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**APLP1 AND APLP2, MEMBERS OF THE APP FAMILY OF PROTEINS, BEHAVE SIMILARLY TO APP IN THAT THEY ASSOCIATE WITH NMDA RECEPTORS AND ENHANCE NMDA RECEPTOR SURFACE EXPRESSION**

Sarah L. Cousins, Wei Dai and F. Anne Stephenson\*

University College London School of Pharmacy

29/39 Brunswick Square

London WC1N 1AX

United Kingdom

\* To whom correspondence should be addressed

Telephone: 44 207 753 5877

FAX: 44 207 753 5964

E-mail: anne.stephenson@ucl.ac.uk

**Abbreviations:** APP, amyloid precursor protein; APLP, amyloid precursor-like protein; ELISA, enzyme linked immunoadsorbent assay; GluN1, GluN2A, GluN2B, NMDA receptor NR1 subunit, NR2A subunit, NR2B subunit; Neto1, neuropilin tolloid-like 1; NMDA, N-methyl-D-aspartate.

**ABSTRACT**

The function of amyloid precursor protein (APP) is unknown, although the discovery that it contributes to the regulation of surface expression of N-methyl-D-aspartate (NMDA) receptors has afforded new insights into its functional significance. Since APP is a member of a gene family that contains two other members, amyloid precursor-like proteins 1 and 2 (APLP1 and APLP2), it is important to determine if the related APP proteins possess the same properties as APP with respect to

their interactions with NMDA receptors. Following expression in mammalian cells, both APLP1 and APLP2 have been shown to associate with NMDA receptors and enhance their surface expression. This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/jnc.13063

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APLP2 behaved similarly to APP in that they both co-immunoprecipitated with the two major NMDA receptor subtypes, GluN1/GluN2A and GluN1/GluN2B, via interaction with the obligatory GluN1 subunit. Immunoprecipitations from detergent extracts of adult mammalian brain showed co-immunoprecipitation of APLP1 and APLP2 with GluN2A- and GluN2B-containing NMDA receptors. Furthermore, similarly to APP, APLP1 and APLP2 both enhanced GluN1/GluN2A and GluN1/GluN2B cell surface expression. Thus all three members of the APP gene family behave similarly in that they each contribute to the regulation of cell surface NMDA receptor homeostasis.

**Running Title: APLP1 AND APLP2 ASSOCIATE WITH NMDA RECEPTORS**

**Key words: NMDA receptors; amyloid precursor protein; amyloid precursor-like 1; amyloid precursor-like 2; Alzheimer's disease**

## **INTRODUCTION**

Amyloid precursor protein (APP) is a ubiquitously expressed type I transmembrane protein. It is implicated in pathogenic mechanisms leading to Alzheimer's Disease (AD) due to its known proteolytic processing via the amyloidogenic pathway to generate the neurotoxic amyloid- $\beta$  peptides. These amyloid- $\beta$  peptides form the extracellular amyloid plaques, key pathological traits of AD. The function of APP itself is unclear, although evidence does support a role as a trophic factor promoting neurite outgrowth, neuronal migration and repair via interaction with extracellular matrix proteins (reviewed in Muller and Zheng, 2012; Hoe *et al.*, 2012). We recently proposed a new role for APP as a regulator of cell surface N-methyl-D-aspartate (NMDA) receptor homeostasis. This is because it was found that APP co-associated with the two major NMDA receptors expressed in adult brain, i.e. GluN1/GluN2A and GluN1/GluN2B, and indeed also GluN1/GluN2A/neuropilin tolloid-like 1 (Neto1) and GluN1/GluN2B/Neto1 complexes (Cousins *et al.*, 2009; 2013; Innocent *et al.*, 2012). Over-expression of APP resulted in an enhanced cell surface NMDA receptor expression. Similar findings were also reported by Hoe *et al.* (2009), thus both groups surmised that APP may be an important contributor to the proper functioning of excitatory post-synaptic mechanisms. This may be

particularly important for familial forms of early onset autosomal dominant AD where gene duplications of the APP gene have been identified (Cabrejo *et al.*, 2006; McNaughton *et al.*, 2012).

