1 **Glutamate and γ -aminobutyric acid differentially modulate glymphatic**
2 **clearance of amyloid β through pulsation- and aquaporin-4 dependent**
3 **mechanisms**

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40 **Abstract**

41 The glymphatic system contributes to a large proportion of brain waste clearance,

42 including removal of amyloid β ($A\beta$). We have demonstrated that glutamate and

43 γ -aminobutyric acid (GABA) influence glymphatic clearance through distinct

44 mechanisms whereby GABA exerts modulatory effects in an aquaporin-4

45 (AQP4)-dependent manner while the actions of glutamate are pulsation-dependent. The

46 efficacy of GABA and glutamate in alleviating A β in APP-PS1 and Angiotensin-II
 47 (Ang-II)-induced hypertension mouse models was further evaluated. Notably, increasing
 48 GABA or inhibiting glutamate levels led to reduced binding of A β to pre-labeled plaques
 49 to similar extents in APP-PS1 mice while GABA appeared more efficient in A β clearance
 50 in hypertensive animals than the glutamate inhibitor. Our findings support the modulation
 51 of neurotransmitters that influence the glymphatic pathway via distinct mechanisms as a
 52 potentially effective therapeutic strategy for clearance of A β deposits from the brain.

53 **Keywords:** brain; pulsation; aquaporin-4; Glutamate; GABA; waste removal

54 **Introduction**

55 β -Amyloid ($A\beta$) accumulation is one of the pathological hallmarks of Alzheimer's
 56 disease (AD). Both genetic and environmental factors are involved in AD development.
 57 Earlier studies have provided substantial evidence supporting an important role of
 58 environmental factors, such as hypertension, in the development of sporadic AD¹.
 59 Cerebral blood vessels provide major $A\beta$ clearance routes from the brain. Hypertension
 60 disrupts the normal function of cerebral blood vessels, promoting vascular $A\beta$ deposition,
 61 which, in turn, impairs vessel function, forming a vicious circle and eventually leads to
 62 cognitive dysfunction^{2,3}.

63 In the CNS, $A\beta$ is released from neurons into brain interstitial fluid (ISF). Recently,
 64 the glymphatic system and dural lymphatic vessels have been proposed as major
 65 extracellular disposal routes of $A\beta$ ⁴⁻⁷.

66 Neurotransmitters play an important role in the pathogenesis of AD. $A\beta$ is reported to
 67 disrupt synaptic transmission of different neurotransmitters⁸⁻¹¹, leading to cognitive
 68 decline. Several pre-clinical and clinical studies have demonstrated that modulation of
 69 neurotransmitter systems by memantine (NMDA receptor antagonist) and etazolate
 70 (GABA-A receptor agonist) can effectively alleviate accumulation of $A\beta$ ^{12,13}. Interestingly,
 71 neurotransmitters are also involved in modulation of glymphatic clearance. For example,
 72 previous studies have shown that noradrenaline mediates glymphatic transport in that
 73 convective exchange is increased in the presence of adrenergic antagonists, resulting in
 74 faster clearance of $A\beta$ ¹⁴. Glutamate and GABA are major stimulatory and inhibitory
 75 neurotransmitters in the CNS, respectively. However, their potential roles in the

glymphatic system are yet to be established. Aquaporin-4 (AQP4) and arterial pulsation are two main driving forces in the glymphatic pathway^{4,15}. The GABA-A receptor co-localizes with AQP4 in brain tissue^{16,17}. In addition, glutamate has been shown to influence vascular smooth muscle cell function, which is associated with pulsation¹⁸. In the current study, we tested the hypothesis that glutamate and GABA play important roles in glymphatic pathway clearance through modulation of pulsation or AQP4. Improper function of these neurotransmitters may differentially contribute to clearance failure of A β .

METHODS

Animals.

C57BL/6J male mice (6–8 weeks, 12–16 weeks and 8 months) were provided by the Sun Yat-sen University Medical Experimental Animal Center (Guangzhou, China). AQP4-deleted male mice (AQP4^{-/-}) 6–8 weeks of age were a gift from the Jiangsu Key Laboratory of Neurodegeneration (Nanjing Medical University, Nanjing, China). Male APP^{swe}/PS1^{dE9} (APP/PS1) mice 7–8 months old were purchased from Guangdong Medical Experimental Animal Center. AQP4^{-/-} and APP-PS1 mice were both backcrossed onto a C57BL/6J background. All animals were kept in temperature- and humidity-controlled rooms under a 12 h/12 h light/dark cycle. Sample size was selected according to previously published reports. Experimental procedures were performed in accordance with the guidelines imposed by Sun Yat-sen University Committee on the Care and Use of Animals.

98 **Reagents and Antibodies.**

99 FITC-dextran 70 kDa (Sigma, FD4, USA) was used to trace CSF movement, and
100 FITC-dextran 2000 kDa (Sigma, FD2000S, USA) and Rhodamine B-dextran 70 kDa
101 (Sigma, R9379, USA) to label vasculature. All reagents were dissolved in artificial CSF
102 (ACSF) at a concentration of 1%. Neurotransmitter agonists/antagonists, including
103 glutamate (Sigma, G3291, USA), NMDA receptor antagonist,
104 2-amino-5-phosphonovaleric acid (APV) (Sigma, A8054 , USA), AMPA/kainate receptor
105 antagonist 6-Cyano-7-nitroquinoxaline-2, 3-dione (CNQX) (Sigma, C127, USA), GABA
106 (Sigma, A2129, USA), GABA-A receptor antagonist and Bicuculline (Bic) (Sigma,
107 285269, USA), were dissolved in CSF tracer before use at a concentration of 0.2 mM.
108 Evans Blue (Sigma, E2129, USA) dissolved in saline was used to label lymph node
109 drainage. Angiotensin-II (Bechem, H-1705, Torrance, USA) was employed to induce
110 hypertension. Fluorescent-labeled A β peptides (Anaspec, 60492-01, Fremont, USA) and
111 FSB (Millipore, 344101, Darmstadt, Germany) were used to examine A β binding. To
112 label amyloid plaques, antibodies specific for A β 1-42 (Biolegend, 805501, San Diego,
113 USA) and A β 1-40 (Biolegend, 805401, USA) were employed. Antibodies for Collagen-I
114 (Co-I) (Abcam, ab34710, Hong Kong, UK) and α -smooth muscle actin (SMA) (Boster,
115 BM0002, Wuhan, China) were used to test vascular structure changes and those for AQP4
116 (Alomone Labs, 300-314, Jerusalem, Israel) and GFAP (Millipore, 2642205, USA) to
117 detect aquaporin and astrocytes, respectively. All secondary antibodies were purchased
118 from Cell Signaling Technology (4408, 4409, 4412, 4413, Carlsbad, USA).

