Dynamic regulation of $GABA_BRs$ by neural activity and phosphatase signaling

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Introduction

Activation of metabotropic GABA type-B receptors ($GABA_BRs$) mediates slow, sustained neural inhibition and critically limits the damage caused by excess excitation, such as occurs during seizure or stroke.

$GABA_BRs$ comprise heterodimers of R1 (GABA-binding) and R2 ($G_{i/o}$-binding) subunits. They act presynaptically to block neurotransmitter release via inhibition of voltage-gated Ca^{2+} channels and postsynaptically to hyperpolarize neuronal membrane potential via activation of inwardly rectifying K^+ (GIRK) channels.

Our lab and others have previously identified a bi-phasic regulation of $GABA_BRs$ by activation of NMDA-type glutamate receptors (NMDARs) and increases in intracellular Ca^{2+}. Initially, 5'AMP-dependent protein kinase (AMPK)-mediated phosphorylation of Ser-783 of the R2 subunit increases, in turn stabilizing $GABA_BRs$ at the plasma membrane, but during prolonged or excitotoxic stimulation, protein phosphatase 2A (PP2A)-dependent dephosphorylation of Ser-783 becomes predominant and $GABA_BRs$ are endocytosed and degraded.

This NMDAR-mediated impairment of $GABA_BR$ signaling may exacerbate excitotoxic neuronal death, and has also been observed in response to chronic stress and psychostimulant administration. **Therefore, preventing R2 Ser-783 dephosphorylation may represent a promising therapeutic intervention for multiple neurological and psychiatric disorders.**

Here, we explore this hypothesis by examining whether **1)** models of excitotoxicity alter R2 levels and phosphorylation in hippocampal slices; **2)** exposure to a kainate-induced seizure model alters hippocampal R2 levels and phosphorylation in vivo; and **3)** knock-in mice bearing a putatively phospho-mimetic mutation of Ser-783 (Ser to Asp; S783D) exhibit augmented $GABA_BR$ signaling. In a parallel series of experiments, we combined affinity purification and mass spectrometry approaches to **4)** determine the specific region of the R1 C-terminus that interacts with PP2A and **5)** identify specific R1-interacting phosphatase isoforms.

Model

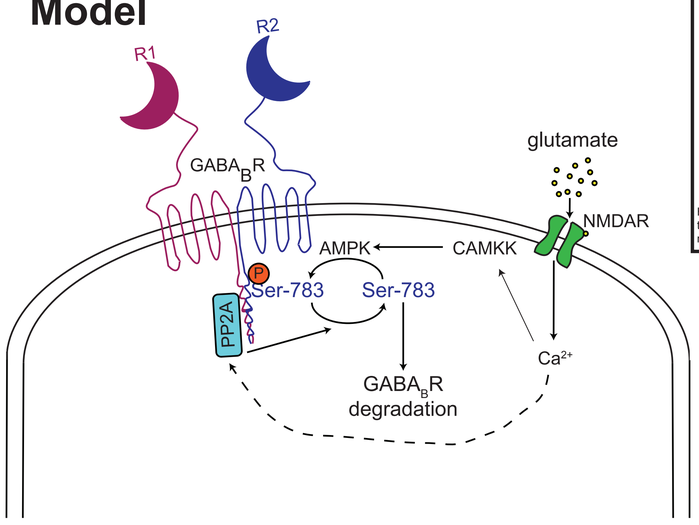
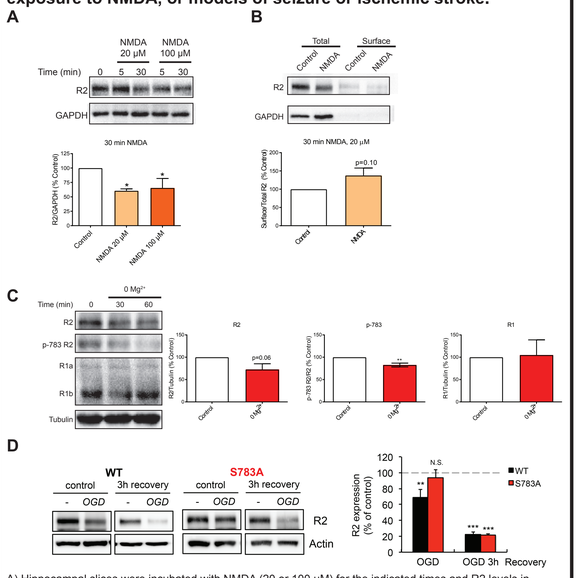
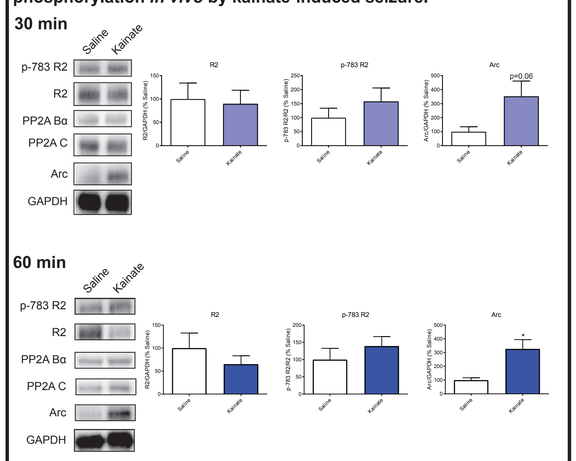


Figure 1: Decreased R2 levels in hippocampal slices in response to exposure to NMDA, or models of seizure or ischemic stroke.



