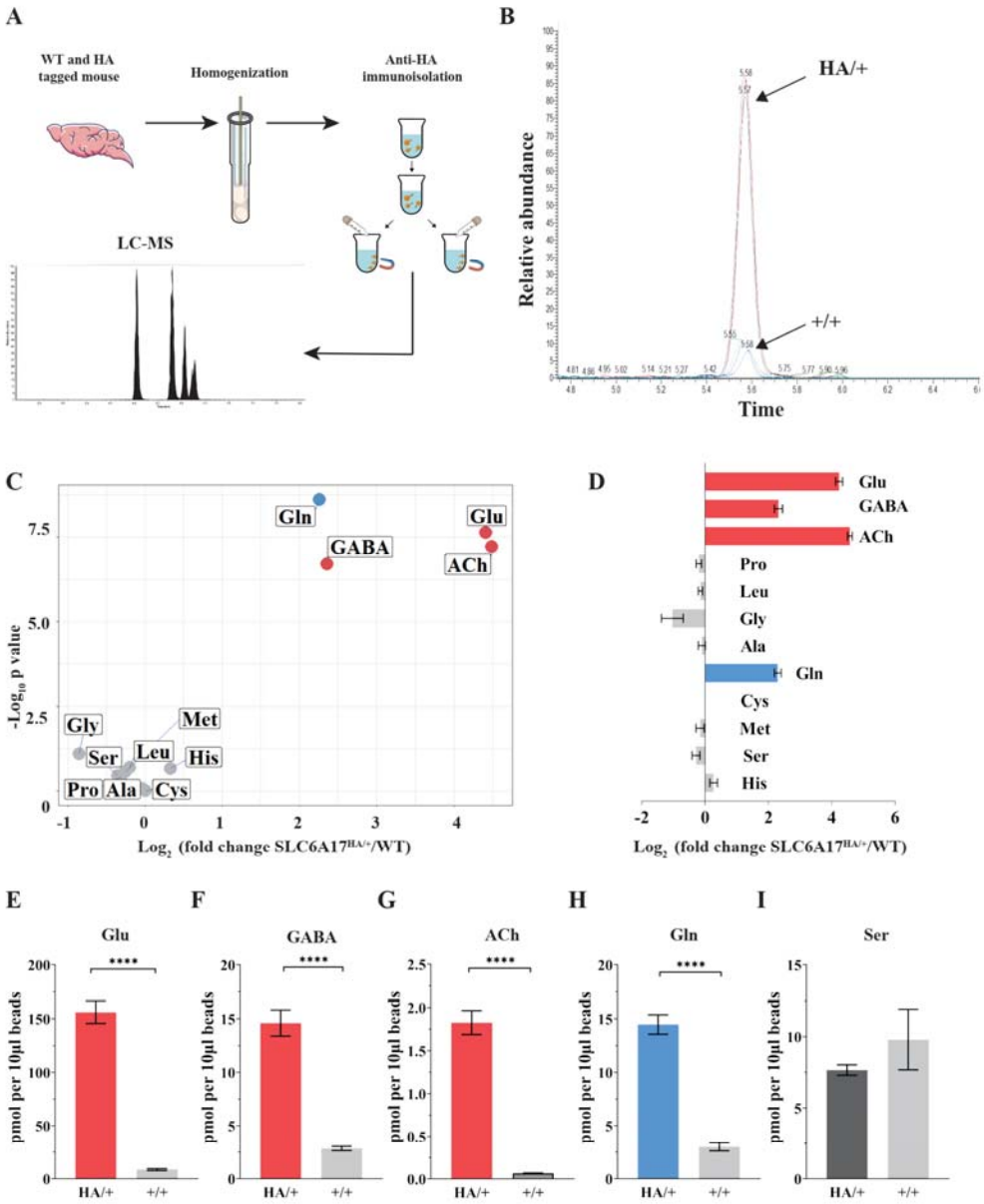

A
WT and HA tagged mouse
Homogenization
Anti-HA immunoisolation
LC-MS
B
HA/+
+/+
Relative abundance
Time
C
Gln
Glu
GABA
ACh
Gly
Met
Ser
Leu
His
Pro
Ala
Cys
-Log10 p value
Log2 (fold change SLC6A17HA/+/WT)
D
Glu
GABA
ACh
Pro
Leu
Gly
Ala
Gln
Cys
Met
Ser
His
Log2 (fold change SLC6A17HA/+/WT)
E
Glu
F
GABA
G
ACh
H
Gln
I
Ser
pmol per 10µl beads
****
HA/+
+/+