119 **Intra-cisternal and interstitial tracer injection.**

Mice were anesthetized with an intraperitoneal injection of 1% pentobarbital (50 mg/kg) and positioned in a stereotaxic frame (RWD Life Science company, Shenzhen, China). A microsyringe (BASi, West Lafayette, USA) was inserted into the cisterna magna (i.c.v.), and 10 μ l CSF tracer injected at a speed of 0.2 μ l/min. For interstitial injection, micro glass pipettes filled with 1 μ l CSF tracer was connected to a microinjection system (BASi, USA). Under a two-photon microscope, the pipette was inserted into the cortex under 200 μ m and the solution infused at a velocity of 0.2 μ l/min.

***In vivo* two-photon imaging of glymphatic pathway clearance.**

A 2×2mm² cranial window was prepared over the right parietal cortex (2 mm caudal from bregma, 1.7 mm lateral from the midline) with a micro-drill and a metal plate glued at the edge of the cranial window^{16,17}. The mouse was fixed on the stage of two-photon microscope (Leica, DM6000, Wetzlar, Germany). ACSF was perfused during the whole surgical procedure to keep the cranial window moist. Throughout the experiment, body temperatures were kept constant at 36.8°C with a feedback-controlled heating pad (RWD Life Science Company, Shenzhen, China). To visualize vasculature, 0.2 ml Rhodamine B-dextran 70 kDa was injected intravenously before imaging. A Leica NA 0.95 together with a 25× magnification water-immersion objective was used at an excitation wavelength of 800 nm. The cerebral vasculature was initially imaged with 512 × 512-pixel frames from the surface to a depth of 200 μ m with 2 μ m z-steps. Tracer movement was detected with dual channels. Imaging panels 100 μ m below the cortical surface were selected for analysis of tracer movement into the paravascular space with Leica Lite software. For paravascular and interstitial movement, circular regions of interest (ROI) 25 pixels in

diameter were centered on the surrounding penetrating arterioles. To define tracer movement into brain tissue, the distribution of CSF tracers in three-dimensional (3D) vectorized reconstruction was analyzed. Mean pixel intensities within these ROIs and 3D reconstructions were measured at 5 min intervals. For interstitial clearance, mean fluorescence of CSF tracers was measured at 15 min intervals.

Evans blue injection and quantification.

With careful separation of muscle and anadesma in the back, the cisterna magna was exposed. In total, 5 μ l of 10% Evans blue (Sigma, E2129) was slowly injected into the cisterna magna at a rate of about 1 μ l/min over 5 min. After 30 min, deep cervical lymph nodes (dcLNs) were dissected for assessment of Evans blue content. The intensity of Evans blue was measured using confocal microscopy, and the same capture parameters maintained during intensity detection.

Pulsation measurement.

Pulsation measurements were conducted as previous experiment described¹⁵. X-T line scan technology was used to measure pulsation. Scans of 2000 ms (0.5 ms per line) were acquired orthogonal to the vessel axis in different vessel types, including surface arteries, surface veins, penetrating arteries and penetrating veins. Vessel fluorescence was extracted from X-T plots and plotted in relation to time using Graphpad prism 6.0. Pulsatility was calculated as standard deviation of the percentage of mean values.

Unilateral internal carotid artery ligation

Unilateral internal carotid artery ligation was performed as previous experiment described¹⁵. The right common, internal, and external carotid arteries were surgically

164 isolated. The internal carotid was ligated by 5-0 silk suture.

165 **Murine models of hypertension.**

166 Hypertension mouse models were generated by Angiotensin-II (1000 ng/kg/min)
167 infusion using osmotic pumps (DURECT, Alzet model 2004), which were implanted
168 subcutaneously in 12–16 weeks-old C57Bl/6 mice for 28 days. Assessment of Ang-II
169 amounts was performed according to previous reports¹⁹. Blood pressure was measured
170 with the tail cuff method every week. Mouse models without hypertension were
171 discounted.

172 ***In vivo* two-photon time-lapse imaging of A β binding.**

173 Mice received FSB injection (I.P. 7.5 mg/kg) two days prior to the experiment.
174 Anesthesia and craniotomy were performed in keeping with previous protocols.
175 Microglass pipettes filled with 1 μ l A β -555 peptide solution were connected to a
176 microinjection system (BASi, USA). A β 40-555 were used among hypertension groups
177 and A β 42 555 were used among APP/PS1 groups. Under a two-photon microscope, the
178 pipette was inserted into the cortex and the solution infused at a velocity of 0.2 μ l/min. *In*
179 *vivo* two-photon imaging of the binding process was conducted about 200 μ m away from
180 the pipette tip with a 20 $\times$ water immersion objective. A β 40/42-555 and FSB were excited
181 at 920 nm and 800 nm, respectively. The recording time was 10 min with 30 s per frame.
182 For administration of the GABA, APV and CNQX, drugs were dissolved in a CSF at a
183 final concentration of 10 μ M in 10 μ l and injected into the cisternal at the velocity of
184 1 μ l/min.

185 **Immunofluorescence.**

186 Immunofluorescence analysis was conducted on 10 μ m paraformaldehyde
187 (PFA)-fixed frozen sections. Slices were blocked for 1 h at room temperature with normal
188 goat serum and 0.3% Triton and incubated overnight with primary antibody at 4°C,
189 followed by secondary antibody for 1 h at room temperature. Slices were stained with
190 DAPI (F6057, Sigma, USA) and immunofluorescence images observed under a confocal
191 microscope (Leica, DM6000, Wetzlar, Germany).

