Identification of an Atypical Calcium-Dependent Calmodulin Binding Site on the C-terminal domain of GluN2A

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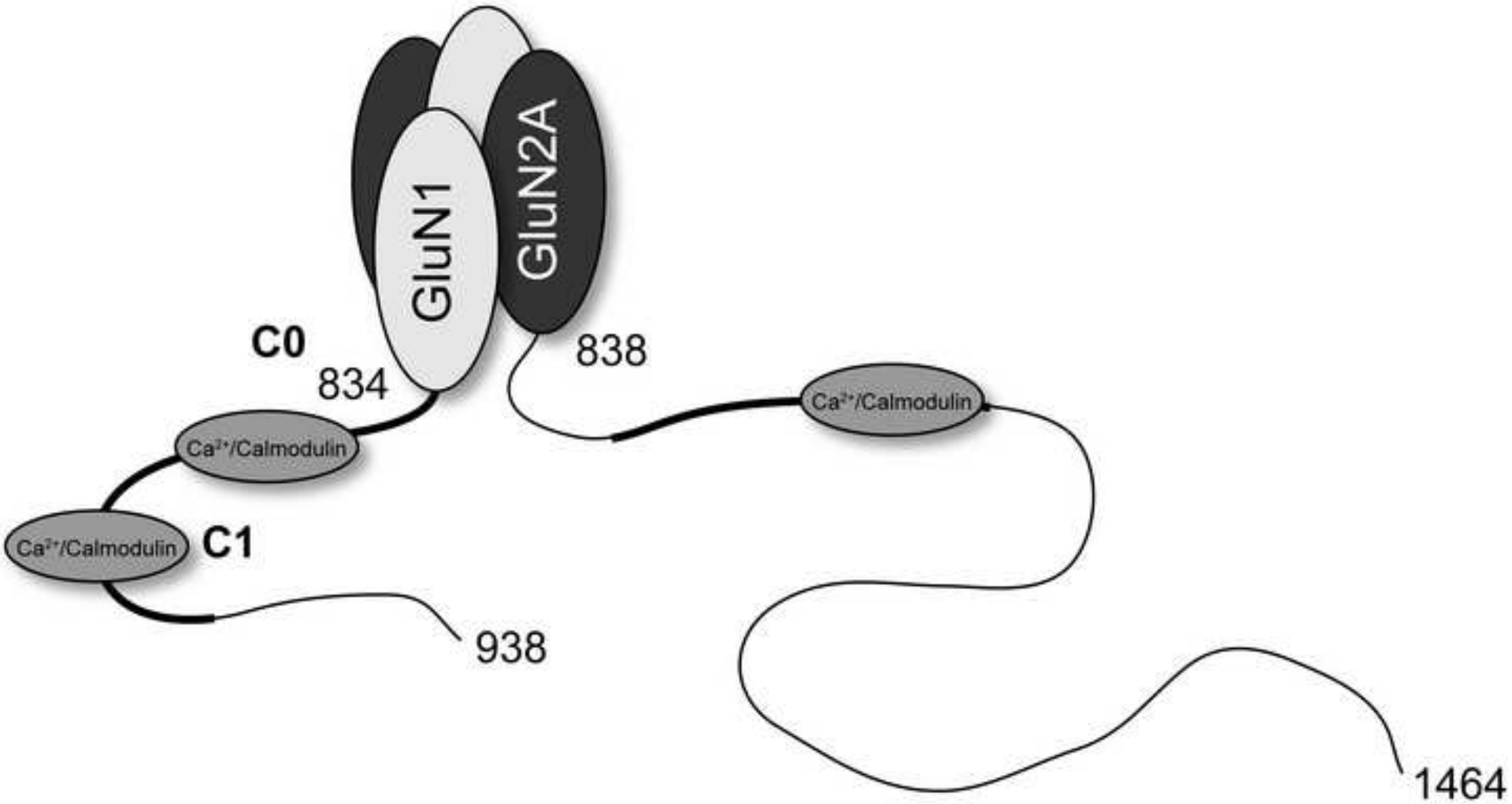
Highlights

- Calmodulin was identified by MS as a GluN2A C-terminus-associated protein
- Ca^{2+}/calmodulin bound directly to GluN2A with high affinity (5.2 $\pm$ 2.4 nM) *in vitro*
- Disruption of classic Ca^{2+}/calmodulin recognition motifs did not affect binding
- Trp-1014 of GluN2A was a critical determinant of Ca^{2+}/calmodulin binding *in vitro*

Graphical Abstract (for review)



Identification of an Atypical Calcium-Dependent Calmodulin Binding Site on the C-terminal domain of GluN2A

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Abstract

N-methyl-D-aspartate (NMDA) receptors are calcium-permeable ion channels assembled from four subunits that each have a common membrane topology. The intracellular carboxyl terminal domain (CTD) of each subunit varies in length, is least conserved between subunits, and binds multiple intracellular proteins. We defined a region of interest in the GluN2A CTD, downstream of well-characterized membrane-proximal motifs, that shares only 29% sequence similarity with the equivalent region of GluN2B. GluN2A (amino acids 875-1029) was fused to GST and used as a bait to identify proteins from mouse brain with the potential to bind GluN2A as a function of calcium. Using mass spectrometry we identified calmodulin as a calcium-dependent GluN2A binding partner. Equilibrium fluorescence spectroscopy experiments indicate that Ca^{2+}/calmodulin binds GluN2A with high affinity (5.2 $\pm$ 2.4 nM) *in vitro*. Direct interaction of Ca^{2+}/calmodulin with GluN2A was not affected by disruption of classic sequence motifs associated with Ca^{2+}/calmodulin target recognition, but was critically dependent upon Trp-1014. These findings provide new insight into the potential of Ca^{2+}/calmodulin, previously considered a GluN1-binding partner, to influence NMDA receptors by direct association.

