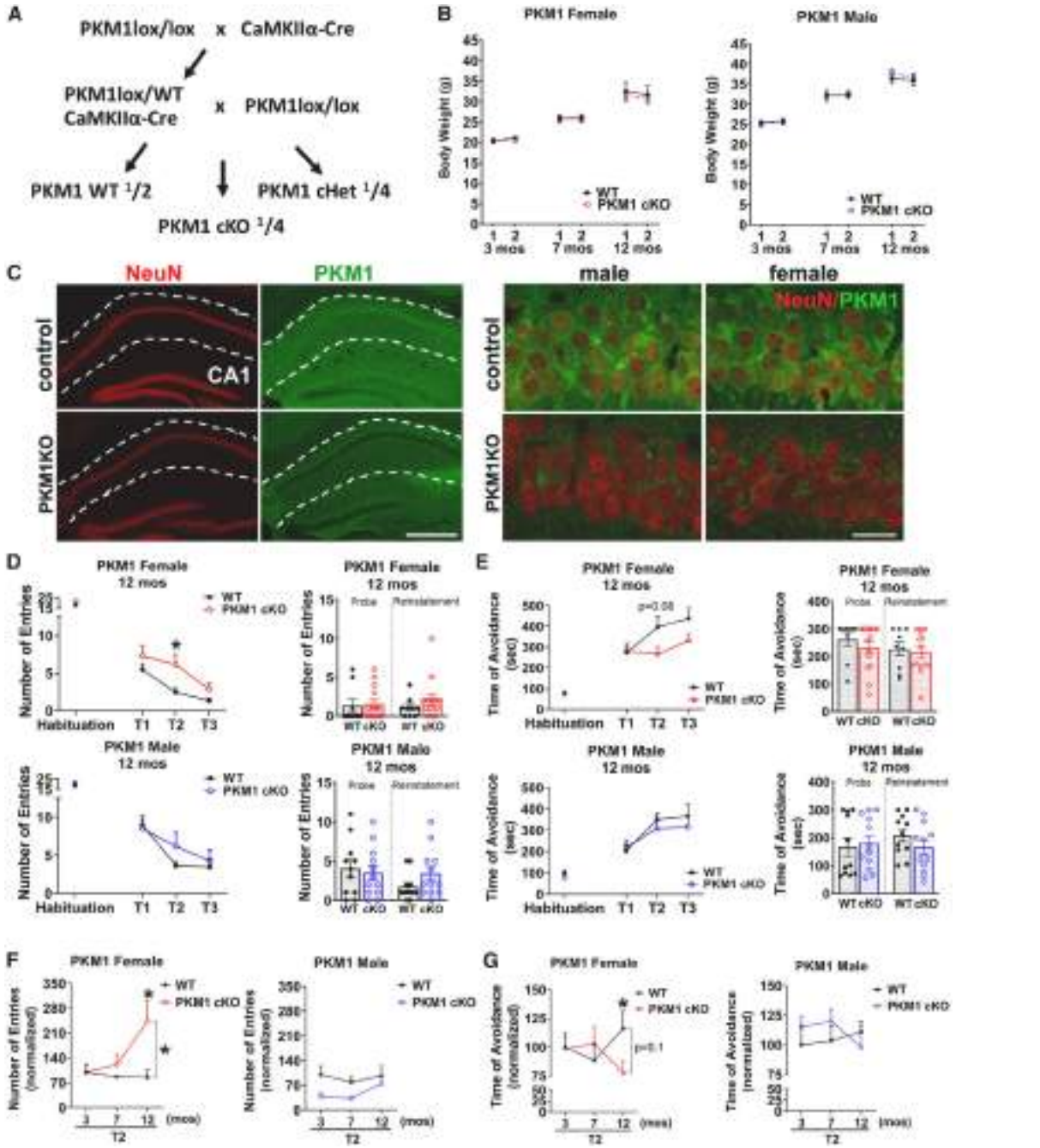

Figure 4. Female mice have an age-dependent neuronal requirement for glycolysis

(A) Breeding scheme to generate mice with conditional postnatal deletion of PKM1 in CA1 and other forebrain neurons.