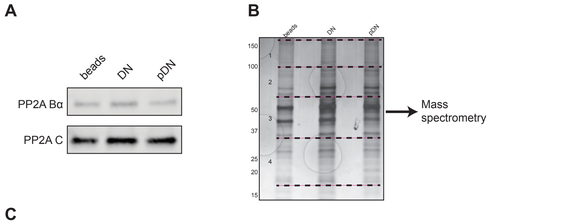
A) Hippocampal slices were incubated with NMDA (20 or 100 µM) for the indicated times and R2 levels in lysates were detected by immunoblot (n=7). B) Hippocampal slices were incubated with NMDA (20 µM) for 30 min, then subjected to surface biotinylation. Lysates were incubated overnight with Neutravidin beads to purify biotinylated proteins and total lysates and eluates were immunoblotted (n=5). C) Hippocampal slices were exposed to Mg2+-free ACSF (which induces seizure-like events) and R2, p-783 R2, and R1 levels were detected by immunoblot. Summary data are for 30 min exposures (n=5). D) Hippocampal slices from WT and S783A mice were exposed to OGD for 30 min, followed by 3 hr perfusion with normal ACSF. R2 levels in lysates were assessed by immunoblot (n= 4-6).

Figure 2: Regulation of hippocampal R2 levels and phosphorylation *in vivo* by kainate-induced seizure.



Mice were injected with kainate (20 mg/kg, i.p.) and sacrificed by focal magnetic irradiation 30 (top panel) or 60 (bottom panel) min later. Whole hippocampi were dissected and lysates were immunoblotted to assess the levels of R2, p-783 R2, Arc, and PP2A Bα and C subunits (n=5 for all groups).

Figure 5: An unbiased proteomic approach to identify R1-binding phosphatase subunit proteins.

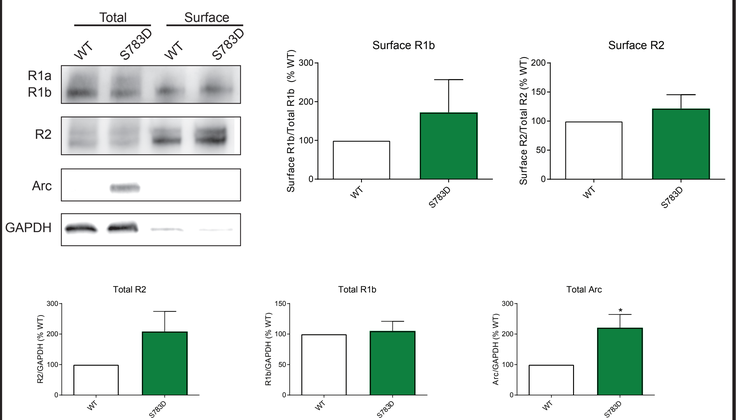


C

Sample	Score	Expectation	Protein ID	Protein Name
DN 2	17957	0	TBA1B_MOUSE	Tubulin alpha-1B chain
pDN 2	16073	0	TBA1B_MOUSE	Tubulin alpha-1B chain
pDN 1a	1068	2.50E-103	DYN1_MOUSE	Dynamin-1
pDN 2	837	3.30E-80	PP1R7_MOUSE	Protein phosphatase 1 regulatory subunit 7
DN 2	634	6.00E-60	PP1R7_MOUSE	Protein phosphatase 1 regulatory subunit 7
pDN 2	502	1.10E-46	2ABA_MOUSE	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha
DN 2	438	2.60E-40	DNJA1_MOUSE	DnaJ homolog subfamily A member 1
DN 2	424	6.50E-39	PRS7_MOUSE	26S protease regulatory subunit 7
DN 2	392	9.40E-36	ARP3_MOUSE	Actin-related protein 3
DN 2	372	1.00E-33	DNJA2_MOUSE	DnaJ homolog subfamily A member 2
pDN 2	351	1.40E-31	FNTA_MOUSE	Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha
DN 2	339	2.20E-30	QCR1_MOUSE	Cytochrome b-c1 complex subunit 1, mitochondrial
pDN 2	331	1.30E-29	ARP3_MOUSE	Actin-related protein 3
DN 2	328	2.90E-29	CSN2_MOUSE	COP9 signalosome complex subunit 2
pDN 2	323	8.00E-29	PLAK_MOUSE	Junction plakoglobin
DN 2	318	2.60E-28	ACOT9_MOUSE	Acyl-coenzyme A thioesterase 9, mitochondrial
pDN 2	303	8.60E-27	ANXA2_MOUSE	Annexin A2
pDN 2	300	1.80E-26	DHE3_MOUSE	Glutamate dehydrogenase 1, mitochondrial
DN 2	298	2.60E-26	FNTA_MOUSE	Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha
pDN 2	294	6.90E-26	CSPG2_MOUSE	Versican core protein