Keywords

Calmodulin; calcium; NMDA; glutamate

1. Introduction

NMDA-type glutamate receptors are calcium-permeable and considered major regulators of calcium entry into the postsynaptic neuron [1]. Synaptic NMDA receptors function in the context of large intracellular multi-protein complexes that, when coupled with their unique activation characteristics, affords them a central role in fast excitatory signaling, synaptic development and neuronal plasticity [2,3]. It is also clear that an inability to control calcium influx via NMDA receptors results in a calcium-mediated excitotoxic cascade that triggers neuronal cell death associated with ischemic stroke, sustained seizures and chronic neurodegeneration [2,3].

Functional NMDA receptors are tetrameric complexes assembled from two GluN1 and two GluN2 subunits [2,3]. There are eight isoforms of the GluN1 subunit, generated by alternative splicing, whereas the GluN2 family includes four main subtypes, GluN2 (A-D), encoded by different genes. The identification of GluN3 (A-B) subunits provides further potential for functional heterogeneity through assembly of different combinations. All ionotropic glutamate receptor subunits share a common membrane topology with an extensive extracellular amino and ligand binding domain, a transmembrane region, and an intracellular CTD. The CTD of each subunit shows greatest diversity with respect to amino acid sequence and harbors multiple phosphorylation sites and specific binding motifs for target proteins that influence regulation, function and location of the receptor [2.]

There is a long-standing interest in determining how distinct physiological and pharmacological characteristics of hetero-oligomeric NMDA receptors are defined by the presence of a particular GluN2 subtype [4,5]. Analysis of CTDs has been a productive approach and led to the identification of membrane-associated guanylate kinase (MAGUK) proteins as direct binding partners [6]. When comparing the relatively long CTDs of GluN2A and GluN2B, the membrane-proximal and extreme distal regions show greatest sequence homology. Postsynaptic density protein of 95 kDa (PSD-95) and related scaffold proteins bind a common distal ESDV motif [6], whereas the membrane-proximal regions of GluN2A and GluN2B share tyrosine-based recognition motifs for the clathrin-dependent, endocytic adapter protein 2 complex (AP-2) [7,8]. We

have previously shown that the same membrane-proximal regions of GluN2A and GluN2B also bind a light chain of the actin-based motor myosin II that likely serves an early trafficking function [9]. As the membrane-proximal regions of the GluN2A and GluN2B C-termini appear to have some functional equivalency, the goal of this study was to analyze the region of GluN2A immediately downstream of the paired endocytic motifs and minimal myosin light chain interaction domain. We utilized affinity chromatography coupled with mass spectrometry (MS) to identify potential binding partners of GluN2A (875-1029). We report that Ca^{2+}/calmodulin binds with high affinity to GluN2A and identify a single tryptophan residue that is critical for direct interaction.

2. Materials and Methods

2.1. Plasmids and constructs

Plasmids containing rat GluN2A, GluN2B and GluN1-1a cDNAs, and the construction of GluN1-C0 (834-864), GluN1 (834-938), GluN2A (838-874), GluN2A (875-1029), GluN2A (1030-1464) and GluN2A (838-1464) in pGEX vectors (GE Healthcare, Piscataway, NJ have been described previously [9,10]. GluN2A (991-1029) was amplified by PCR and inserted into pGEX-6P-3 for fluorescence spectroscopy experiments. Point mutants were constructed in GluN2A (875-1029) using the QuickChange II XL site-directed mutagenesis protocol (Agilent Technologies, Santa Clara, CA). All sequences were confirmed by the Center for Genome Research and Biocomputing Core Facility, Oregon State University (OSU).

2.2. Protein purification, antibodies and reagents

Glutathione-S-transferase **(**GST)-fused CTDs were expressed in *Escherichia coli* (BL21-Gold (DE3)pLysS; Stratagene, La Jolla, CA), and purified by column chromatography using immobilized glutathione (Pierce, Rockford, IL). Bound GST fusion protein was eluted using a buffer containing 10mM reduced glutathione and 50mM Tris-HCl (pH 7.5) followed by dialysis against a buffer containing 50mM Tris-HCl and 150mM NaCl (pH 7.5). The anti-calmodulin antibody, secondary antibodies and bovine calmodulin were purchased from Calbiochem (EMD Millipore, Billerica, MA); anti-PSD-95 was from Affinity Bioreagents Inc. (Golden, CO). Alexa-488 labeled bovine calmodulin was a kind gift from Dr. Sonia Anderson (Department of Biochemistry and Biophysics, OSU) [11]. General reagents were from Sigma-Aldrich Corp. (St. Louis, MO).