(B) PKM1cKO mice have similar weights to controls. Repeated weight measurements at 3, 7, and 12 months of age. Data are means ± SEM; n = 10–11 PKM1 WT and 14 KO females, and 11 or 12 WT and 13 KO males at each time point.

(C) PKM1 immunofluorescence shows loss of PKM in CA1 neurons in PKM1cKO mice. Sections from 12-month-old mice are stained with NeuN (red) and PKM1 (green). Scale bar, 400 μm (left), 40 μm (right).

(D–G) Female PKM1cKO mice develop age-dependent spatial learning and memory deficits as shown by active place avoidance.

(D and E) Female PKM1cKO mice have increased entrances into the aversive zone (D), and a trend of decreased maximal time of avoidance of this zone (E) at 12 months of age (p = 0.06). No deficits were observed in males.

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levels,[36] we found no significant changes in blood glucose levels between GLUT3cKO and WT mice (Figure S3C).

In vivo metabolic and anatomical imaging shows sex-specific requirement for neuronal GLUT3

To determine the impact of GLUT3cKO on glucose metabolism *in vivo*, we first assessed total brain glucose uptake using [^{18}F]FDG-PET. GLUT3cKO did not affect the [^{18}F]FDG-PET signal in either the hippocampus or CA1 of 11- to 15-month-old male or female mice (Figures 3D and 3E), consistent with prior findings that postnatal day 15 pups lacking GLUT3 in the brain also have normal FDG-PET.[7] This suggests that glucose uptake by glia may constitute the majority of the [^{18}F]FDG-PET signal, precluding detection of neuronal glucose uptake *in vivo*. We therefore used HP ^{13}C MRSI to measure pyruvate conversion into lactate. Although the contributions of neurons and glia to the HP ^{13}C signal also cannot be directly distinguished by ^{13}C MRSI, combining this method with neuron-specific transgenic models enabled us to assess how changing glucose metabolism within neurons affects metabolism in different brain regions. In the hippocampus, HP [1-^{13}C] lactate-to-pyruvate ratios were significantly lower in female GLUT3cKO versus WT mice (Figures 3F and S3D). Males showed a similar trend. No significant differences were found in the thalamus (Figure S3E), where GLUT3 is not deleted in neuronal cell bodies.

To investigate the molecular basis for decreased pyruvate-to-lactate conversion in CA1, we examined expression of proximal genes that regulate pyruvate metabolism. Pyruvate flux toward lactate can be modulated by lactate dehydrogenase (LDH)[37–39] and pyruvate dehydrogenase (PDH),[40,41] as well as monocarboxylate transporters (MCTs),[42] which are responsible for pyruvate uptake and lactate efflux. However, we did not observe any changes in the expression of LDH, PDH, or MCT isoforms (Figures S3F–S3H). The conversion of pyruvate into lactate by LDH may also depend on the coenzyme NAD(H),[43–45] with an increased NADH/NAD+ ratio associated with increased HP [1-^{13}C]pyruvate-to-lactate flux. However, no change in the expression of GAPDH, which plays a prominent role in NAD metabolism,[46] was detected (Figure S3I).

Neuronal requirement for glycolysis *in vivo*

The finding that GLUT3cKO mice develop severe memory loss provides strong *in vivo* evidence that neurons must import glucose directly to function normally. However, this does not prove they require glycolysis, as neurons have been hypothesized to metabolize glucose primarily through the PPP under physiologic conditions.[8,9] To gain insight into the neuronal requirement for glycolysis, we analyzed the effect of postnatal deletion of PKM1,[47] the predominant neuronal isoform of PK[48,49] that catalyzes the final step in glycolysis.