APP is a member of a gene family that contains two other members, amyloid precursor proteins 1 and 2 (APLP1 and APLP2). The three proteins are highly homologous, sharing ~ 38 - 51 % amino acid sequence identity (reviewed in Walsh *et al.*, 2007; Ali *et al.*, 2013). APLP1 and APLP2 both undergo proteolytic processing similarly to APP; however, the amyloid- $\beta$  sequence is not conserved between APP and APLP1 or APLP2. APLP2, similarly to APP, is ubiquitous and is expressed in neuronal and non-neuronal tissues, whereas APLP1 is found primarily in cells of the nervous system (Lorent *et al.*, 1995). The study of APP and APLP1/2 knock-out mice suggests functional differences between the three family members and, significantly, a particular importance for APLP2 in the physiology of the central nervous system (reviewed in Aydin *et al.*, 2012).

To gain further insight into the significance of APP/NMDA receptor interactions, it was of interest to investigate if APLP1 and APLP2 behaved similarly to APP with regard to their respective association and receptor cell surface trafficking. The results are reported in this paper.

## **EXPERIMENTAL PROCEDURES**

### **Constructs and antibodies**

The constructs, pCISGluN1-1a, pCISGluN2A, pCISGluN2B and pCI-neoAPP695<sup>FLAG</sup> were as previously described (Cik, *et al.*, 1993; Hawkins *et al.*, 1999; Perkinson, *et al.*, 2004). Note that only the GluN1-1a splice variant was used thus pCISGluN1 and GluN1 are used throughout the manuscript to denote the GluNR1-1a splice variant. pCMV6APLP1 (variant 2, accession number NM\_005166) and pCMV6APLP2 (variant 2 accession number NM\_001142276) were purchased from OriGene (Rockville, Maryland, USA). Each clone generates APLP1 and APLP2 with a C-terminal c-Myc tag.

Anti-GluN1 C2, anti-GluN2A (44–58), anti-GluN2A (1381–1394), anti-GluN2A/2B (raised against the amino acid sequence NR2A 1454-1464 but recognizes GluN2A and GluN2B), anti-GluN2B (46–60) and anti-APP (679–695) antibodies (all antibody amino acid sequences correspond to numbering for the respective immature proteins) were raised, generated and affinity-purified as previously described (Chazot, *et al.*, 1992; Hawkins *et al.*, 1999; Groc, *et al.*, 2006; Cousins *et al.*, 2009). Anti-GluN2B antibodies were from Millipore (Massachusetts, USA). Anti-His antibodies were from Life Technologies Ltd (Paisley, Scotland). Anti-c-Myc clone 4A6 mouse monoclonal antibodies were from Upstate (Charlottesville, VA). Anti-APLP1 antibodies raised against human APLP1 200-300 and anti-APLP2 raised against human APLP2 262-491 were from AbCam (Cambridge, UK).

### **Mammalian cell transfections**

Human embryonic kidney (HEK) 293 cells (purchased for the American Type Culture Collection (ATCC), Virginia, USA) were transfected using the calcium phosphate method as described (Cik *et al.*, 1993). For double transfections 10 µg DNA were used at a 1:1 ratio. For triple transfections a total of 20 µg DNA were used with a ratio of 1:3 GluN1: GluN2A or GluN1: GluN2B (10 µg total) with 10 µg APP695<sup>FLAG</sup>, 10 µg APLP1<sup>c-Myc</sup> or 10 µg APLP2<sup>c-Myc</sup>. HEK 293 cells expressing GluN1/GluN2 were cultured post-transfection in the presence of 1 mM ketamine to prevent cell cytotoxicity. Transfected cells were incubated for 24 h post-transfection and either assayed for cell surface NMDA receptor expression or they were harvested and used in immunoprecipitation assays.

### **Immunoprecipitation assays**

Immunoprecipitations were carried out as previously described from HEK 293 transfected cells extracted with either 1 % (v/v) Triton X-100 or, from brain membranes prepared from adult Sprague Dawley male rats from Biological Services, University College London, culled according to Schedule 1 procedures of the Animals (Scientific Procedures) Act with full institutional approval, extracted

with 1 % (w/v) sodium deoxycholate. Triton X-100 or sodium deoxycholate detergent extracts were centrifuged at 100,000 xg and the resultant supernatants used for the immunoprecipitations (Cousins *et al.*, 2009). Immunoprecipitating antibodies used were anti-GluN1 C2, anti-GluN2A (1381–1394) and anti-GluN2A/2B. The resulting immune pellets were analysed by SDS-PAGE followed by immunoblotting.