2.3. Protein identification by mass spectrometry

GST-GluN2A (875-1029), or GST alone, were incubated with mouse brain homogenates extracted in a buffer containing: 10mM HEPES pH 7.5, 100mM NaCl, 1mM dithiothreitol (DTT), 0.1% NP-40 and 10% glycerol in the presence of: 1 mM calcium chloride (+ Ca^{2+}), or 1mM EDTA in the absence of added calcium (- Ca^{2+}). Reactions were allowed to proceed in the same binding buffer (+ Ca^{2+} or - Ca^{2+}) with

rotation overnight at 4°C. Unbound proteins were removed by three sequential washes with binding buffer (+ Ca^{2+} or - Ca^{2+}); bound proteins eluted from the beads by boiling in sample buffer. Proteins were separated on a pre-cast 4–12% SDS-polyacrylamide gradient gel (BioRad Laboratories, Hercules, CA) and visualized by a mass spectrometry-friendly Coomasie stain (BioRad). Bands were carefully excised and subjected to an in-gel tryptic digest as described in [12]. Samples were then desalted using a microC18 ZipTip (EMD Millipore), dried and resuspended in 7 µL of 0.1% formic acid, 5 µL was then analyzed by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC/ESI MS/MS) with an LCQ DECA XP mass spectrometer (ThermoScientific) using an instrument configuration described by [13]. Data collection was performed in a data dependent mode in which a MS precursor scan was followed by MS/MS scans of the three most abundant ions in the preceding MS scan. Ions selected for MS/MS were subjected to dynamic exclusion for 45 seconds. Proteins were identified from MS data with the protein database search algorithm COMET (Institute for Systems Biology) using a mouse protein database from the International Proteome Index (24 January 2005). Peptide validation was performed using Peptide Prophet [14], and peptides filtered with a Peptide Prophet probability greater than or equal to 0.8.

2.4. Glutathione-S-transferase pull-down assays

Assays with purified calmodulin were conducted as previously described [9], except reactions were initiated with 250 nM calmodulin and allowed to proceed for 1 hour at 4°C. Unbound protein was removed by three sequential washes with binding buffer and bound proteins were eluted from the beads by boiling in sample buffer. In all studies, binding buffer and wash buffer contained: 1 mM calcium chloride (+ Ca^{2+}), or 1 mM EDTA in the absence of added calcium (- Ca^{2+}). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and processed for immunoblot analysis as described previously [9].

2.5. Fluorescence spectroscopy

Fluorescence measurements were taken in 300 µl of buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, 20 nM Alexa-CaM plus 1 mM calcium. Purified GST-GluN2A (991-1029), or GST-GluN1-C0 (834-864), was added from a single stock solution and allowed to equilibrate in the dark for 5 min prior to fluorescence determinations. Additions of purified GST-CTD proteins were made sequentially from a single stock solution, and the fluorescence emission corrected for dilution. All experiments were performed on a SLM 8000C spectrofluorometer (SLM Instruments, Urbana, IL). Data were fit to equation (1) by weighted nonlinear least squares using the computer program Scientist (Micromath Inc., St. Louis, MO) where the weighting factors were equal to the square of the reciprocal of the standard deviation for each data point,

$$F = a - b\left[\frac{2\,[R_0][L_0]}{[R_0]+[L_0]+K+\sqrt{([R_0]+[L_0]+K)^2-4\,[R_0][L_0]}}\right] \qquad (1)$$

In equation (1), derived in the Supplementary Material, F is the observed fluorescence, a is the fluorescence of the unquenched Alexa-CaM, $[R_0]$ is the total Alexa-CaM concentration, $[L_0]$ is the total GST-GluN2A (991-1029), or GST-GluN1-C0 (834-864), K is the equilibrium dissociation constant for Alexa-CaM complex formation with either GST-GluN2A or GST-GluN1-C0 and b is the fluorescence of the Alexa-CaM-protein complex.

3. Results

3.1. Identification of Ca^{2+}/Calmodulin as a direct binding partner of GluN2A

A specific region of the GluN2A CTD (Fig. 1A), downstream of known membrane-proximal binding motifs, was used as bait to pull down proteins from mouse brain with the potential to interact with GluN2A in a calcium-regulated manner. GST-fused GluN2A (875-1029) and GST alone were expressed in bacteria and immobilized on glutathione-Sepharose beads. Immobilized fusion proteins were then used as affinity matrices to identify proteins in mouse brain homogenates with the potential to bind, or dissociate, as a function of calcium. Proteins were resolved by SDS-PAGE and visualized by Coomassie staining. Six bands changing in a calcium-sensitive pattern were excised and submitted for MS analysis. A major band (Fig. 1B, band 5) retained by the GST-GluN2A (875-1029) affinity matrix in the presence of calcium and migrating between 15 and 20 kDa was identified as mouse calmodulin; eight unique peptides, of a total of 63, gave 48% coverage of the calmodulin sequence (Fig. 1B). Based on the known biology of the NMDA receptor and other ion channels [15], this interaction was prioritized for further validation. MS data also revealed peptides matching GluN2A (bands 1 and 2) and alpha and beta tubulin subunits (bands 3, 4 and 6). GluN2A peptides matched the "bait" protein, whereas tubulin subunits are known NMDA interactors [16] and were ranked as low interest for further validation.

To test the specificity of the observed interaction between Ca^{2+}/calmodulin and GluN2A (875-1029) we performed GST pull down assays using the full-length CTD of GluN2A. The full-length CTD of GluN1, which contains two Ca^{2+}/calmodulin binding motifs [17], was also used as a positive control. For these studies, GST alone or GST-fused GluN1 (834-938) and Glu2NA (838-1464) were tested for their ability to pull down calmodulin from mouse brain homogenates in the presence and absence of calcium (Fig. 2A). Immunoblot analysis revealed mouse calmodulin to be retained on GST-GluN2A and GST-GluN1 affinity matrices, but only in the presence of calcium (compare lanes 7 and 8, with lanes 3 and 4; Fig. 2A). To confirm appropriate binding specificity of bacterially-expressed CTD proteins, we also probed all samples for PSD-95. As anticipated, native PSD-95 was retained only by GST-GluN2A and bound strongly in the absence and presence of calcium (lanes 4 and 8; Fig. 2B). PSD-95 was not retained by GST-GluN1

or by glutathione-Sepharose beads charged with GST alone (Fig 2B). These findings demonstrate appropriate binding specificity of PSD-95 in this assay, and indicate that Ca^{2+}/calmodulin is also a GluN2A-interacting protein.