192 **Statistical analysis.**

193 Data were analyzed with SPSS Statistics 20 and GraphPad Prism 6.0 and presented as
194 mean $\pm$ s.e.m or mean $\pm$ S.D. Different treatment groups were evaluated using one-way
195 ANOVA with LSD test or two-way ANOVA with Tukey's test for multiple comparisons to
196 determine differences among individual groups. The unpaired t-test was used when
197 comparing two individual groups. A probability of $p < 0.05$ was indicative of significant
198 differences between groups. Regardless of the method used, the results were equivalent in
199 magnitude and statistically significant. Data from all statistical analyses are presented in
200 Supplementary Table 1.

201

202 **RESULTS**

203 **GABA promotes while glutamate suppresses glymphatic clearance**

204 To explore the roles of GABA and glutamate in glymphatic pathway clearance, specific
205 agonists/antagonists for these neurotransmitters dissolved in CSF tracer were injected into
206 the cisterna magna (Fig. 1e). Cerebral vasculature was labeled with 70 kDa Rhodamine B,
207 which allows visualization of arteries based on blood flow direction and morphology.

Measurements were taken 5 min after infusion when the fluorescent tracer could be stably visualized. The tracer rapidly entered the cortex through the paravascular spaces surrounding the surface along penetrating arteries but was absent in venules at the beginning (Fig. 1a–d). To quantitatively evaluate the movement of para-arterial CSF tracer, mean fluorescence intensity (ROI) was measured at 100 μ m below the cortical surface over 30 min with intervals of 5 min (Fig. 1b). During the measurements, mean fluorescence intensity in the sham group increased constantly up to ~300% at 30 min (Fig. 2a–c). A large proportion of para-arterial CSF tracer gradually fluxed into this space. Interestingly, glutamate significantly inhibited paravascular penetration (Glutamate vs. vehicle, two-way ANOVA, for interaction factor, $P < 0.001$) while both the GABA_A receptor agonist and antagonist had no effect on paravascular movement (GABA vs. vehicle, two-way ANOVA, for interaction factor, $P = 0.6027$; bicuculline vs. vehicle, two-way ANOVA, for interaction factor, $P = 0.2211$) (Fig 2b). The neurotransmitter glutamate has two major ionotropic receptors (NMDA and AMPA/kainate receptors), which are widespread in the cerebral cortex and other brain regions. Notably, upon separate blockage of these two receptors, APV (NMDA receptor antagonist) significantly accelerated paravascular movement (APV vs. vehicle, two-way ANOVA, for interaction factor, $P < 0.001$) whereas CNQX (AMPA/kainate receptor antagonist) had no effect (CNQX vs. vehicle, two-way ANOVA, for interaction factor, $P = 0.9303$). Since water and small solutes freely enter the brain interstitium from paravascular spaces via bulk flow, we additionally analyzed tracer influx into the surrounding interstitium via 3D vectorized reconstruction of tracer distribution in the parenchyma (Fig. 2b–c). The mean

230 fluorescence intensity in the sham group increased to ~3.0-fold at 30 min, indicating
 231 influx of tracer from the paravascular space. As expected, glutamate reduced CSF tracer
 232 penetration into the interstitium (Glutamate vs. vehicle, two-way ANOVA, for interaction
 233 factor, $P<0.001$) (Fig. 2d). Unexpectedly, APV and CNQX robustly accelerated tracer
 234 influx into parenchyma (APV vs. vehicle, two-way ANOVA, for interaction factor,
 235 $P=0.1346$; CNQX vs. vehicle, two-way ANOVA, for interaction factor, $P<0.001$) (Fig.
 236 2d). Moreover, GABA induced marked enhancement whereas bicuculline (GABA_A
 237 receptor antagonist) led to significant inhibition of influx (GABA vs. vehicle, two-way
 238 ANOVA, for interaction factor, $P<0.05$; bicuculline vs. vehicle, two-way ANOVA, for
 239 interaction factor, $P<0.001$) (Fig. 2e). Therefore, our results suggest that glutamate slows
 240 paravascular movement though the NMDA receptor and inhibits tracer influx into the
 241 parenchyma though the AMPA/kainate receptor. GABA, which did not influence
 242 paravascular movement, accelerated tracer influx into the parenchyma.

243 In the glymphatic pathway, CSF enters the brain interstitium though paravascular
 244 movement and interstitial fluid in parenchyma is subsequently cleared from paravenous
 245 routes. To further explore the role of neurotransmitters in modulating interstitial fluid
 246 clearance, CSF tracers were injected directly into brain parenchyma 150-200 μm below
 247 the skull surface and two-photon imaging of tracer clearance performed over 60 minutes
 248 with intervals of 15 minutes (Fig. 3a). The mean fluorescence intensity in the region of
 249 interest (ROI) at 200 μm below the cortical surface was measured to quantify interstitial
 250 fluid clearance. Following injection, mean fluorescence intensity in the vehicle group
 251 constantly decreased and was reduced by ~60% at 60 minutes (Fig. 3b, c). Notably,

252 glutamate strongly inhibited while CNQX significantly accelerated interstitial fluid
253 clearance (glutamate vs. vehicle, two-way ANOVA, for interaction factor, $P<0.001$;
254 CNQX vs. vehicle, two-way ANOVA, for interaction factor, $P<0.001$). However, APV did
255 not promote interstitial fluid clearance (APV vs. vehicle, two-way ANOVA, for
256 interaction factor, $P=0.6069$). In contrast, GABA greatly promoted whereas bicuculline
257 robustly inhibited interstitial fluid clearance (GABA vs. vehicle, two-way ANOVA, for
258 interaction factor, $P<0.001$; bicuculline vs. vehicle, two-way ANOVA, for interaction
259 factor, $P<0.01$) (Fig. 3b, c). Based on these findings, we concluded that glutamate impairs
260 interstitial fluid clearance though the AMPA/kainate receptor and GABA facilitated
261 drainage.