### **Immunoblotting**

Immunoblotting was carried out using 7.5% (w/v) polyacrylamide slab minigels all as described previously (Papadakis *et al.*, 2004). Primary antibodies used were as follows: anti-GluN1 C2; anti-GluN2A (1381–1394); anti-GluN2A 1435–1445; anti-APP (679–695); anti-APLP1; anti-APLP2 and anti-c-Myc. Rabbit or mouse horseradish-linked secondary antibodies were used at a final dilution of 1:2000 and immunoreactivities were detected using the ECL Western blotting system (Amersham Biosciences Ltd., Little Chalfont, Bucks., UK). Immunoblots with brain tissue used the above secondary antibodies at a 1:10,000 dilution and subsequent development using the SuperSignal West Femto ECL substrate (Perbio Science UK Ltd., Northumberland, UK). For all the immunoblots of immunoprecipitates, for double transfections, 50% of immune pellets were analysed by the precipitating primary antibody and 50% with the second different specificity antibody; for triple transfections and in brain samples where three different antibodies were used for immunoblotting of immune pellets, 14% of the immune pellets were analysed by the precipitating primary antibody and 42% of the pellets for each of the additional two specificity antibodies.

### **Determination of NMDA receptor cell surface expression by ELISA**

The surface expression of GluN1/GluN2A and GluN1/GluN2B receptors was measured using anti-GluN2A (44–58) and anti-GluN2B (46–60) antibodies respectively since for GluN1-1a-containing NMDA receptors, cell surface expression occurs when GluN1-1a is co-expressed with GluN2A or

GluN2B receptor subunits (McIlhinney *et al.* 1998). Cell surface ELISA was carried out as previously described (Papadakis *et al.*, 2004; Cousins *et al.*, 2008).

### **Analysis of results**

All results are the means  $\pm$  SEM and were analysed by the Student's two tailed paired t test.

## **RESULTS AND DISCUSSION**

### **APLP1 and APLP2 associate with GluN1/GluN2 NMDA receptors via association with GluN1 subunits**

APP is a member of a gene family which contains two other members, APLP1 and APLP2, that share significant, i.e. at least 57 %, amino acid sequence similarity. Thus to gain insight into the functional significance of APP/NMDA receptor association, the potential interaction of NMDA receptors with these other family members was investigated initially in a model heterologous expression system. In the first instance, the co-immunoprecipitation of APLP1 and APLP2 with GluN1, GluN2A and GluN2B single subunits was investigated. The results shown in Figure 1 reveal that similarly to APP695<sup>FLAG</sup>, both APLP1<sup>c-Myc</sup> ( $M_r = 87 \pm 1$  kDa  $n = 18$ ) and APLP2<sup>c-Myc</sup> ( $141 \pm 3$  kDa  $n = 18$ ) co-immunoprecipitate with GluN1 single subunits (Figure 1A and 1D) but not with GluN2A (Figure 1B and 1E) nor GluN2B (Figure 1C and 1F) single subunits. Since when expressed alone, GluN1 is retained in the endoplasmic reticulum, this suggests that similarly to APP, APLP1 and APLP2 associate with GluN1 in this subcellular compartment.

To determine if APLP1 and APLP2 co-immunoprecipitate with assembled GluN1/GluN2 NMDA receptors, either GluN1/GluN2A or GluN1/GluN2B combinations were co-expressed with APLP1<sup>c-Myc</sup> or APLP2<sup>c-Myc</sup> in HEK 293 cells, homogenates were collected, detergent solubilised and immunoprecipitations carried with anti-GluN2A or anti-GluN2A/B antibodies. Since neither APLP1<sup>c-Myc</sup> nor APLP2<sup>c-Myc</sup> co-immunoprecipitate with GluN2 subunits, if either is detected in immunoprecipitates, it implies that they would associate with GluN1/GluN2 assembled heteromeric

receptors. The results shown in Figure 2 show that indeed this is the case. Thus immunoprecipitation with anti-GluN2A antibodies results in the detection of GluN1 and APLP1<sup>c-Myc</sup> or APLP2<sup>c-Myc</sup> in the respective immune pellets (Figure 2A and 2C). Similarly, immunoprecipitation with anti-GluN2A/2B antibodies to precipitate GluN2B subunits, results in the detection of GluN1 and APLP1<sup>c-Myc</sup> or APLP2<sup>c-Myc</sup> in the respective immune pellets (Figure 2B and 2D).