To rule out the possibility of an indirect association of Ca^{2+}/calmodulin with GluN2A we performed GST pull down assays with purified calmodulin and a series of deletion mutants representing the full CTD of the GluN2A subunit. GST-fused GluN2A (838-874), GluN2A (875-1029), GluN2A (1030-1464) and GluN1-C0 (834-864) were examined for interaction with purified calmodulin in the presence and absence of calcium (Fig. 2C). Calmodulin bound strongly and specifically to GST-GluN2A (875-1029) and GST-GluN1-C0 (834-864) in the presence of calcium (Fig. 2C, compare lanes 4 and 6 with 9 and 11), yet failed to interact with GST-GluN2A (838-874), GST-GluN2A (1030-1464) or GST alone. Calmodulin was not retained on any of the CTD affinity matrices in the absence of calcium (lanes 7, 8, and 10). Together these findings indicate that the interaction of Ca^{2+}/calmodulin with GluN2A (875-1029) occurs *in vitro* via a direct protein-protein interaction and is not easily explained by a bridging protein(s) present in mouse brain homogenates.

3.2. Tryptophan-1014 of GluN2A is critical for Ca^{2+}/calmodulin binding

Ca^{2+}/calmodulin typically binds target proteins via conserved motifs that have been classified according to the position of hydrophobic residues within the target sequence [18]. We therefore analyzed the amino acid sequence of GluN2A (875-1029) for such binding patterns and identified five potential regions of interest with spacing of hydrophobic residues in either a 1-8-14, 1-14, 1-16 or a 1-10 arrangement (Fig. 3A). Towards the goal of disrupting the observed GluN2A-Ca^{2+}/calmodulin interaction, we used site-directed mutagenesis to systematically replace key hydrophobic residues within the putative GluN2A binding motifs with alanine. We initially reasoned that GluN2A (875-1029) could, like GluN1, contain more than one Ca^{2+}/calmodulin-binding site and therefore prepared a series of six mutants (mutant 1-6), with successive

disruptions made in the background of the previous one. All mutants were expressed as GST-fusion proteins, immobilized on glutathione-Sepharose and tested for interaction with purified calmodulin in the presence of calcium. The first four GluN2A mutants, all bound Ca^{2+}/calmodulin in a manner that was essentially comparable to wild-type GluN2A (lanes 3 to 7, Fig. 3A). Disruption of Trp^{1014} and Ile^{904} however, dramatically reduced (mutant 5), or ablated (mutant 6), Ca^{2+}/calmodulin binding relative to wild-type GluN2A (lanes 8 and 9; Fig. 3A). These results indicated that some of the putative Ca^{2+}/calmodulin-binding motifs that we targeted within GluN2A are not binding sites, but allowed us to identify two regions of interest, approximately 100 amino acids apart, for further evaluation.

We next constructed point mutations of Trp^{1014} (mutant 7) and Ile^{904} (mutant 8), and a double mutant (mutant 9), in the wild-type GluN2A (875-1029) background (Fig. 3B). These three mutants, wild-type GluN2A and GST alone were tested for interaction with Ca^{2+}/calmodulin. Purified Ca^{2+}/calmodulin interacted strongly and specifically with wild-type GluN2A and mutant 8 (lanes 3 and 5, Fig. 3B), but failed to interact with mutants 7 and 9 (lanes 4 and 6, Fig. 3B) or GST alone. These data indicate that Trp^{1014} of GluN2A is critical for direct binding of Ca^{2+}/calmodulin.

3.3. Ca^{2+}/calmodulin binds GluN2A (991-1029) with high affinity.

The GluN1 subunit harbors two distinct Ca^{2+}/calmodulin binding sites: one within the C0 region of all splice variants, and a second within the alternatively spliced C1 cassette [17]. A single tryptophan residue is considered to be the anchor residue for Ca^{2+}/calmodulin binding to C0 [19,20], therefore given our identification of Trp^{1014} as a critical determinant of Ca^{2+}/calmodulin-GluN2A binding, we compared the relative affinity of Ca^{2+}/calmodulin for both targets. We constructed a GluN2A deletion mutant (991-1029) for bacterial expression, centered upon Trp^{1014} and of comparable length to the GluN1-C0 region. Equimolar (100 pmols) GST and GST-fused GluN2A (991-1029) or GluN1-C0 (834-864) were incubated with increasing concentrations of CaM (0.01 - 1 μM) in the presence of calcium and investigated for their ability to retain Ca^{2+}/calmodulin. Both GST-GluN2A (991-1029) and GluN1-C0 (834-864) affinity matrices retained Ca^{2+}/calmodulin in a concentration-dependent manner (Fig. 4).

Ca^{2+}/calmodulin was detected at 100 nM Ca^{2+}/calmodulin and above in GluN2A reactions (compare lanes 3-5 and 6-9, Fig. 4) and showed comparable affinity to the interaction of Ca^{2+}/calmodulin with GluN1-C0 (834-864) in these assays.