We bred PKM1$^{lox/lox}$ mice with CamKIIα (CamKCre) mice (Figure 4A). PKM1cKO (PKM1$^{lox/lox}$; CamKCre) mice were the progeny of PKM1$^{lox/lox}$ and PKM1$^{wt/lox}$; CamKCre. PKM1WT included control mice (PKM1$^{wt/lox}$ and PKM1$^{lox/lox}$) lacking the Cre transgene. PKM1cKO mice were born in roughly normal Mendelian proportions (control 42.4%, PKM1 heterozygotes 29.3%, PKM1cKO 28.3%, n = 191 mice total), and no differences in survival were noted (Figure S4A). PKM1cKO mice of both sexes had similar body weights compared with controls through 12 months of age (Figure 4B). Loss of PKM1 expression in essentially all CA1 neurons was confirmed by immunofluorescence (Figure 4C).

We next examined how PKM1cKO affects spatial learning and memory assessed by active place avoidance. Both male and female PKM1cKO mice had normal memory at 3 and 7 months (Figures S4B–S4E). However, female (but not male) PKM1cKO mice developed learning and memory loss by 12 months, as indicated by increased entrances to the aversive zone (Figures 4D and 4F) and decreased maximal time of avoidance of the aversive zone (Figures 4E and 4G).

Female (but not male) PKM1cKO mice also showed deficits on elevated plus maze testing. PKM1cKO female mice showed a trend of increased time in the open arm (Figure S4F), and total distance moved was increased at 7 months (Figure S4G). Female PKM1cKO had more open and closed arm entries than controls (Figures S4G and S4H), suggesting the difference in open arm time could be driven by hyperactivity. Indeed, female PKM1cKO moved a greater distance in open field testing and had increased rearing (Figure S4I). In contrast, male and female PKM1cKO mice had normal withdrawal latency on hot plate testing (Figure S4J), indicating preserved sensory function. Therefore, complete PKM1 loss worsens memory and produces hyperactivity in female mice, providing *in vivo* evidence that postnatal neurons must metabolize glucose by glycolysis and suggesting a sex-specific difference in the requirement and/or mechanism of glycolysis.

To determine how decreased glycolysis can compromise the function of CA1 neurons, we first examined the impact of PKM1cKO on neuronal survival. However, there was no change in the density of NeuN-positive cells within CA1 at 12 months in either female or male PKM1cKO mice (Figure S5A). In addition, total brain volume and hippocampus, thalamus, and ventricle size assessed by *in vivo* T_2-weighted MRI in live 11- to 15-month-old mice did not differ between female or male PKM1cKO and PKM1WT mice (Figures 5A and S5B). Therefore, postnatal loss of PKM1 disrupts neuronal function rather than survival in female mice.

PKM1-mediated glycolysis is critical in neurons from female mice

[^{18}F]FDG-PET imaging did not detect any significant differences in glucose uptake in the total hippocampus, or specifically in

(F and G) Longitudinal analysis shows change in second time point (T2) of active place avoidance testing, with each mouse normalized to the mean control value at 3 months. PKM1cKO females are equivalent to controls at 3 and 7 months of age, but 12-month-old PKM1cKO mice enter the aversive zone more frequently than controls (F), and avoid it for less time (G), whereas no deficits were observed in males (see Figure S4 for full data from 3 to 7 months). n = 10–11 WT, 14 KO females, and 11 or 12 WT and 13 KO males, each compiled from three cohorts.

*$p \leq 0.05$ by Welch ANOVA with Dunnett's T3 multiple comparisons test (D and E) and Welch's t tests (F and G). Brackets in graphs (F and G) show significance of linear mixed modeling for the interaction of genotype and age (F and G).

