

Supplementary Material

Glutamate Gradually Elevates $[Zn^{2+}]_i$ via the CaM–CaMKII–NOS Cascade in
Primary Cultured Rat Embryonic Cortical Neurons

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ionotropic glutamate receptor, nitric oxide synthase, Zn^{2+}**

22 Statistical report of each figure:

23 Fig. 2b F value = 19.69799, N = 9, t(df) = 4 for each comparison

24 P value for each set of comparison

	Mock	Glu	AMPA	NMDA
Glu	1.05596E-9			
AMPA	5.37055E-5	0.04191		
NMDA	1.5233E-4	0.01826	0.99731	
AMPA+NMDA	1.60593E-7	0.82198	0.35352	0.20312

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26 Fig 2d:

27 F value = 12.17185, N =4, t(df) = 6 for each comparison

28 p value for each set of comparison

	Mock	Glu	AP5	AP5+ Glu	DNQX	DNQX +Glu
Glu	1.96726E-5					
AP5	1	2.03146E-5				
AP5+Glu	0.16604	0.00729	0.17051			
DNQX	1	2.3255E-5	1	0.19034		
DNQX+Glu	0.99728	6.37349E-5	0.99813	0.39205	0.99908	
AP5+DNQX +Glu	0.92424	2.16934E-4	0.92867	0.71744	0.94547	0.9976

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30 Fig.2f

31 F value = 29.37139, N =4, t(df) = 4

	Mock	Glu	DHPG	4R- APDC
Glu	2.86737E-6			
DHPG	1	3.00123E-6		
4R-APDC	0.99994	3.45352E-6	0.99998	
L-AP4	1	3.12124E-6	1	0.99999

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36 Fig 3b
 37 F value = 48.58848, N = 4, t(df) = 6 for each comparison
 38 p value for each set of comparison

	Mock	Glu	W7	W7+ Glu	KN62	KN62 +Glu
Glu	638192E-8					
W7	1	6.45505E-8				
W7+Glu	0.33914	5.13284E-8	0.41823			
KN62	1	6.35491E-8	0.99999	0.31208		
KN62+Glu	0.00145	4.1167E-7	0.00205	0.16229	0.00128	
W7+KN62 +Glu	0.9149	6.97171E-8	0.95524	0.92869	0.89556	0.01829

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 40 Fig 3d
 41 F value = 15.08868, N = 5, t(df) = 6 for each comparison
 42 p value for each set of comparison

	Mock	Glu	NIO	NIO+ Glu	NPA	NPA +Glu
Glu	1.63542E-6					
NIO	1	1.29203E-6				
NIO+Glu	0.01032	0.03415	0.00813			
NPA	1	1.34342E-6	1	0.00846		
NPA+Glu	0.014	0.02558	0.01107	1	0.01151	
NIO+NPA +Glu	0.043	0.00802	0.03457	0.99643	0.03586	0.99902

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 44 Fig 4b
 45 F value = 8.77412, N = 4, t(df) = 4 for each comparison
 46 P value for each set of comparison

	Mock	Glu	AMPA	NMDA
Glu	0.00666			
AMPA	0.00319	0.99509		
NMDA	0.03903	0.88559	0.69655	
AMPA+NMDA	5.54591E-4	0.69123	0.88196	0.22328

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49 Fig 4d

50 F value = 6.37236, N =4, t(df) = 6 for each comparison

51 *p* value for each set of comparison

	Mock	Glu	W7	W7+ Glu	KN62	KN62 +Glu
Glu	0.00379					
W7	0.98381	6.26408E-4				
W7+Glu	1	0.00473	0.97107			
KN62	1	0.00511	0.96541	1		
KN62+Glu	0.90869	0.04685	0.48671	0.93816	0.94664	
W7+KN62 +Glu	0.9955	9.24287E-4	1	0.99017	0.98753	0.58976

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53 Fig 4f

54 F value = 13.12325, N =4, t(df) = 6 for each comparison

55 *p* value for each set of comparison

	Mock	Glu	NIO	NIO+ Glu	NPA	NPA +Glu
Glu	0.0001					
NIO	0.94496	0.0001				
NIO+Glu	0.1261	0.0018	0.58113			
NPA	0.99806	0.0001	0.9988	0.31023		
NPA+Glu	0.02047	0.01343	0.15957	0.97085	0.06273	
NIO+NPA+Glu	0.46495	2.77101E-4	0.95968	0.97996	0.77821	0.61928