262 Interstitial solutes are ultimately cleared into peripheral lymph nodes outside the brain
263 parenchyma. Accordingly, we injected Evans blue i.c.v. and examined for the presence of
264 the dye in deep cervical lymph nodes (dcLNs) in animals receiving different treatments.
265 Consistent with previous findings, Evans blue was detected in dcLNs 30 min after
266 injection. Mean fluorescence of Evans blue in lymph nodes was analyzed for each group
267 (Fig. 3e). Unexpectedly, Evans blue staining was barely detectable in dcLNs of animals
268 receiving glutamate (Fig. 3b) but evident in lymph nodes of animals receiving APV and
269 CNQX, respectively (Glutamate vs. vehicle, $t=9.976$, $P<0.001$; APV vs. vehicle, $t=8.737$,
270 $P<0.001$; CNQX vs. vehicle, $t=6.811$, $P<0.001$) (Fig. 3d), suggesting that a
271 glutamate-related clearance mechanism is involved in this drainage route of cerebrospinal
272 fluid (CSF). Consistently, GABA accelerated whereas bicuculline inhibited ultimate
273 drainage of the tracer dye (GABA vs. vehicle, $t=5.27$, $P<0.001$; bicuculline vs. vehicle,

274 $t=10.85$, $P<0.001$) (Fig. 3e). Our findings suggest that glutamate and GABA modulate
275 drainage of interstitial fluid from the parenchyma via distinct routes. Based on the
276 collective findings, we propose that glutamate and GABA play opposite roles in CSF
277 tracer movement. Specifically, glutamate inhibits paravascular movement though the
278 NMDA receptor and facilitates interstitial fluid clearance though the AMPA/kainate
279 receptor while GABA does not influence paravascular movement but accelerates
280 interstitial fluid drainage.

281 **Glutamate-mediated inhibition of paravascular movement though the NMDA receptor** 282 **is pulsation-dependent**

283 Paravascular movement of CSF is reported to driven by arterial pulsation. Our previous
284 experiments showed that glutamate inhibits paravascular movement though the NMDA
285 receptor. Accordingly, we proposed that glutamate-mediated inhibition of paravascular
286 movement was pulsation-dependent.

287 To further examine this hypothesis, two-photon line scanning to visualize pulsation was
288 performed. Vascular pulsatility was measured at different levels of the cerebrovascular
289 tree, specifically, surface arteries, penetrating arteries, ascending veins and surface veins
290 (Fig. 4a). In the vehicle group, penetrating arteries and ascending veins showed
291 significantly higher pulsatility, compared with surface arteries and veins (Fig. 4b, c).
292 Upon administration of glutamate and its antagonist (i.c.v.), glutamate strongly inhibited
293 whereas APV promoted pulsatility among surface and penetrating arteries (Among
294 surface arteries, Glutamate vs. vehicle, $t=6.498$, $P<0.001$; APV vs. vehicle, $t=5.613$,
295 $P<0.001$; among penetrating arteries, Glutamate vs. vehicle, $t=6.437$, $P<0.001$; APV vs.

296 vehicle, $t=13.400$, $P<0.001$) (Fig. 4d). However, CNQX, GABA, and bicuculline had no
297 significant influence on pulsatility (Fig. 4e). We concluded from this experiment that
298 glutamate reduced pulsatility through the NMDA receptor while GABA did not influence
299 pulsatility.

300 Based on pulsatility measurements, we further reduced cerebral arterial pulsatility by
301 unilateral ligation of the internal carotid artery and injected the CSF tracer (i.c.v.), which
302 dissolves glutamate and the GABA agonist/antagonist. After arterial ligation, we observed
303 significantly reduced pulsatility in the vehicle group (Fig. 5b). Moreover, administration
304 of glutamate and GABA agonist/antagonist failed to induce significant changes in
305 pulsatility (Fig. 5c). Next, we quantified paravascular tracer movement 100 μm below the
306 cortex surface as described previously. The vehicle group showed markedly slower
307 paravascular movement after arterial ligation (Fig. 5a). Moreover, glutamate and APV
308 failed to either inhibit or facilitate paravascular movement (Glutamate vs. vehicle,
309 two-way ANOVA, for interaction factor, $P=0.3392$; APV vs. vehicle, two-way ANOVA,
310 for interaction factor, $P=0.2709$) (Fig. 5b). Meanwhile, administration of CNQX, GABA,
311 and bicuculline did not lead to significant changes in paravascular movement, compared
312 with the vehicle group (CNQX vs. vehicle, two-way ANOVA, for interaction factor,
313 $P=0.4799$; GABA vs. vehicle, two-way ANOVA, for interaction factor, $P=0.8563$;
314 bicuculline vs. vehicle, two-way ANOVA, for interaction factor, $P=0.9873$) (Fig. 5c). The
315 mean fluorescence intensity in parenchyma was additionally measured. The vehicle group
316 showed markedly slower tracer accumulation in parenchyma (Fig. 5d, e). However,
317 glutamate and APV groups displayed almost no CSF tracer penetration into parenchyma

318 (Glutamate vs. vehicle, two-way ANOVA, for interaction factor, $P=0.9991$; APV vs.
319 vehicle, two-way ANOVA, for interaction factor, $P=0.9693$) (Fig. 5d). Interestingly,
320 GABA and CNQX remarkably facilitated tracer influx (GABA vs. vehicle, two-way
321 ANOVA, for interaction factor, $P<0.001$; CNQX vs. vehicle, two-way ANOVA, for
322 interaction factor, $P<0.05$) (Fig. 5e).

323 These two experiments further confirmed that glutamate inhibits paravascular
324 movement through the NMDA receptor in a pulsation-dependent manner while GABA
325 has no significant influence on this process.

326 **The influence of Glutamate and GABA on tracer penetration into parenchyma is**
327 **AQP4-dependent.**

328 Paravascular movement of CSF and ISF is reported to be driven by arterial pulsation
329 and facilitated by an astroglial AQP4 water channel. Given that GABA and glutamate
330 inhibitor accelerated paravascular clearance to significant extents but had distinct effects on
331 dcLN drainage, we propose that AQP4 influences GABA and glutamate activities in
332 different ways.