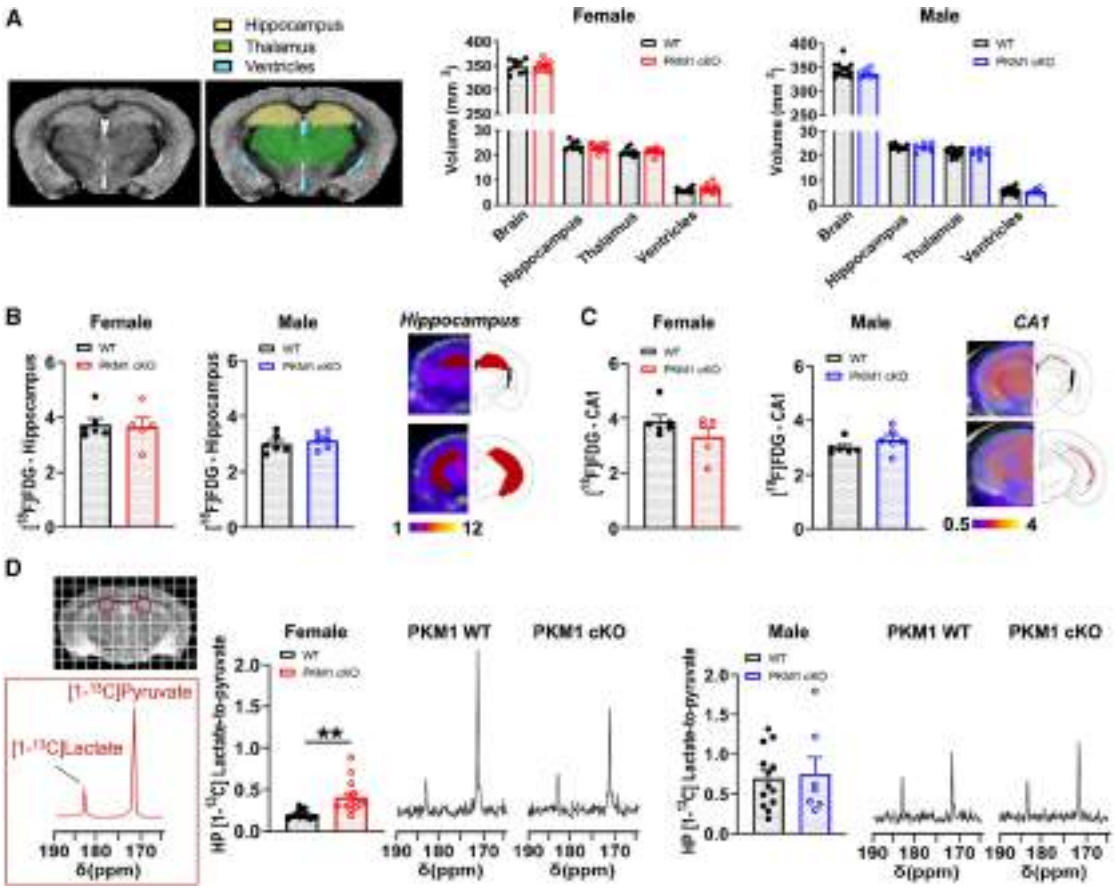

Figure 5. Female PKM1cKO mice have increased metabolic conversion of HP pyruvate to lactate

(A) Representative *in vivo* T_2-weighted images of 11- to 15-month-old mice used for volumetric analyses showed no differences between female or male PKM1cKO and PKM1WT mice for the entire brain, hippocampus, thalamus, or ventricles. Data are means ± SEM. n = 9 PKM1WT, 14 KO females, and n = 7 PKM1WT, 13 KO males.

(B and C) There were no differences in [^{18}F]FDG-PET signal between 11- and 14-month-old mice PKM1WT and PKM1cKO mice in the hippocampus (B) or CA1 (C). n = 5–6 mice/group.

(D) Representative ^{13}C spectra of 11- to 15-month-old mice showing HP [1-^{13}C]pyruvate and HP [1-^{13}C]lactate levels from a region containing CA1 (red square). HP [1-^{13}C]lactate-to-pyruvate ratios were markedly higher in female PKM1cKO versus PKM1WT mice, but were similar in males. n=9 PKM1WT, 14 KO females and seven PKM1WT, 13 KO males. **p ≤ 0.01 by unpaired t tests.

