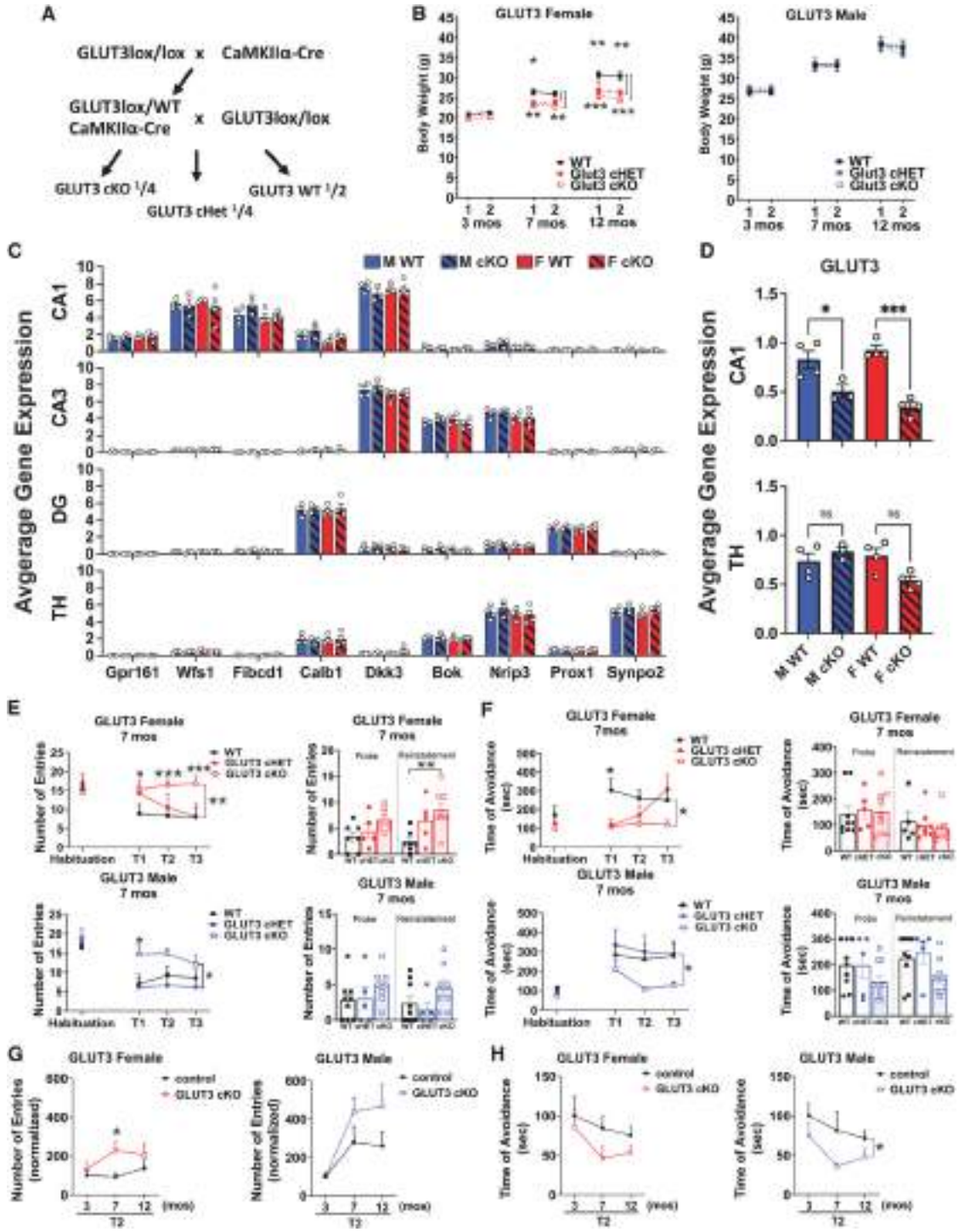

Figure 2. Neurons have an age-dependent requirement for neuronal glucose uptake *in vivo*

(A) Breeding scheme for GLUT3cKO mice.

(B) Weight measurements at age 3, 7, and 12 months. Data are means ± SEM; n = 9 GLUT3 WT, 5 Het, and 8 KO females, and n = 8–9 WT, 5 Het, and 8 KO males at each time point.