333 Initially, AQP4 knockout mice were employed to determine whether GABA and glutamate
334 participate in AQP4-dependent clearance (Fig. 6a). Fluorescence in the paravascular space
335 was slowly increased in AQP4-null mice (Fig. 6b, c), accompanied by little influx into the
336 interstitium (Fig. 6d and e), indicating obvious blockage of para-arterial and interstitium
337 exchange. Glutamate did not influence draining of the tracer into the para-vascular space
338 while APV restored impaired paravascular movement in AQP4-null animals to a remarkable
339 extent (Glutamate vs. vehicle, two-way ANOVA, for interaction factor, $P=0.9875$; APV vs.

vehicle, two-way ANOVA, for interaction factor, $P < 0.05$) (Fig. 6b). CNQX, GABA, and bicuculline did not alter paravascular movement relative to the vehicle group (CNQX vs. vehicle, two-way ANOVA, for interaction factor, $P = 0.8122$; GABA vs. vehicle, two-way ANOVA, for interaction factor, $P = 0.6682$; Bicuculline vs. vehicle, two-way ANOVA, for interaction factor, $P = 0.8934$) (Fig. 6c). Glutamate almost impaired tracer influx with nearly no penetration of fluorescence into the parenchyma and CNQX failed to facilitate tracer penetration into the parenchyma. APV accelerated paravascular movement but did not promote tracer penetration into the parenchyma (Glutamate vs. vehicle, two-way ANOVA, for interaction factor, $P = 0.6300$; CNQX vs. vehicle, two-way ANOVA, for interaction factor, $P = 0.3194$; APV vs. vehicle, two-way ANOVA, for interaction factor, $P = 0.5353$) (Fig. 6d). Moreover, GABA and bicuculline did not alter the dynamic pattern of the fluorescent signal in parenchyma (GABA vs. vehicle, two-way ANOVA, for interaction factor, $P = 0.4193$; Bicuculline vs. vehicle, two-way ANOVA, for interaction factor, $P = 0.2425$;) (Fig. 6e).

Based on the collective data, we concluded that glutamate inhibited paravascular movement through the NMDA receptor, which was pulsation-dependent, and suppressed tracer penetration into the parenchyma and interstitial fluid drainage through the AMPA/kainate receptor which was AQP4-dependent. GABA did not appear to influence paravascular movement but accelerated glymphatic clearance in an AQP4-dependent manner.

Hypertension impairs glymphatic clearance of A β through reduction of arterial pulsation

Given that glymphatic clearance of interstitial solutes is highly dependent on vascular

pulsatility, we hypothesized that hypertension influences pulsatility, in turn, impairing lymphatic clearance. The Ang-II hypertension model was applied to test this theory. After chronic infusion of Ang-II for 28 days, mice displayed a marked increase in systolic blood pressure (SBP) and diastolic blood pressure (DBP), compared to vehicle-infused mice (Fig. 7g, h) (vehicle vs. hypertension, for SBP, $t=4.429$, $P<0.01$, for DBP, $t=5.326$, $P<0.001$). As expected, pulsatility was significantly reduced among surface/penetrating artery and surface/ascending veins (hypertension vs. vehicle, for surface artery, $t=5.485$, $P<0.001$; for penetrating artery, $t=4.263$, $P<0.01$; for surface vein, $t=12.320$, $P<0.001$; for ascending vein, $t=5.257$, $P<0.001$) (Fig. 7s) in parallel with glymphatic clearance impairment (hypertension vs. vehicle, for paravascular movement, two-way ANOVA, for interaction factor, $P<0.05$; for interstitial movement, two-way ANOVA, for interaction factor, $P<0.001$) (Fig. 7k-m) and obvious enlargement of Virchow-Robin Space (VRS) around the arteries (Fig. 7e). Moreover, we observed no significant differences in GFAP expression and AQP4 polarization (Fig. 7q, t, u). To examine cerebral A β deposition *in vivo*, we performed two-photon imaging of fibrillar amyloid plaques pre-labeled with the fluorescent fibrillar amyloid-binding dye, FSB, while simultaneously labeling cerebral vessels with Rhodamine B. *In vivo* imaging revealed significant A β deposition on cerebral vessel walls, and to some extent, in surrounding tissues (Fig. 7a-d). This finding was in keeping with the strong staining observed for A β 1–40 and weak staining for A β 1–42 (Fig. 7p, lower panel). The vehicle group displayed no cerebral FSB-positive amyloid plaques or A β staining. Moreover, severe cerebrovascular warp (Fig. 7f) and collagen deposition (vehicle vs. hypertension, for collagen staining, $P<0.05$) (Fig. 7p, r) were

384 detected in hypertensive animals, indicating that hypertension induces substantial changes
385 in vascular structure.

386 **Impairment of glymphatic clearance is associated with A β plaque deposition in** 387 **APP-PS1 mice**

388 Two-photon imaging revealed fibrillar amyloid plaques in the parenchyma, but no
389 evidence of CAA in 7–8-month-old APP-PS1 mice (Fig. 8a), consistent with strongly
390 positive A β 1–42 and negative A β 1–40 immunofluorescence staining in slices (Fig. 8b).
391 APP-PS1 mice showed significant impairment of glymphatic clearance as indicated by
392 normal paravascular movement but little influx into the interstitium (APP-PS1 vs. WT,
393 two-way ANOVA, for paravascular movement, for interaction factor, $P=0.2461$; for
394 interstitial movement, for interaction factor, $P<0.001$) (Fig. 8d and e), similar to that in
395 AQP4^{-/-} mice. Despite no distinct changes in pulsatility in APP-PS1 mice, increased
396 number of GFAP-positive astrocytes and impaired AQP4 polarization were observed,
397 compared with age-matched WT mice (APP-PS1 vs. WT, for GFAP expression, $t=7.527$,
398 $P<0.001$; for AQP4 polarization, among large vessels, $t=4.215$, $P<0.01$; among small
399 vessels, $t=0.9817$, $P=0.1372$) (Fig. 8f-i), indicating that inflammatory responses are
400 activated in the APP-PS1 group that influence the function of AQP4.