We used GST-fused NR2A (991-1029) and GluN1-C0 (834-864), to determine the binding affinity of Alexa-488 labeled calmodulin (Alexa-488-CaM) for these targets using equilibrium fluorescence spectroscopy. Alexa-488-CaM alone exhibited maximum excitation at 495 nm and maximum emission at 520 nm (data not shown). Titration of GluN2A (991-1029) quenched the fluorescence of Alexa-488-CaM in a concentration-dependent manner in the presence of calcium (Fig. S1). These data were best fit by a single hyperbolic curve using nonlinear, curve-fitting analysis. We derived a binding affinity for GluN2A (991-1029) for a half maximal quenching of Alexa-488-CaM fluorescence of 5.2 ± 2.4 nM. Titration of GluN1-C0 (834-864) produced a similar concentration-dependent response and quenched Alexa-488-CaM fluorescence in the presence of calcium (Fig. S1). The binding affinity of GluN1-C0 (834-864) for half maximal quenching of Alexa-488-CaM fluorescence was 5.9 ± 5.5 nM. These results lend further support for the existence of a high affinity GluN2A binding site that binds Ca^{2+}/calmodulin.

4. Discussion.

Distinct differences between GluN2A- versus GluN2B-containing NMDA receptors have been revealed using a variety of experimental approaches that have together contributed to an overall understanding of how subunit composition may influence neuronal signaling in heteromeric receptors. In the present study we defined a region of interest in the GluN2A CTD, that shares only 29% sequence similarity with the GluN2B subunit. Using affinity chromatography coupled with MS we identified calmodulin as a GluN2A-interacting protein. The direct interaction of calmodulin with GluN2A was specific and required calcium. Ca^{2+}/calmodulin binding to GluN2A was not affected by disruption of classic sequence motifs associated with Ca^{2+}/calmodulin target recognition, but was critically dependent upon tryptophan-1014. This residue is absent in the GluN2B subunit but conserved in the human GluN2A sequence. Equilibrium fluorescence spectroscopy experiments indicate that the affinity of Ca^{2+}/calmodulin for GluN2A is high *in vitro,* and thus may warrant further study in cell-based models. These results provide new insight into the potential of Ca^{2+}/calmodulin to influence the structure and function of the GluN2A subunit by a direct association.

Our identification of a tryptophan residue within the GluN2A recognition sequence is intriguing as a tryptophan is the presumed anchor for Ca^{2+}/calmodulin recognition of GluN1-C0 [19,20]. Ca^{2+}/calmodulin binding to GluN1-C0 is functionally significant for calcium-dependent inactivation of NMDA receptors [17,21], and may also be of structural importance [22]. The interaction of Ca^{2+}/calmodulin with GluN1-C1 appears to regulate cell surface expression of certain GluN1 splice variants [23]. However, a subsequent crystal structure of Ca^{2+}/calmodulin bound to a GluN1-C1 peptide now supports a more complicated mechanism than originally proposed as ER retention and phosphorylation sites on GluN1-C1, identified as functionally important in cell-based assays [24], were not masked in the Ca^{2+}/calmodulin-bound structure [20]. The crystal structure also indicted that a 1-7 motif on GluN1-C1 was critical for Ca^{2+}/calmodulin recognition [20]. Thus it appears that Ca^{2+}/calmodulin recognition of all three GluN target sequences occurs via alternate motifs rather than classic motifs characterized by conserved patterns of hydrophobic residues.

Ca^{2+}/calmodulin bound GluN2A with relatively high affinity (5.2 $\pm$ 2.4 nM), as determined by equilibrium fluorescence spectroscopy, and this was comparable to the affinity derived for Ca^{2+}/calmodulin binding to GluN1-C0 *in vitro*. Although less sensitive, our GST-pulldown studies also indicated that the affinity of Ca^{2+}/calmodulin for both targets was similar. The affinity of Ca^{2+}/calmodulin for GluN1-C0 (5.9 ± 5.5 nM) was somewhat higher than previously calculated, potentially due to differences in methodology. Previous estimates have ranged from 21 to 87 nM for the GluN1-C0 site [17,21], whereas Ca^{2+}/calmodulin has been shown to have higher affinity (2 to 4 nM) for GluN1-C1 [17,20]. Taken together, our results provide a preliminary ranking of the relative affinity of Ca^{2+}/calmodulin for NMDA receptor subunits as follows: GluN1-C1 < GluN2A $\leq$ GluN1-C0.

In summary, we have identified a region of GluN2A that binds Ca^{2+}/calmodulin with high affinity *in vitro*. Biochemical identification of glutamate receptor CTD binding proteins has frequently provided a starting point for interrogation of the potential functional consequences of such interactions. For example, it has been proposed that Ca^{2+}/calmodulin binding to GluN1-C1 may either directly, or indirectly, influence local intracellular signaling events [20]. In the case of GluN2A, earlier physiological studies have shown that some forms of NMDA receptor desensitization are GluN2 subunit specific, and involve residues near the GluN2A Ca^{2+}/calmodulin binding site identified herein [25,26]. Other residues in this region are subject to post-translational modification, yet details of the enzyme and mechanism are lacking [2]. Further characterization of the GluN2A subunit may expand our understanding of the full extent to which Ca^{2+}/calmodulin can modulate NMDA receptor signaling.

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Figure Legends.

Figure 1. Identification of calmodulin as a GluN2A-associated protein.