Figure 7

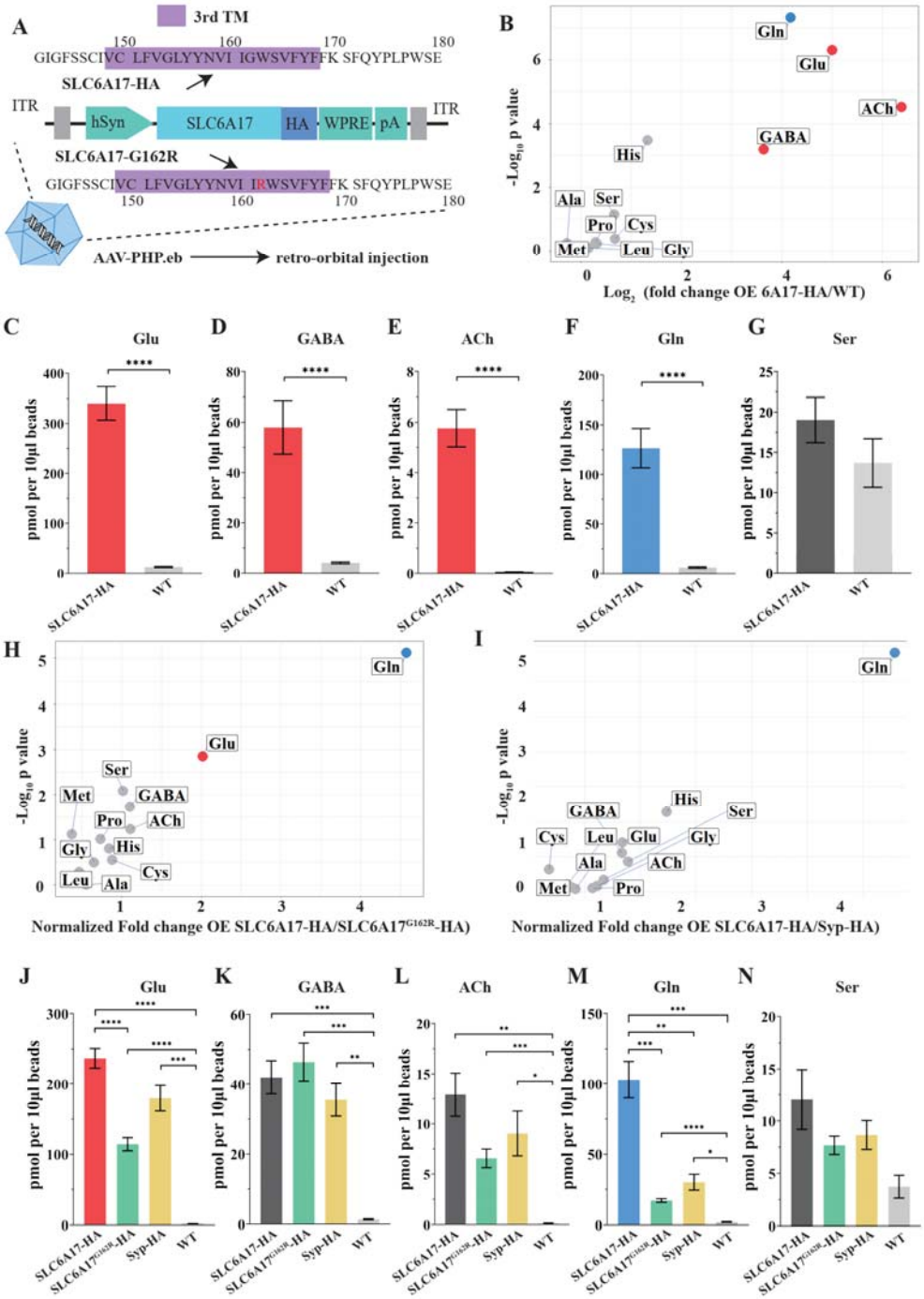

A
3rd TM
GIGFSSCIVC LFVGLYYNVI IGWSVFYFFK SFQYPLPWSE
SLC6A17-HA
ITR
hSyn
SLC6A17
HA
WPRE
pA
ITR
SLC6A17-G162R
GIGFSSCIVC LFVGLYYNVI IRWSVFYFFK SFQYPLPWSE
AAV-PHP.eb
retro-orbital injection
B
-Log10 p value
Log2 (fold change OE 6A17-HA/WT)
Gln
Glu
ACh
GABA
His
Ala
Ser
Pro
Cys
Met
Leu
Gly
C
Glu
D
GABA
E
ACh
F
Gln
G
Ser
pmol per 10µl beads
SLC6A17-HA
WT
H
Normalized Fold change OE SLC6A17-HA/SLC6A17G162R-HA)
I
Normalized Fold change OE SLC6A17-HA/Syp-HA)
J
K
L
M
N
SLC6A17-HA
SLC6A17G162R-HA
Syp-HA
WT