401 **Inhibition of glutamate and increase in GABA differentially affect soluble A β** 402 **binding to pre-existing plaques in APP-PS1 and hypertension models**

403 Previously, a method for microinjection of soluble A β was developed to assess A β
404 binding ability in the brain. Condello and colleagues²⁰ showed that A β 40–555 infusions
405 led to homogeneous binding to amyloid plaques, indicating a direct correlation between

soluble A β clearance and total binding quantity. We employed this method to examine the roles of glutamate and GABA in glymphatic clearance of soluble A β in FAD and hypertension models. Consistently, soluble A β microinjected into the parenchyma (Fig. 9b) diffused throughout the hemisphere and rapidly bound to FSB-labeled fibrillar plaques within 10 min (Fig. 9a). After the administration of neurotransmitter into the cisternal, GABA, APV and CNQX inhibited the binding process. Notably, we observed nearly no soluble A β binding to FSB-labeled plaques 10 min after administration of GABA, APV and CNQX in both FAD and hypertension models (two-way ANOVA, APP-PS1 vs. GABA, for interaction factor, $P < 0.001$; APP-PS1 vs. APV, for interaction factor, $P < 0.001$; APP-PS1 vs. CNQX, for interaction factor, $P < 0.001$; hypertension vs. GABA, for interaction factor, $P < 0.001$; hypertension vs. APV, for interaction factor, $P < 0.001$; hypertension vs. CNQX, for interaction factor, $P < 0.001$.), supporting the theory that inhibition of glutamate or activation of GABA induces rapid clearance of soluble A β (Fig. 9c and d).

Discussion

In this study, we have examined the roles of two main neurotransmitters, GABA and glutamate, in modulation of the glymphatic clearance of A β . Our experiments suggest that these two neurotransmitters exert opposite effects on glymphatic clearance through distinct pathways. GABA enhanced glymphatic clearance in an AQP4-dependent manner while glutamate suppressed clearance in a manner dependent on arterial pulsation and AQP4 though distinctive receptors.

Arterial pulsation is a main driving force for solute movement in the brain. For

example, cerebral arterial pulsation can drive paravascular CSF-interstitial fluid exchange¹⁵. In addition, arterial pulsation facilitates the exit of ISF solutes in the opposite direction to blood flow along cerebral arteries²¹. In the present study, GABA, APV and CNQX were shown to promote glymphatic clearance. Interestingly, however, APV accelerated paravascular movement, but not GABA or CNQX. In addition, APV promoted clearance of fluorescent tracers in the para-arterial space in AQP4-deleted mice, suggesting that enhancement of glymphatic clearance by APV was not dependent on AQP4 and was more likely acts on blood vessels. Indeed, glutamate is reported to constrict pial arterioles whereas inhibition of glutamate by an ionotropic receptor antagonist dilates arterioles¹⁸. Because APV and CNQX are both ionotropic receptor antagonists, the issue of why APV but not CNQX promotes paravascular movement of fluorescent tracers was unclear. One possible explanation was that endothelial cells expressed NMDA but not AMPA/kainate. Activation of endothelial NMDA could increase Ca²⁺ influx, which, in turn, reduced pulsatility.

The involvement of AQP4 in solute movement in the brain has been well established^{4,6,22}. Consistent with previous observations, significant reduction of interstitial clearance was evident in AQP4^{-/-} mice. CNQX-mediated interstitial clearance was proposed to rely heavily on AQP4, in view of the finding that increase in glymphatic clearance was abolished in AQP4^{-/-} mice. Although astrocytes expressed ionotropic receptors, including AMPA/kainate and NMDA, only AMPA was widely expressed in many brain regions and has been confirmed to act as a fully functional receptor. The AMPA receptor functions through mediation of calcium and sodium influx in astrocytic

processes²³. However, the mechanism underlying AQP4 involvement in CNQX-mediated clearance is currently unclear. This process may be mediated indirectly because no direct interactions have been detected between AMPA and AQP4. Glutamate exerts depolarizing effects on astrocytes through ion influx. Upon depolarization of astrocytes, molecules such as AQP4 that normally localize in endfoot membranes are redistributed to non-endfoot membranes, thereby compromising AQP4 function²⁴. By inhibiting ion influx, CNQX may therefore counteract glutamate-mediated astroglial depolarization to maintain AQP4 in perivascular endfeet.

Unlike glutamate, GABA had no effect on pulsation. Inhibition of GABA by the GABA_A receptor antagonist, bicuculline, led to a considerable decrease in glymphatic clearance. GABA enhanced glymphatic clearance in wild-type but not AQP4^{-/-} mice, indicating that GABA-mediated clearance was AQP4-dependent. Earlier studies suggested that the GABA receptor co-localizes with AQP4^{16,25}. Further research is warranted to ascertain whether direct interactions between GABA and AQP4 promote glymphatic clearance.

Failure to clear A β deposits is linked to a number of A β -associated diseases, such as FAD and hypertension^{8,26,27}. However, brain distribution of A β is variable among different diseases²⁸. For example, A β mainly localizes in brain parenchyma in AD but is deposited along arteries in hypertension^{1,3,19}. These findings highlighted the complexity of the mechanisms underlying failure of A β deposit clearance. Accordingly, we further explored the potential roles of glutamate and GABA neurotransmitters in clearance of A β deposits in FAD and hypertension.

Transgenic APP/PS1 mice, a commonly used model of AD, can generate substantial amounts of β -amyloid. Consistent with previous observations, significant levels of $A\beta$ were deposited in the parenchyma in APP/PS1 mice. Similar to AQP4^{-/-} mice, APP-PS1 mice exhibited marked impairment of glymphatic clearance, specifically, an increase in paravascular fluorescence but little influx into the interstitium. While no significant alterations in pulsatility were observed, AQP4 polarization was remarkably impaired and GFAP expression increased in APP-PS1 mice. These findings may explain the impaired interstitium clearance in APP-PS1 mice and shed further light on the mechanisms underlying $A\beta$ deposition in Alzheimer's disease patients.