(A) Schematic representation of the GluN2A subunit. A region of interest was targeted in GluN2A (875-1029) downstream of known AP-2 and myosin regulatory light chain (MRLC) binding domains. A conserved terminal motif binds MAGUK proteins.

(B) Analysis of GluN2A associated proteins. GST fused GluN2A (875-1029), and GST alone, were immobilized on glutathione-Sepharose beads and used as baits to pull down proteins from mouse brain extracts with or without calcium (1 mM). Retained proteins were separated on a 4–12% SDS-polyacrylamide gradient gel and visualized by Coomasie stain. Six bands were submitted for MS analysis; mouse calmodulin (NP_033920) was identified in band 5.

Figure 2. Ca^{2+}/calmodulin is a direct binding partner of GluN2A.

(A) Schematic representation of the NMDA receptor. The location of known Ca^{2+}/calmodulin binding sites in the GluN1 C-terminus, at C0 and C1, is shown relative to GluN2A (875-1029) that was found to harbor a Ca^{2+}/calmodulin binding site.

(B) Ca^{2+}/calmodulin binds the CTD of GluN1 and GluN2A subunits. Deletion mutants of the GluN1 and GluN2A were fused to GST and used as baits to pull down calmodulin and PSD-95 from mouse brain homogenates with or without calcium (1 mM). Bound proteins were resolved by PAGE, blotted to nitrocellulose, and immune complexes revealed with anti-calmodulin or anti-PSD-95 antibodies.

(C) Ca^{2+}/calmodulin binds directly to GluN2A. Deletion mutants representing the full C-terminus of the GluN2A subunit were fused to GST and used as baits to test the specificity of GluN2A interaction with purified calmodulin. GST-fused proteins or GST were immobilized on glutathione-Sepharose beads and incubated with calmodulin with or without calcium (1 mM). Bound protein was resolved by PAGE, blotted to nitrocellulose, and immune complexes revealed with an anti-calmodulin antibody.

Figure 3. Trp-1014 of GluN2A is critical for Ca^{2+}/calmodulin binding.

(A) Strategy for sequential disruption of putative Ca^{2+}/calmodulin binding sites in GluN2A (875-1029). Putative Ca^{2+}/calmodulin binding sites were targeted by site-directed mutagenesis to yield six mutants. GST-fused proteins, or GST, were used as baits to test binding of purified calmodulin with calcium (1 mM).

(B) Targeted disruption of Ca^{2+}/calmodulin binding. Tryptophan-1014 and isoleucine-904 were disrupted by site-directed mutagenesis; single and double mutants were compared with wild-type GluN2A. GST-fused proteins, or GST, were used as baits to test binding of purified calmodulin with calcium (1 mM). Bound protein was resolved by PAGE, blotted to nitrocellulose, and immune complexes revealed with an anti-calmodulin antibody.

Figure 4. GluN2A harbors a high-affinity Ca^{2+}/calmodulin binding site.

Titration of calmodulin in the presence of (A) GluN2A and (B) GluN1-C0. GST-fused GluN2A (991-1029), GST-GluN1-C0 (834-864) or GST alone, were immobilized on glutathione-Sepharose beads and incubated with rotation for 1 h at 4°C with calcium (1 mM) and increasing concentrations of calmodulin (0.01, 0.03, 0.05, 0.1, 0.3, 0.5 and 1 μM). Bound protein was resolved by PAGE, blotted to nitrocellulose, and immune complexes revealed with an anti-calmodulin antibody.

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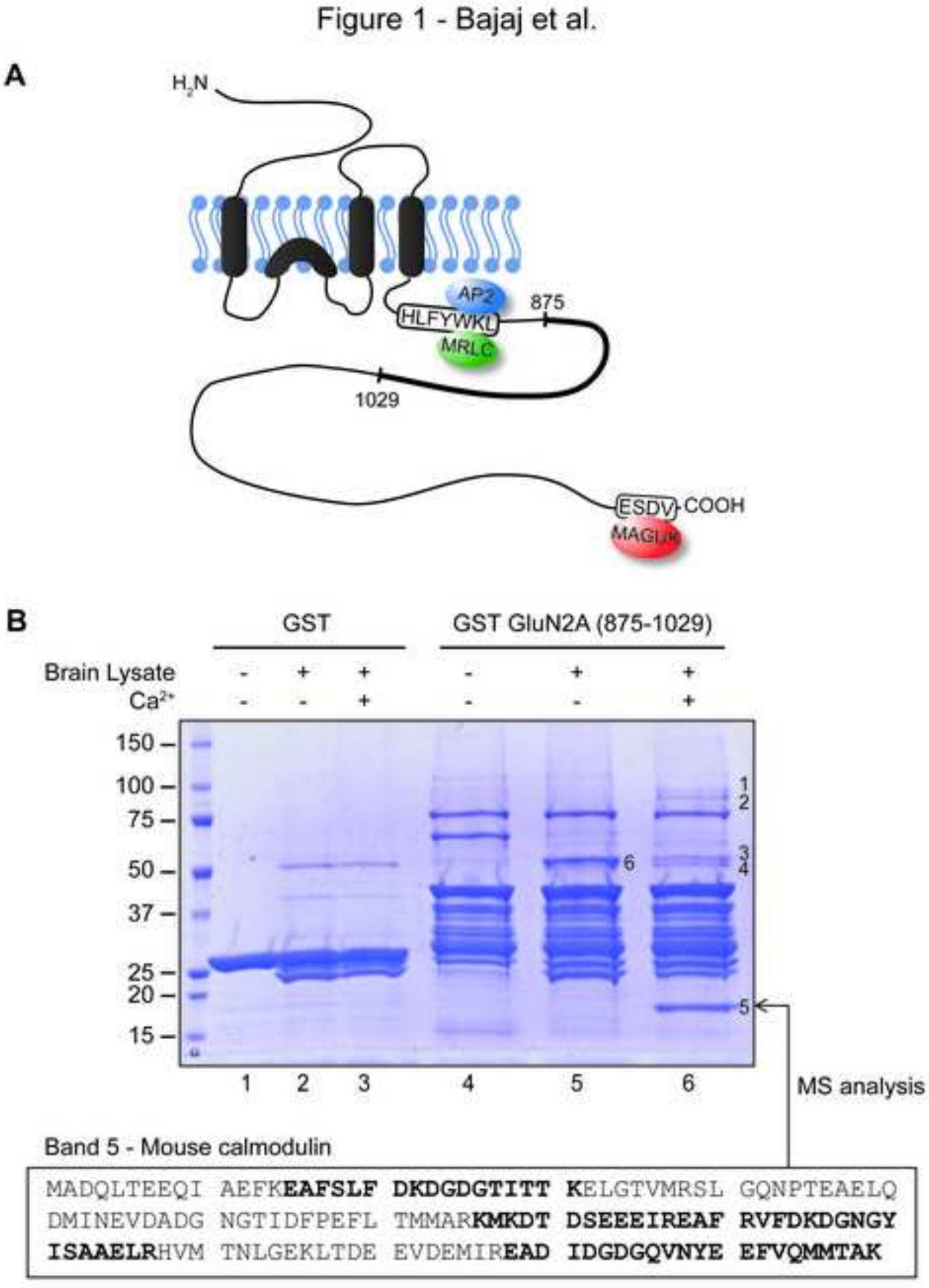