Figure 8

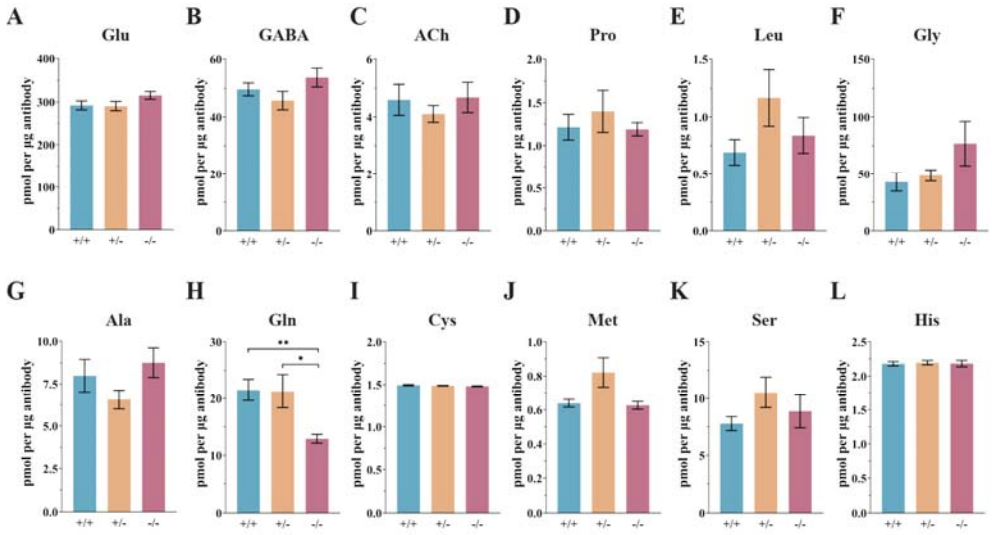

A
Glu
B
GABA
C
ACh
D
Pro
E
Leu
F
Gly
G
Ala
H
Gln
I
Cys
J
Met
K
Ser
L
His
pmol per µg antibody
+/+
+/-
-/-
**
*