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57 Fig 5b

58 F value = 50.08772, N =3, t(df) = 2 for each comparison

59 *p* value for each set of comparison

	Mock	Glu
Glu	6.8125E-4	
High K	1.95239E-4	0.21789

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65 Fig 5d

66 F value = 4.71057, N =7, t(df) = 2 for each comparison

67 *p* value for each set of comparison

	Mock	Glu
Glu	0.03202	
High K	0.96696	0.05266

68 Fig 5f

69 F value = 10.70992, N =4, t(df) = 2 for each comparison

70 *p* value for each set of comparison

	Mock	Glu
Glu	0.00412	
High K	0.53162	0.02148

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72 Fig 6b

73 **For the nNOS^{S847}**

74 F value = 0.82241, N =4, t(df) = 2 for each comparison

75 *p* value for each set of comparison

	Mock	Glu 15 min
Glu 15 min	0.61083	
Glu 30 min	0.47696	0.96994

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77 **For the nNOS^{S1417}**

78 F value = 5.23375, N =4, t(df) = 2 for each comparison

79 *p* value for each set of comparison

	Mock	Glu 15 min
Glu 15 min	0.04066	
Glu 30 min	0.96251	0.06136

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81 Fig 7b

82 F value = 3.87514, N =6, t(df) = 3 for each comparison

83 *p* value for each set of comparison

	Mock	Glu	TPEN
Glu	0.03246		
TPEN	0.82105	0.17516	
TPEN+Glu	0.9993	0.04204	0.87792

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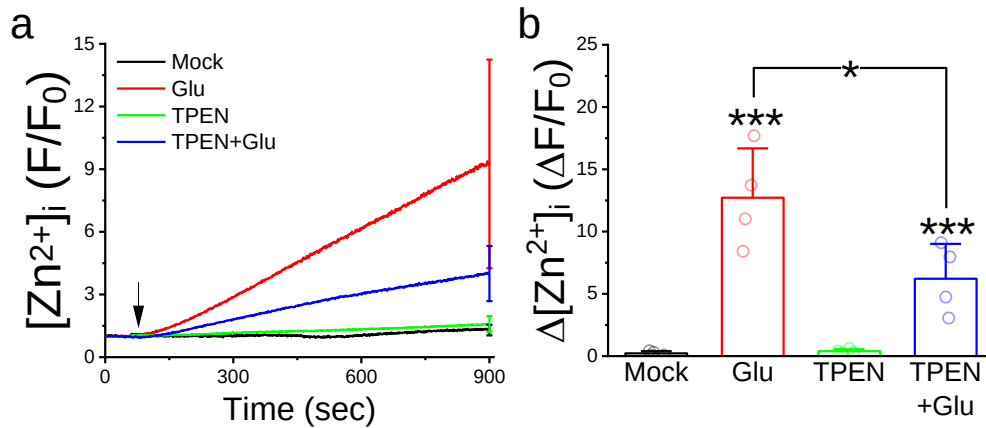


Fig. S1. TPEN suppresses the glutamate-induced elevation of $[Zn^{2+}]_i$ in cultured neurons. We loaded the neurons with FluoZin-3 and added glutamate (100 μ M) into the recording chamber with or without TPEN (0.4 μ M). (a) The representative normalized average of fluorescence traces from one batch of the neurons. The arrow indicates the application of glutamate. (b) The average changes in the normalized fluorescence intensities. The data presented were Mean $\pm$ SD (N = 4). *: $p < 0.05$, ***: $p < 0.001$ when analyzed with one-way ANOVA with Tukey *post hoc* test.

F value = 23.72309, N = 4, t(df) = 3 for each comparison

p value for each set of comparison

	Mock	Glu	TPEN
Glu	5.04039E-5		
TPEN	0.99958	5.80293E-5	
TPEN+Glu	0.02018	0.01199	0.0242