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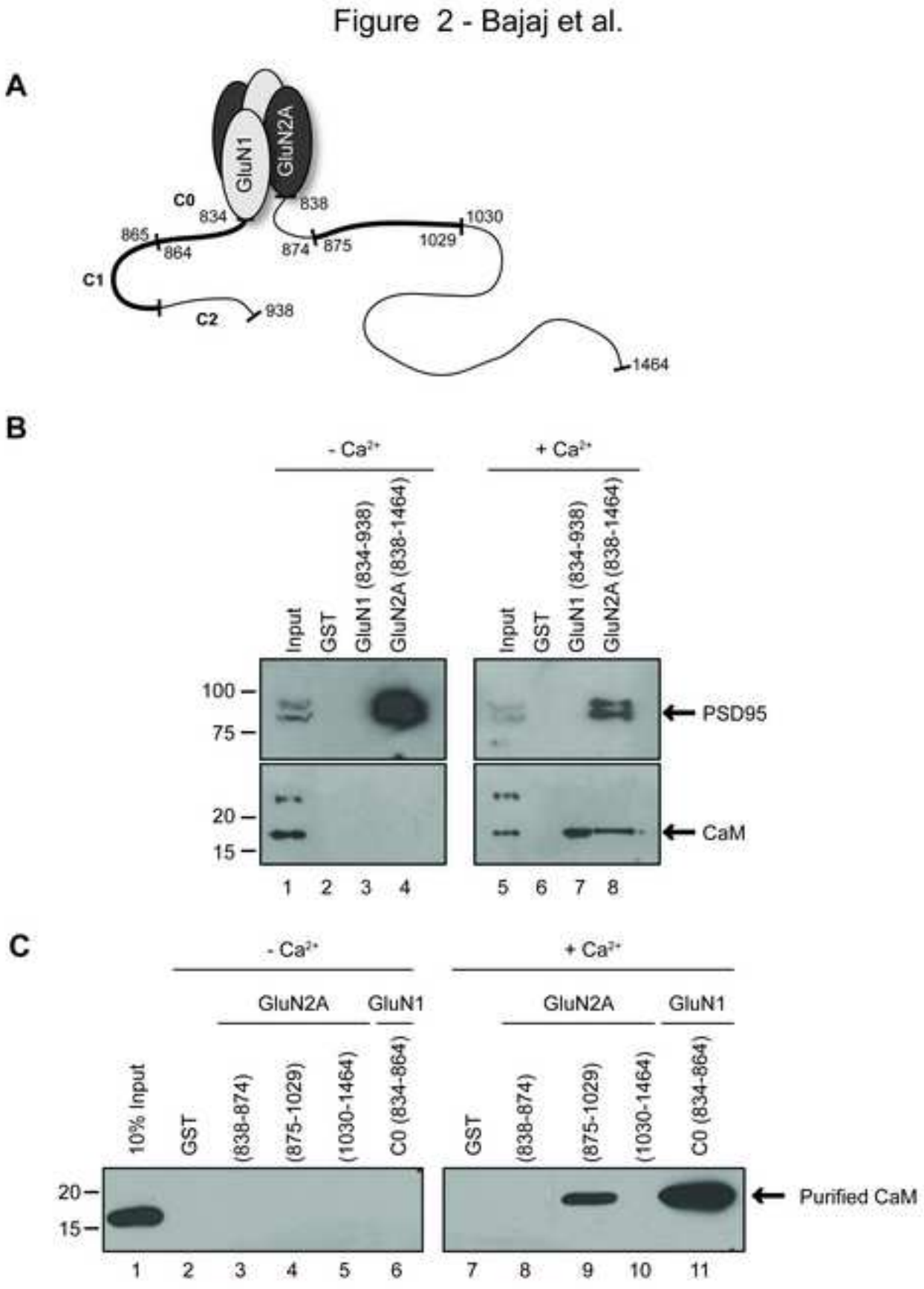