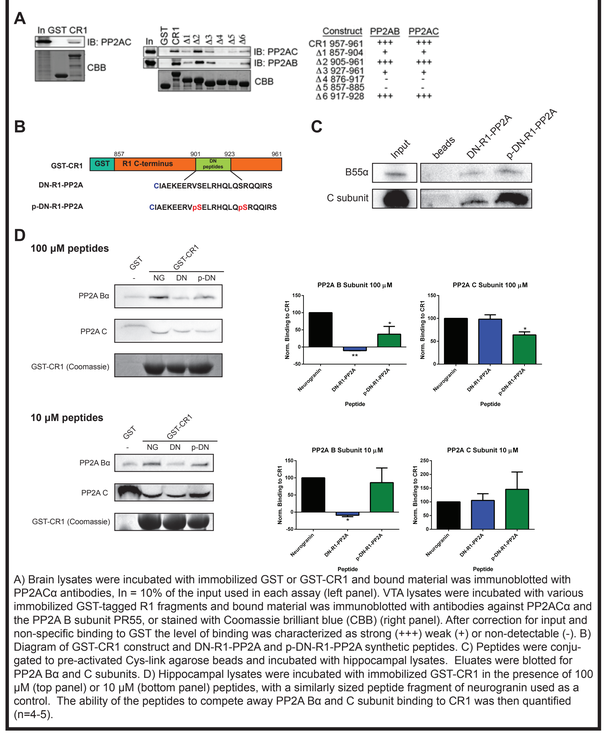
A) Pull-downs were performed on hippocampal lysates using peptide-conjugated beads as described above. B) Eluted proteins were run on SDS-PAGE and gels were silver stained and submitted for mass spectrometry. C) Table of the top (sorted by expectation score) identified proteins that specifically interact with either peptide and not beads alone (summary of 2 independent experiments).

Figure 3: No change in surface $GABA_BRs$, but increased Arc levels, in hippocampal slices from S783D mice.



Hippocampal slices from WT or S783D mice were subjected to surface biotinylation, and lysates were incubated with Neutravidin beads to isolate biotinylated proteins. Total lysates and eluates were immunoblotted to determine levels of R2, R1b, and Arc (n=4). Similar results were obtained using a BS3 surface cross-linking approach (data not shown).

Figure 4: Identification of a PP2A-binding motif in the C-terminus of R1.



A) Brain lysates were incubated with immobilized GST or GST-CR1 and bound material was immunoblotted with PP2ACα antibodies, In = 10% of the input used in each assay (left panel). VTA lysates were incubated with various immobilized GST-tagged R1 fragments and bound material was immunoblotted with antibodies against PP2ACα and the PP2A B subunit PR55, or stained with Coomassie brilliant blue (CBB) (right panel). After correction for input and non-specific binding to GST the level of binding was characterized as strong (+++) weak (+) or non-detectable (-). B) Diagram of GST-CR1 construct and DN-R1-PP2A and p-DN-R1-PP2A synthetic peptides. C) Peptides were conjugated to pre-activated Cys-link agarose beads and incubated with hippocampal lysates. Eluates were blotted for PP2A Bα and C subunits. D) Hippocampal lysates were incubated with immobilized GST-CR1 in the presence of 100 µM (top panel) or 10 µM (bottom panel) peptides, with a similarly sized peptide fragment of neurogranin used as a control. The ability of the peptides to compete away PP2A Bα and C subunit binding to CR1 was then quantified (n=4-5).

Conclusions

Exposure to NMDA or models of seizure or ischemic stroke decreases R2 levels in hippocampal slices.

Exposure to a kainate-induced seizure model in vivo resulted in a trend toward decreased hippocampal R2 levels.

Phosphomimetic mutation of Ser-783 to Asp in S783D mice does not significantly alter $GABA_BR$ surface expression, but does increase steady-state levels of Arc in hippocampal slices.

We have identified a sub-region of the R1 C-terminus that is sufficient to bind to PP2A and to compete away binding to GST-CR1. We have also performed unbiased proteomic screens to identify proteins that bind to this sequence in the hippocampus.

Interfering with $GABA_BR$ dephosphorylation and degradation represents a promising neuroprotective strategy for the treatment of seizure disorders and ischemic stroke.