Hypertension is a strong risk factor for age-related dementia that is characterized by both beta amyloid ($A\beta$) deposition and vascular dysfunction³. Consistently, $A\beta$ deposition was evident in Ang-II induced hypertensive animals as well as glymphatic clearance impairment. In parallel, pulsatility was significantly decreased whereas the GFAP and AQP4 levels remained unchanged. Thus, the hypertension-induced decrease in arterial pulsation was possibly responsible for $A\beta$ deposition. Interestingly, GABA, APV and CNQX promoted rapid clearance of soluble $A\beta$ in both FAD and hypertension models, supporting the theory that modulation of neurotransmitters may serve as an effective therapeutic strategy for removal of $A\beta$ deposits in the brain. However, it should be noted that CNQX was less effective than GABA in soluble $A\beta$ clearance in the hypertension model, which may be attributable to differences in their mechanisms of action. Unlike GABA, CNQX does not interact directly with AQP4.

Our study had a number of limitations that need to be addressed. Previous reports

494 suggest that A β can be removed from the brain via various clearance systems (most
495 importantly, the blood–brain barrier (BBB)). As the glymphatic pathway flushes A β
496 towards the perivascular space, which may influence clearance through BBB^{1,15,28}, the
497 issue of whether GABA and glutamate modulate BBB permeability or act via interactions
498 with receptors for advanced glycation end products (RAGE) or low density
499 receptor-related protein-1 (LRP1) requires further investigation.

500 In conclusion, the neurotransmitters glutamate and GABA exerted distinct
501 modulatory effects on glymphatic clearance. The collective data from our study presented
502 novel insights into the mechanisms underlying A β drainage that may be effectively
503 applied for treatment of Alzheimer’s disease and hypertension.

504

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593

594 **Author contributions**

595 Yi-wei Feng, Qun Zhang, Xiao-fei He, Dong-xu Liu, Dan Wu, Ge Li, Cheng Wu and
596 Feng-yin Liang performed the experiments. Yi-wei Feng, Qun Zhang and Xiao-fei He
597 drafted the manuscript. Guang-qing Xu, Yue Lan and Zhong Pei conceived and designed
598 the research. Zhong Pei and Guang-qing Xu edited and revised the manuscript.
599 Guang-qing Xu, Yue Lan and Zhong Pei approved the final version of the manuscript.

600

601 **Competing financial interests**

602 All authors declare no conflict of financial interests.

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608 **Figure legends**

609 **Fig. 1.** *In vivo* two-photon imaging of CSF tracer clearance through the glymphatic
610 system. **(a–d)** *In vivo* imaging of CSF tracer movement along the surface **(a)** and
611 penetrating arteries **(b)**. Cerebral vasculature was visualized with Rhodamine B (red).
612 CSF tracer (green) moved along the paravascular space of arteries but not veins (A:
613 surface artery; V: surface vein; PA: penetrating artery; PV: penetrating vein; Dark blue
614 circles, arterioles; light blue circles, veins). Magnification of penetrating vessels **(c)** and
615 **(d)**. **(e)** Schematic diagram of the imaging setup and intra-cisternal injection of the CSF
616 tracer. **(f)** Schematic depiction of the glymphatic system. In this brain-wide pathway, CSF
617 enters the brain along para-arterial routes and is cleared along paravenous routes. **(g–h)**
618 3D reconstruction of distribution of the CSF tracer in brain at 10 and 30 min. The tracer

619 moved rapidly into the parenchyma over time.

620

621 **Fig. 2.** GABA promotes while glutamate suppresses glymphatic clearance. **(a)** *In vivo*
 622 two-photon imaging of tracer clearance through the para-vascular glymphatic pathway
 623 after intra-cisternal injection of GABA/glutamate and the respective antagonists. **(b, c)**
 624 Quantification of CSF tracer influx into the surrounding parenchyma via 3D
 625 reconstruction and paravascular CSF tracer clearance at 100 μ m below the cortical surface
 626 **(d, e)**. Glutamate strongly inhibited whereas APV accelerated paravascular movement. No
 627 significant changes in paravascular movement were observed in mice receiving GABA,
 628 bicuculline and CNQX. Glutamate inhibited penetration of the tracer into the interstitium.
 629 APV did not influence the dynamic pattern of tracer penetration while CNQX
 630 significantly promoted tracer influx. GABA promoted whereas bicuculline inhibited tracer
 631 influx to a significant extent (n=6 mice per group). Present and following data are
 632 presented as means $\pm$ S.D. * P <0.05, ** P <0.01 and *** P <0.001.

633

634 **Fig. 3.** Glutamate inhibits while GABA accelerates ISF clearance. **(a)** *In vivo* two-photon
 635 imaging of tracer clearance in the interstitium after injection of tracer containing
 636 glutamate/GABA agonist/antagonist. **(b, c)** Quantification of mean tracer fluorescent
 637 changes in mice receiving glutamate/GABA agonist/antagonist. Glutamate significantly
 638 inhibited whereas CNQX accelerated tracer clearance. ISF clearance was promoted by
 639 GABA and conversely inhibited by bicuculline. **(e)** Representative fluorescent images of
 640 Evans blue staining indicative of drainage into dcLNs after injection of GABA/glutamate

641 and the respective antagonists. dcLNs in each group are magnified by 50×. **(d)**
 642 Quantification of dcLN fluorescence in each group. Glutamate significantly inhibited
 643 whereas CNQX and APV promoted drainage. Moreover, drainage was enhanced by
 644 GABA and inhibited by bicuculline (n=6 mice per group).

645
 646 **Fig. 4.** Measurement of vascular pulsatility in mouse cortex. **(a)** Cortical surface arteries
 647 and veins and penetrating arteries and veins. X–T line scans (red lines) were generated
 648 orthogonal to the vessel axis. **(b)** Vascular pulsatility was defined as absolute changes in
 649 fluorescence values over 4000 ms with intervals of 0.5 ms. **(c)** Vascular pulsatility in
 650 cortical surface arteries (SA), penetrating arteries (PA), ascending veins (AV) and surface
 651 veins (SV). Pulsatility was greatest in penetrating arteries and veins, compared with
 652 surface vessels. **(d-e)** Analysis of pulsatility in different groups. Glutamate strongly
 653 inhibited whereas APV significantly accelerated pulsatility among surface and penetrating
 654 arteries. GABA and bicuculline did not affect pulsatility. **(f-g)** Analysis of pulsatility in
 655 different groups after deep cerebral artery ligation. Artery ligation abolished pulsatility
 656 changes by glutamate and APV (n=8-9 vessels per group).