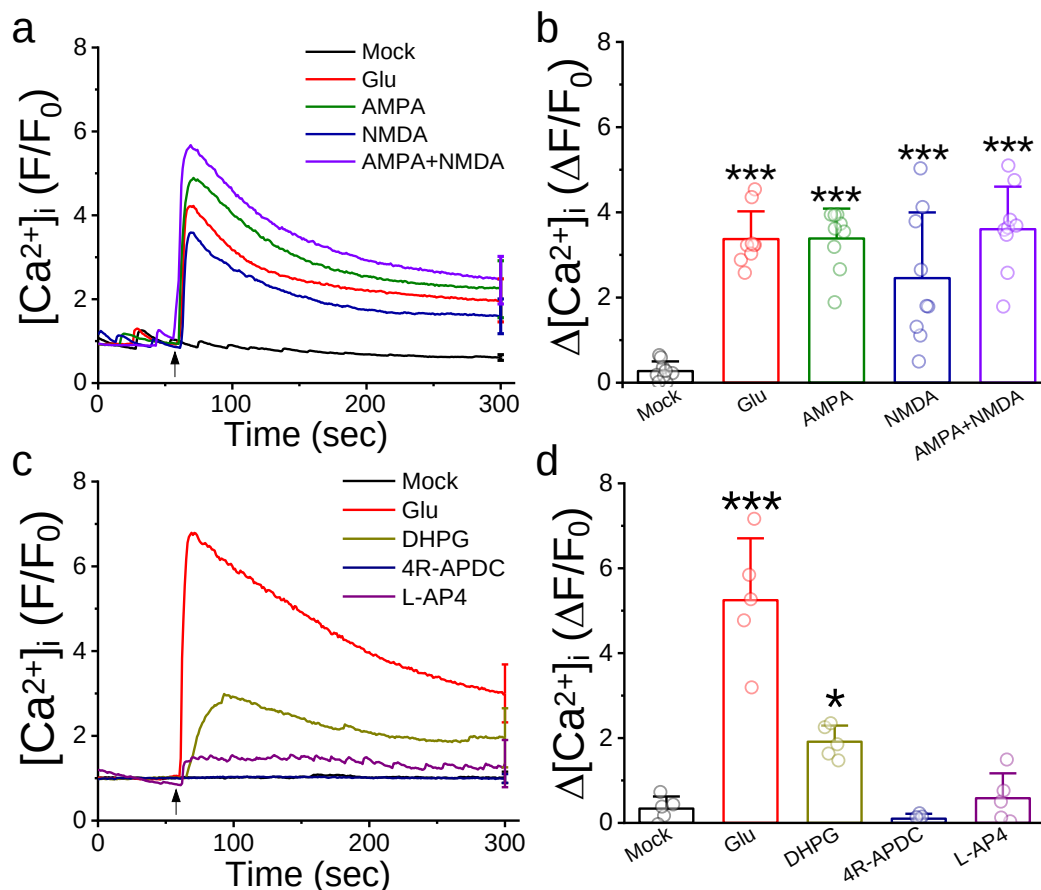


Fig.S2. Agonists of glutamate receptors elevate the $[Ca^{2+}]_i$ in cultured neurons. We loaded neurons with Fluo2, a Ca^{2+} -sensitive fluorescence dye, and monitored the $[Ca^{2+}]_i$ represented by normalized fluorescence intensities (F/F_0). Neurons were stimulated with glutamate, AMPA, NMDA, AMPA+NMDA, DHPG, APDC, and L-AP4 (100 μ M each). (a. & c) Representative averaged F/F_0 from one batch of neurons. SD was displayed at the last data point, and the arrows indicate the chemicals used. (b & d) Changes in F/F_0 ($\Delta F/F_0$). * and ***: Student's t -test with $p < 0.05$ and 0.001, respectively, compared with the Mock group.

Fig. S2b F value = 19.81578, N = 9, t(df) = 4 for each comparison
P value for each set of comparison

	Mock	Glu	AMPA	NMDA
Glu	1.79901E-7			
AMPA	1.66017E-7	1		
NMDA	1.23617E-4	0.24594	0.23393	
AMPA+NMDA	2.89272E-8	0.98397	0.98698	0.08684

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111 Fig.S2d

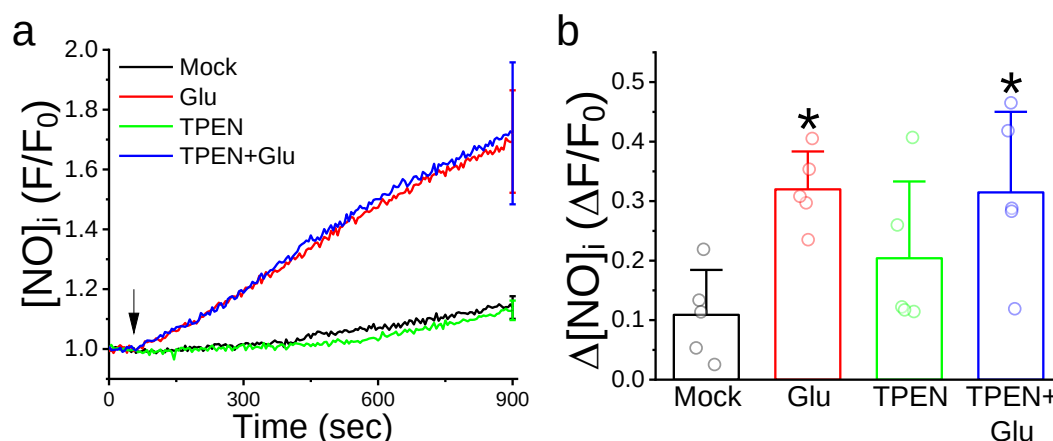
112 F value = 42.2731, N =5, t(df) = 4

	Mock	Glu	DHPG	4R-APDC
Glu	0.0001			
DHPG	0.0218	0.0001		
4R-APDC	0.98514	0.0001	0.00706	
L-AP4	0.98348	0.0001	0.06539	0.83416