(C) Graphs show regional signatures unique to neurons in CA1 (20–36 capture areas/mouse), CA3 (11–30 capture areas/mouse), dentate gyrus (DG; 13–34 capture areas/mouse), and thalamus (TH; 65–170 capture areas/mouse). n = 3–4 mice/group.

(D) GLUT3 expression in CA1 and thalamic neurons. n = 3–4 mice/group.

(E and F) GLUT3cKO mice develop age-dependent spatial learning and memory deficits as shown by active place avoidance. At 7 months, GLUT3cKO female and male mice have increased entrances into the aversive zone (E) and decreased maximal time of avoidance of this zone (F).

(legend continued on next page)

and downstream pyruvate metabolites including lactate (Lac) and malate (Mal) (Figures 1A and S1B).

The media levels of metabolites downstream of PKM also markedly decreased upon PKM KD at 4 and 24 h, again with little or no change in fractional labeling (Figures 1B, 1C, and S1F), consistent with decreased production and release of these metabolites. This decrease in cellular and media metabolites downstream of PKM supports that decreased neuronal glycolysis decreases production of downstream TCA metabolites necessary for bioenergetic production. These data show that neurons can derive the majority of pyruvate from glycolysis, and do so, at least in the absence of glia.

Neurons require glucose uptake *in vivo*

Although the above data show that neurons are capable of metabolizing glucose through glycolysis, it remains possible that they either do not or do not need to take up glucose in the presence of glia *in vivo*. To test the neuronal requirement for glucose uptake *in vivo*, we selectively deleted GLUT3 from CA1 hippocampal neurons (Figure 2A). We bred floxed GLUT3 mice[24] with CamKIIα (CamKCre) mice, which express Cre recombinase from p19 in a subset of hippocampal neurons, including nearly all CA1 neurons, and in cortical and other neurons scattered throughout the forebrain.[25,26] GLUT3cKO ($GLUT3^{lox/lox}$;CamKCre) mice were the progeny of $GLUT3^{lox/lox}$ and $GLUT3^{wt/lox}$;CamKCre. GLUT3WT included control mice ($GLUT3^{wt/lox}$ and $GLUT3^{lox/lox}$) lacking the Cre transgene. GLUT3cKO mice were born in Mendelian proportions (control 50.0%, GLUT3 heterozygotes [GLUT3cHET] 25.2%, GLUT3cKO 24.8%, n = 238), and there were no differences in survival through more than 1 year (Figure S2A). GLUT3cKO male mice had similar body weights compared with controls through 12 months of age (Figure 2B). GLUT3cKO and control females had similar weight at 3 months, but both GLUT3cKO and GLUT3cHET females had progressively decreased weight at 7 and 12 months.

To confirm loss of GLUT3 expression in CA1 neurons, we used Visium Spatial Gene Expression from 10X Genomics. We validated our capacity to detect gene expression in CA1 neurons, where Cre is expressed in essentially all neurons.[27] CA1 neurons showed high expression of expected marker genes (Calb1, Wfs1, Fibcd1, Gpr161, and Dkk3), which enabled their clear differentiation from neurons in adjacent regions such as dentate gyrus (Calb1 and Prox1), CA3 (Dkk3, Bok, and Nrip3), and thalamus (Nrip3 and Synpo2) (Figure 2C).[28] In both male and female GLUT3cKO mice, GLUT3 expression was decreased specifically in CA1, but not in the thalamus where Cre is not expressed (Figure 2D).

To determine if GLUT3 is required for normal hippocampal function, we used the active place avoidance task to examine how GLUT3cKO affects visual-spatial learning and memory.[29] Both male and female GLUT3cKO mice displayed normal learning and memory at 3 months (Figures S2B and S2C), but developed severe deficits by 7 months (Figures 2E–2H). GLUT3cKO mice had an increase in the total number of entrances and a decrease in maximum time of avoidance to the aversive zone compared with controls. These deficits were also apparent, although less prominent, at 12 months of age (Figures S2D and S2E). In contrast, GLUT3cHET mice performed similarly to controls (Figures 2E, 2F, and S2B–S2E).

GLUT3cKO mice displayed no abnormalities in elevated plus maze testing at any age, indicating no gross changes in anxiety level (Figures S2F–S2H). GLUT3cKO mice had similar overall activity levels compared with controls, as shown by the number of open and closed arm entries and total distance in the plus maze (Figures S2G and S2H), along with a similar total number of ambulatory movements and rearing on open field activity testing (Figure S2I). There was no difference in withdrawal latency on hot plate testing (Figure S2J), further indicating intact sensorimotor function in GLUT3cKO mice. Therefore, GLUT3 loss in CA1 neurons markedly worsens spatial learning and memory, providing strong *in vivo* evidence that neurons must import glucose directly to function normally.