657
 658 **Fig. 5.** Glutamate inhibits paravascular movement through the NMDA receptor, which is
 659 pulsation-dependent. **(a)** *In vivo* two-photon imaging of tracer clearance through the
 660 para-vascular glymphatic pathway after intra-cisternal injection of GABA/glutamate and
 661 the respective antagonists. Deep cervical artery was unilaterally ligated during imaging.
 662 **(b, c)** Quantification of CSF tracer influx into the surrounding parenchyma via 3D

663 reconstruction and paravascular CSF tracer clearance at 100 μ m below the cortical surface
 664 **(d, e)**. After ligation, no significant differences were observed in mice receiving glutamate
 665 and GABA agonists/antagonists with regard to paravascular movement. CNQX and
 666 GABA significantly accelerated tracer penetration into the interstitium (n=5-6 mice per
 667 group)
 668

669 **Fig. 6.** Effects of Glutamate and GABA on tracer penetration into parenchyma are
 670 AQP4-dependent. **(a)** *In vivo* two-photon imaging of tracer clearance through the
 671 glymphatic pathway after intracisternal injection of GABA/glutamate and the respective
 672 antagonists in AQP4^{-/-} mice. **(b, c)** Quantification of CSF tracer influx into the
 673 surrounding parenchyma via 3D reconstruction and paravascular CSF tracer clearance at
 674 100 μ m below the cortical surface **(d, e)**. APV strongly accelerated paravascular movement
 675 while no significant changes were observed in mice receiving glutamate and CNQX.
 676 GABA and bicuculline did not affect paravascular movement. No significant changes in
 677 tracer penetration into the interstitium were observed among the experimental groups,
 678 compared with the vehicle group (n=6 mice per group).
 679

680 **Fig. 7.** Hypertension induces brain A β deposition and glymphatic clearance impairment.
 681 **(a-d)** *In vivo* imaging of A β deposition in the cerebral cortex. A β deposition (FSB, green)
 682 was distinct along the vessels. **(e)** Enlarged VRS were observed. **(f)** 3D reconstruction of
 683 the vasculature in hypertension mice. The warping vessel is magnified on the right panel.
 684 **(g-j)** Ang-II evoked a significant increase in systolic blood pressure (SBP) and diastolic

685 blood pressure (DBP) with no changes in heart rates (HR) and body weight **(k-m)**.
 686 Glymphatic clearance impairment was evident in hypertension models. **(n-o)** Arterial
 687 diameters remained unchanged in hypertension models while vascular pulsatility was
 688 severely reduced (n=8-9 vessels per group) **(s)**. **(q)** Representative images of GFAP
 689 expression and AQP4 polarization in the cortex. No distinctive changes in AQP4
 690 polarization **(t)** and GFAP expression **(u)** were observed in the hypertension model. **(p,**
 691 **upper panel)** Representative images of smooth muscle actin (SMA) and collagen
 692 expression in the cortex. No significant changes in SMA expression **(r)** and greater
 693 deposition of collagen **(r)** in vascular walls were observed in hypertension models. **(p,**
 694 **lower panel)** Immunology of A β 1–40 and A β 1–42 in hypertension model mice.
 695 Significant deposition of A β 1–40, but not A β 1–42, in vessels was observed. **(p, lower**
 696 **panel)** Co-labeling of collagen and A β 1–40 in hypertension. A β 1–40 co-localized with
 697 collagen (n=5-6 mice per group).

698

699 **Fig. 8.** Impairment of glymphatic clearance and deposition of A β plaques in APP-PS1
 700 mice. **(a)** *In vivo* imaging of A β deposition in the cerebral cortex (FSB: green). A β
 701 deposition was evident in the parenchyma with no distinct CAA. **(b)** Immunology of A β
 702 1–40 and A β 1–42 in APP-PS1. Significant numbers of A β 1–42-labeled amyloid plaques
 703 were observed in the parenchyma, but no marked deposition of A β 1–40. **(c)**
 704 Representative images of paravascular CSF tracer clearance at 100 μ m below the cortical
 705 surface in APP-PS1 indicating severe impairment in penetration of fluorescence tracer **(e)**
 706 while no changes in paravascular movement was observed **(d)**. **(h-i)** Expression of AQP4

707 and GFAP in cortex and hippocampus. Compared with WT control mice, APP-PS1 mice
708 displayed significant decrease in AQP4 polarization and exhibited a marked increase in
709 GFAP expression in the cortex (n=6 mice per group). No significant pulsatility changes
710 were observed between APP-PS1 and WT control mice (**f**) (n=7-8 vessels per group).

711

712 **Fig. 9.** *In vivo* brain imaging of A β 40–555 binding to existing A β deposits. (**a**) High
713 magnification of two-photon images of homogeneous binding of A β 40–555 (green) to
714 existing amyloid deposits pre-labeled with FSB (blue). (**b**) Schematic diagram of injection
715 of A β 40–555 into the cortex under a microscope. Quantification of co-labeling of A β 40–555
716 with pre-existing amyloid deposits following injection in hypertension (**c**) and APP-PS1 (**d**)
717 mice. A β 40–555 bound rapidly to pre-existing amyloid deposits and reached a plateau
718 within 5 min (green curve). In the hypertension mouse model (**c**), APV, CNQX and GABA
719 inhibited binding. However, the CNQX-treated group showed greater binding than the
720 GABA-treated and APV-treated group at 540, 560 and 600 s. In APP-PS1 mice (**d**), APV,
721 CNQX and GABA inhibited binding with no significant differences (n=5-6 mice per group).
722

Figure 1.

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Figure 6.

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a Figure 8.

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Figure 9.

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