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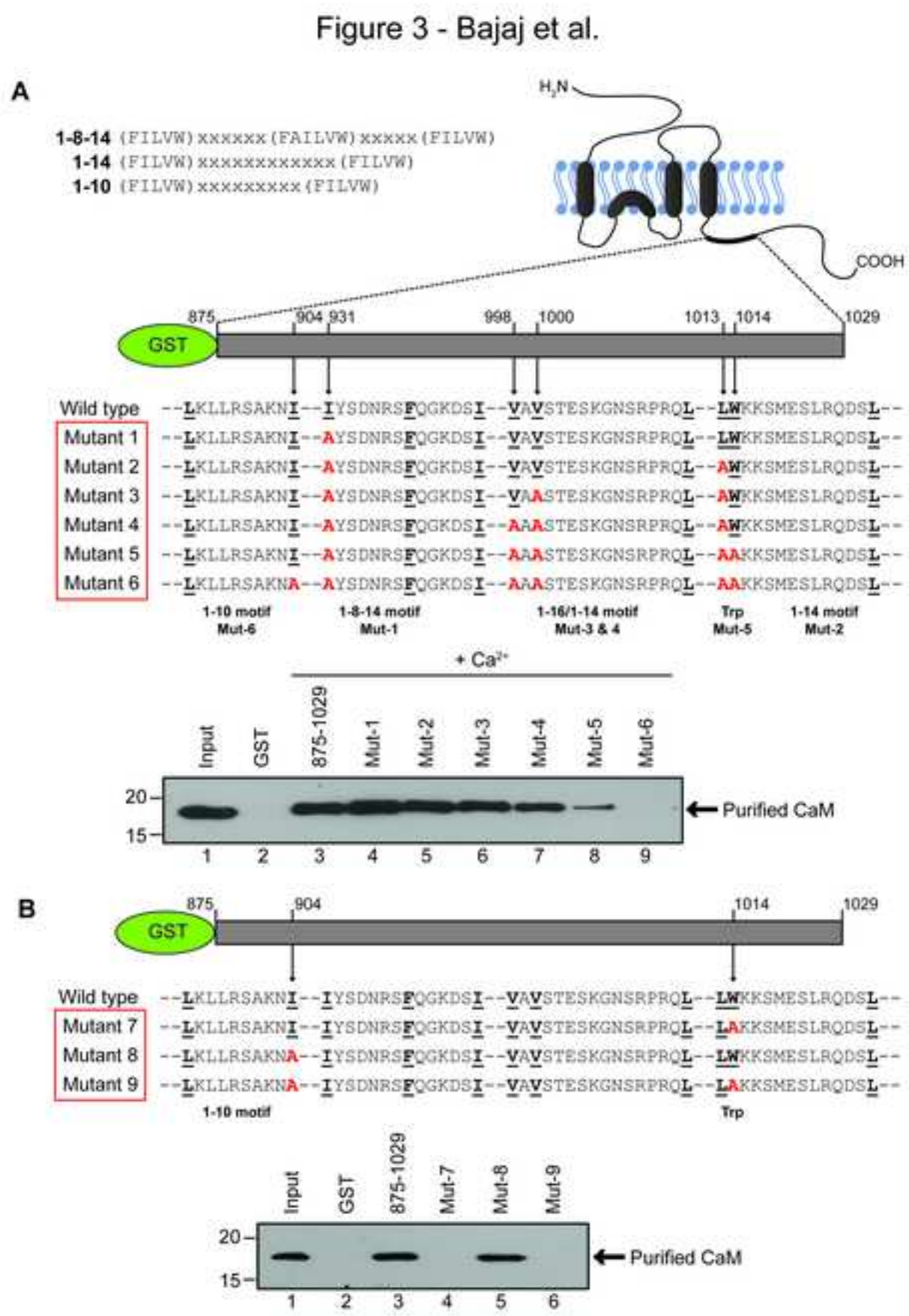

Figure 4

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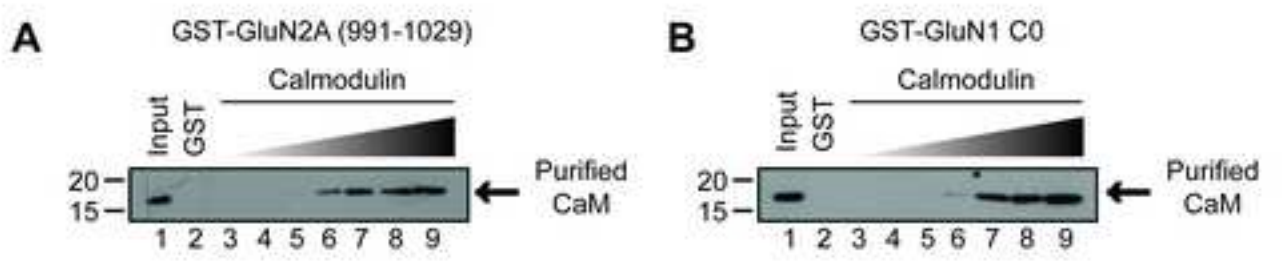

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