To begin to determine how decreased glucose uptake compromises neuronal function and health, we examined the impact of GLUT3cKO on neuronal survival. Loss of GLUT3 did not alter the density of NeuN-positive cells within CA1 in 15-month-old-mice (Figure S3A). However, analysis of brain volume using T_2-weighted MRI in live 10- to 14-month-old mice (Figure 3A) showed that female GLUT3cKO mice had decreased hippocampal volume versus controls, while GLUT3cKO had no impact in males. The difference in hippocampal volume was independent of mouse age (Figure S3B). To further assess this change, we performed high-resolution T_2-weighted MRI of postmortem brains in 19-month-old mice (Figure 3B), imaged in the intact skull to preserve overall brain structure and minimize potential experimental damage to the brain during dissection. Once again, GLUT3cKO female mice had smaller hippocampal and CA1 volumes than controls. The small decrease in CA1 volume in female GLUT3cKO mice may reflect synaptodendritic rarefaction in addition to some neuronal death.[30–32] Nonetheless, these findings indicate that postnatal loss of GLUT3 does not have large effects on the survival of CA1 neurons, suggesting that GLUT3cKO primarily disrupts neuronal function rather than survival. Total brain volume and thalamus volume were also smaller in female GLUT3cKO mice compared with wild-type (WT) controls, while ventricle size was similar (Figure 3C).

Although loss of Cre-expressing cortical neurons might explain the decrease in total brain volume, GLUT3KO mice also displayed decreased thalamus volume, a region where GLUT3 was not

(G and H) Longitudinal analysis shows change in second time point (T2) of active place avoidance, with each mouse normalized to the mean control value at 3 months. Although GLUT3cKO mice are equivalent to controls at 3 months, 7- and 12-month-old GLUT3cKO mice of both sexes enter the aversive zone more frequently than controls (G; see Figure S2 for full data from 3 and 12 months) and avoid it for less time (H). n = 9 WT, 5 Het, 8 KO females, and n=8-9 WT, 5 Het and 8 KO males at each time point, compiled from three cohorts.

ns, not significant.

*$p \leq 0.05$; **$p < 0.01$; ***$p < 0.001$ by Welch ANOVA with Dunnett's T3 multiple comparisons test (B, E, F), two-way ANOVA with Tukey's multiple comparison test (C), one-way ANOVA with Sidak's multiple comparison test (D), and Welch's t test (G and H). Brackets in graphs (E–H) show significance of linear mixed modeling for genotype (E and F) or the interaction of genotype and age (H).