CA1, in either male or female PKM1cKO mice (Figures 5B and 5C). We used HP ^{13}C MRSI to measure pyruvate conversion into lactate. In female PKM1cKO mice, a significant increase in the rate of pyruvate-to-lactate conversion (i.e., increased HP [1-^{13}C]lactate-to-pyruvate ratio) was observed in the CA1 area (Figure 5D), while no changes were detected in male PKM1cKO mice. We also observed an increased rate of pyruvate-to-lactate conversion in the thalamus of female PKM1cKO mice (Figure S5D), even though thalamic neurons had grossly normal levels of PKM1 expression (Figure S5E). This likely reflects contributions of neurites from Cre-expressing neurons projecting to the thalamus,[34] although non-cell-autonomous effects could also contribute.

Notably, PKM1cKO had no impact on cerebral blood volume and flow as measured by MRI methods, suggesting that these parameters had no impact on the HP metabolite levels (Figure S5F). Decreased pyruvate production from glucose in PKM1cKO mice may lead to more import of HP [1-^{13}C] pyruvate into cells or an upregulation of LDH activity, either of which would increase HP [1-^{13}C] pyruvate-to-lactate conversion. In addition, compensation by the PKM2 isoform could also lead to a relative increase in HP [1-^{13}C] pyruvate-to-lactate conversion.[50] However, the relative contributions of these factors to increasing HP [1-^{13}C] pyruvate-to-lactate conversion remain to be delineated.

To gain insight into how male and female neurons may differentially compensate for the absence of PKM1, we assessed PKM2 levels. Although PKM2 is normally expressed in glia rather than neurons,[51] it can be upregulated *in vivo* to compensate for loss of PKM1.[47] While PKM1 levels were decreased in both male and female PKM1cKO mice, no significant changes in PKM2 levels were detected in either sex (Figure S5G), although this does not exclude an increase in PKM2 activity or a compensatory increase in PKM2 in astrocytes.