Figure 9

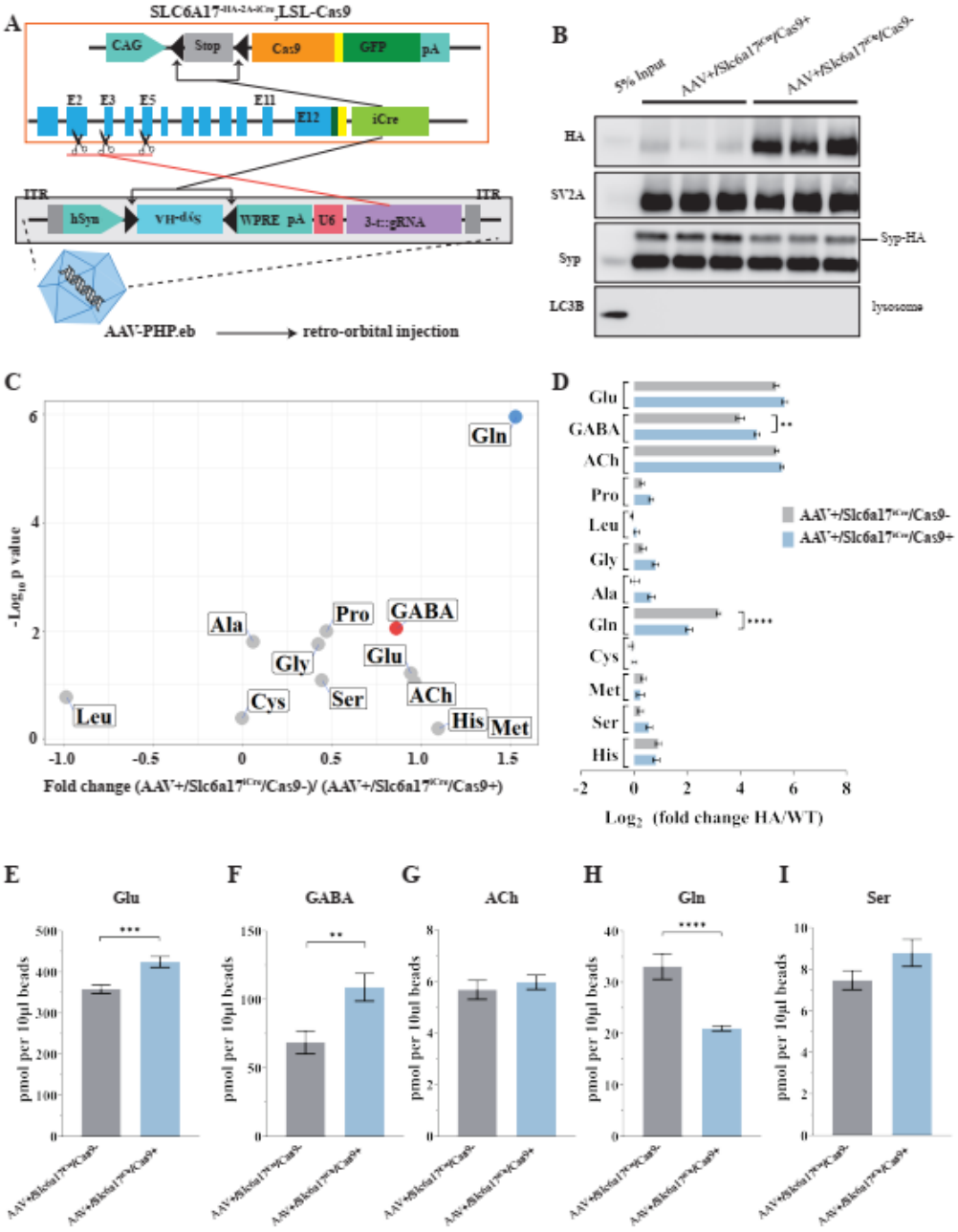
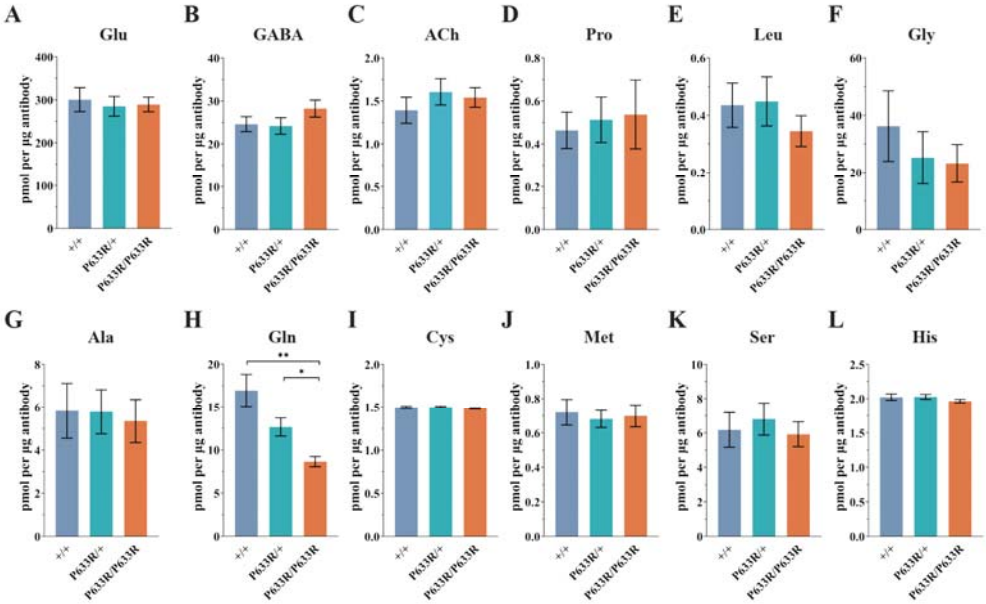

A
SLC6A17-HA-2A-iCre,LSL-Cas9
CAG
Stop
Cas9
GFP
pA
E2
E3
E5
E11
E12
iCre
ITR
hSyn
WPRE
pA
U6
3-n::gRNA
AAV-PHP.eb
retro-orbital injection
B
5% Input
AAV+/Slc6a17iCre/Cas9+
AAV+/Slc6a17iCre/Cas9-
HA
SV2A
Syp
Syp-HA
LC3B
lysosome
C
-Log10 p value
Fold change (AAV+/Slc6a17iCre/Cas9-)/ (AAV+/Slc6a17iCre/Cas9+)
Gln
Ala
Pro
GABA
Gly
Glu
Cys
Ser
ACh
Leu
His
Met
D
Glu
GABA
ACh
Pro
Leu
Gly
Ala
Gln
Cys
Met
Ser
His
AAV+/Slc6a17iCre/Cas9-
AAV+/Slc6a17iCre/Cas9+
Log2 (fold change HA/WT)
E
Glu
F
GABA
G
ACh
H
Gln
I
Ser
pmol per 10µl beads

Figure 10


A
Glu
B
GABA
C
ACh
D
Pro
E
Leu
F
Gly
G
Ala
H
Gln
I
Cys
J
Met
K
Ser
L
His
pmol per µg antibody
+/+
P633R/+
P633R/P633R
**
*