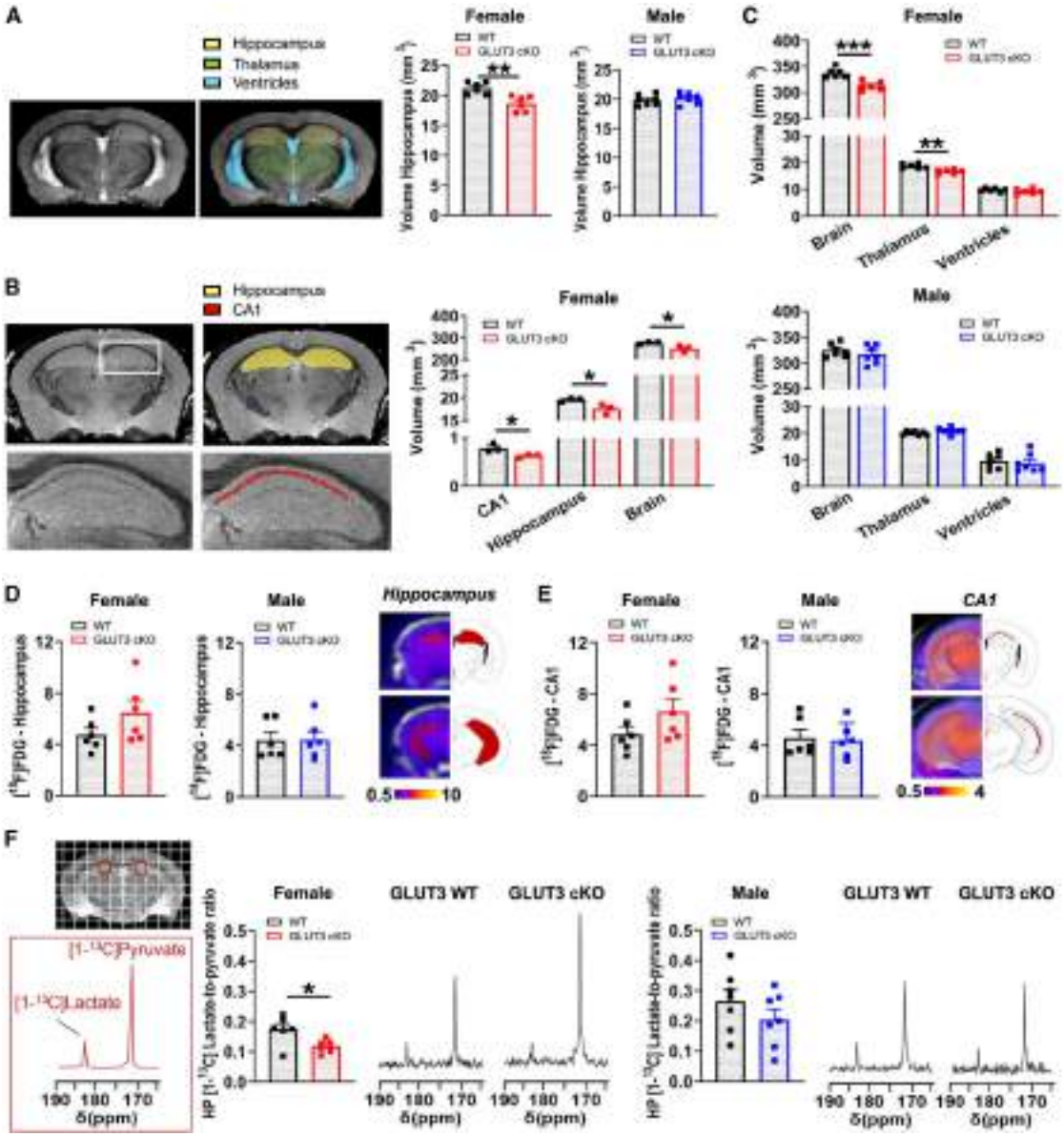

Figure 3. Female GLUT3cKO mice have smaller CA1 and total brain volumes, and decreased HP [1-^{13}C]lactate-to-pyruvate ratios

(A) Representative *in vivo* T_2-weighted images of 10- to 14-month-old mice used for volumetric analyses. The hippocampal volume was significantly smaller in female GLUT3cKO mice. Data are means ± SEM; n = 6–7 mice/group.

(B) Total brain and thalamus volumes calculated from *in vivo* T_2-weighted images of 10- to 14-month-old mice were smaller in female GLUT3cKO mice, while ventricle volume was unchanged. n = 6–7 mice/group.

(C) Representative *ex vivo* T_2-weighted images of 19-month-old mice used for volumetric analyses. CA1, hippocampal, and entire brain volumes were smaller in female GLUT3cKO mice. n = 3 mice/group.

(D and E) [^{18}F]FDG-PET signal from the hippocampus (D) and specifically from the CA1 area (E) was similar between 12- and 14-month-old females and males. n = 6 mice/group.

(F) Representative ^{13}C spectra of 8- to 14-month-old mice showing HP [1-^{13}C]pyruvate and HP [1-^{13}C]lactate levels from a region containing CA1 (red square) for female and male mice. HP [1-^{13}C]lactate-to-pyruvate ratios were significantly lower in female GLUT3cKO mice. n = 6–7 mice/group.

*p ≤ 0.05,**p ≤ 0.01,***p ≤ 0.001 by unpaired t tests.

knocked out in neuronal cell bodies.[33] This suggests either volume loss due to shrinkage of neurites from Cre-expressing neurons that project to the thalamus[34] or a non-cell-autonomous effect. Moreover, since the CamKCre mice express Cre from day P19,[25,26] at roughly the same time that the mouse brain reaches adult size (≈P20),[35] this presumably represents volume loss rather than a developmental change. Finally, although changes in the activity of CA1 hippocampal neurons can influence peripheral glucose