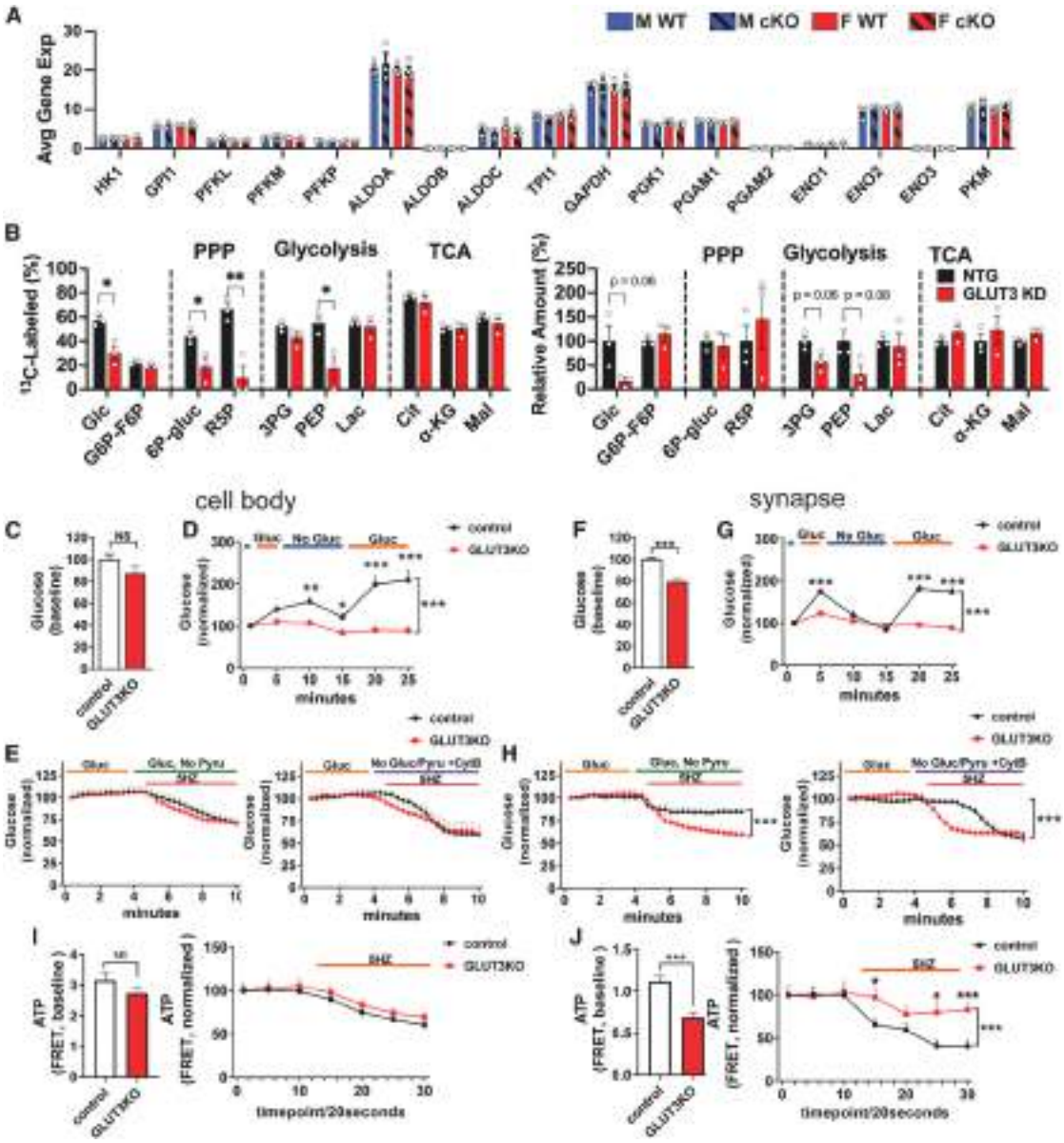

Figure 6. Neurons require glucose uptake and glycolysis to maintain ATP at the synapse

(A) Expression of glycolytic genes in GLUT3cKO CA1 neurons. Data are means ± SEM. n = 3–4 mice/group, compiled from 20 to 36 capture areas/mouse in CA1.

(B) Effect of GLUT3 KD in iPSC-derived neurons incubated for 24 h with 1.5 mM [U-^{13}C]glucose on percentage of glucose-derived metabolites and total metabolites. The corresponding isotopologue data are shown in Figure S7C. Identical NTG controls (1.5 mM [U-^{13}C]glucose) are shown in Figures S7B and S7C. n = 3 samples/group.

(C–H) GLUT3 KO disrupts glucose homeostasis in individual neurons. GLUT3$^{lox/lox}$ neurons were co-transfected with a fluorescent glucose sensor (iGlucoSnFR-mRuby) and either Cre (to delete GLUT3, GLUT3KO) or empty vector (control), as well as BFP-synaptophysin to identify synaptic boutons.

(C and D) GLUT3KO neurons had similar basal glucose levels to controls at the cell body (C), but their glucose levels were less responsive to changes in the extracellular glucose (D).

(E) Glucose levels decreased similarly in GLUT3KO and control neurons with electrical stimulation (5 Hz, 5.5 min) to increase neural activity (left), and the speed and extent of decrease was somewhat greater when glucose uptake was blocked with cytochalasin B (right) n = 8–10 coverslips/group (two or three cells/coverslip) from three independent experiments.

(D and E) Control and GLUT3cKO glucose values are normalized to the starting point.

(F and G) The synapses of GLUT3KO neurons had lower basal glucose levels (F), and their glucose levels were less responsive to changes in the extracellular glucose than controls (G).

(H) Glucose levels in GLUT3KO synapses decreased to a greater extent in GLUT3cKO versus control synapses, in response to electrical stimulation (left). Blocking all glucose uptake with cytochalasin B caused glucose levels in controls to drop to GLUT3KO levels (right). n = 8–10 coverslip/group, three to five synapses/coverslip from three independent experiments.

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