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117 **Fig. S3. TPEN did not inhibit glutamate-induced NO generation in cultured**
 118 **neurons.** We loaded neurons with NO indicator, DAF-FM (5 μ M), and stimulated them
 119 with glutamate (100 μ M) in the presence or absence of TPEN (0.4 μ M). (a)
 120 Representative fluorescence traces from one batch of neurons. The data presented were
 121 Mean with SD displayed at the last time point. The arrow indicates the application of
 122 glutamate. (b) The average glutamate-induced changes ($\Delta F/F_0$). Data were reported as
 123 the Means $\pm$ SD and analyzed using one-way ANOVA with the Tukey *post hoc* test (*:
 124 $p < 0.05$ compared with the Mock group or as indicated).

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127 F value = 4.52549, N =5, t(df) = 3 for each comparison
 128 p value for each set of comparison

	Mock	Glu	TPEN
Glu	0.02834		
TPEN	0.50444	0.34112	
TPEN+Glu	0.03297	0.99983	0.3787

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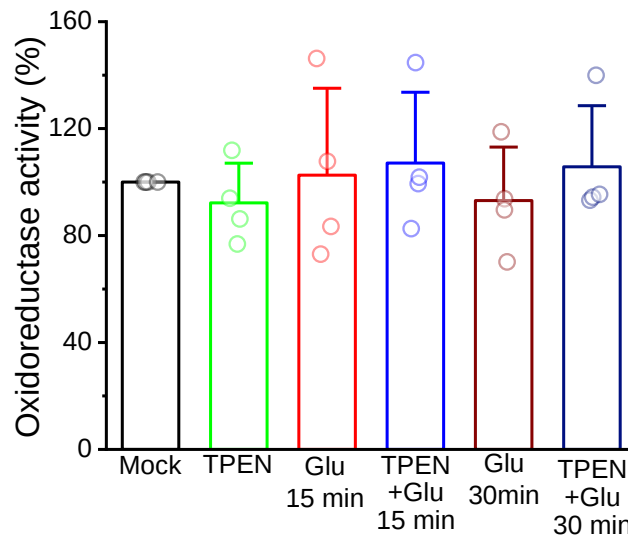
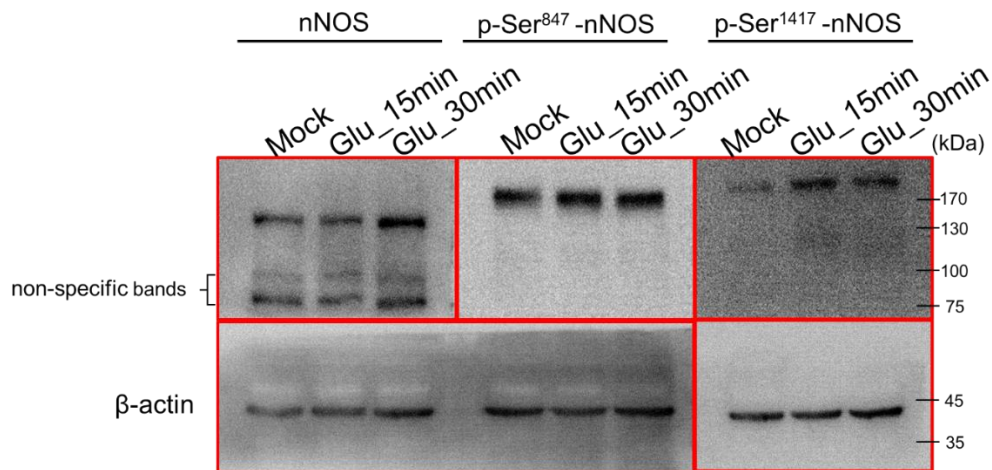


Fig. S4. Glutamate does not affect the function of mitochondria in 30 min in cultured cortical neurons. We stimulated the neurons with glutamate (100 μ M) for 15 and 30 min, both in the absence and presence of TPEN (0.4 μ M), with subsequent monitoring of oxidoreductase activity. The data were Mean $\pm$ SD derived from 4 independent experimental sets. Significance was assessed via one-way ANOVA, followed by Tukey *post hoc* test, with statistical significance determined at $p < 0.05$.

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142 **Fig S5 Original blot images shown in Fig. 6.** Cell lysates isolated from cultured
 143 neurons treated with glutamate (100 μM) for 0 (Mock), 15, and 30 min were loaded
 144 into the wells of a polyacrylamide gel as indicated. After transblotting, the membrane
 145 was cut into 5 pieces as indicated by the dashed lines for staining with different
 146 antibodies.

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