### **APLP1 and APLP2 associate with NMDA receptors in native brain tissues**

The co-association of APLP1 and APLP2 with native NMDA receptors was also investigated. However in order to do this, it was necessary to validate the specificity of anti-APLP1 and anti-APLP2 antibodies since each was raised against a respective fusion protein which had amino acid similarity with the other family members (see Methods). Thus APP695<sup>FLAG</sup>, APLP1<sup>c-Myc</sup> and APLP2<sup>c-Myc</sup> were each expressed alone in HEK 293 cells, and the resultant transfected solubilized lysates probed with anti-APP (679–695), anti-APLP1 (200–300) and anti-APLP2 (262–491) antibodies. Figure 3A shows that anti-APLP1 antibodies recognize a major band with  $M_r = 99 \text{ kDa} \pm 2$  ( $n = 3$ ) consistent with the predicted size of APLP1; anti-APLP2 antibodies recognize a major band with  $M_r = 134 \text{ kDa} \pm 5$  ( $n = 3$ ) consistent with the predicted size of APLP2. Interestingly, anti-APP (679–695) antibodies recognized APP  $M_r = 103 \text{ kDa} \pm 2$  ( $n = 3$ ) but in addition, also both APLP1 and APLP2. This is not unexpected given the identity between the three isoforms at the respective C-termini. However, the antibody is valid for the recognition of APP since the molecular sizes of APP, APLP1 and APLP2 are distinct (Figure 3A). Despite the fact that the three antibodies recognize immunoreactive species with distinct molecular weights in immunoblots, there is a caveat to be aware of in that in the co-immunoprecipitation assays, the antibodies are targeting native, i.e. folded APLP1, APLP2 and APP. Thus there is still a possibility of cross-immunoreactivity between the three antibodies. Ultimately, the optimum control for the specificity of these antibodies would be the use of the appropriate knock-out mice.

Immunoprecipitations from detergent extracts of adult rat brain were carried out using anti-GluN1 C2 antibodies and immune pellets were analysed for GluN1 immunoreactivity to prove that the immunoprecipitation was successful; for GluN2A and GluN2B immunoreactivities to demonstrate the pelleting of an assembled GluN1/GluN2 NMDA receptor and, anti-APP, anti-APLP1 and anti-APLP2 antibodies to determine possible co-immunoprecipitation with APP family members. It was found that GluN1 was always immunoprecipitated as was GluN2A and GluN2B (Figure 3). Furthermore, APP, APLP1 and APLP2 were also all co-immunoprecipitated by anti-GluN1 C2 antibodies (Figure 3). The immunoreactive bands were strongest for APP and APLP1. It was difficult to detect APLP2 in detergent extracts reflecting a low level of APLP2 expression in adult brain. Nevertheless, a clear immunoreactive species was found in anti-GluN1 C2 immunoprecipitates. As always, control non-immune Ig was used as a negative control and no APP, APLP1 nor APLP2 immunoreactivities were found in these samples (Figure 3).

#### **APLP1 and APLP2 enhance GluN1/GluN2A and GluN1/GluN2B NMDA receptor cell surface expression with no concomitant change in receptor subunits**

In addition to interacting with NMDA receptors, we and others (Hoe *et al.*, 2009) showed that APP enhanced the cell surface expression of NMDA receptors. To determine if this was the case for APLP1 and APLP2, GluN1/GluN2A and GluN1/GluN2B NMDA receptors were co-expressed in parallel with either APLP1, APLP2 and with APP as a positive control and cell surface NMDA receptor expression was measured by ELISA 24 h post-transfection. Similarly to APP, APLP1 and APLP2 both enhanced GluN1/GluN2A and GluN1/GluN2B cell surface receptor expression. There was no significant difference in the fold enhancement when APP was compared to that elicited by APLP1 and APLP2 i.e. for each, an approximate two-fold increase in cell surface expression was observed (Figure 4). Again, similarly to APP the increase in cell surface expression was not accompanied by any observed change in the total expression of GluN1, GluN2A or GluN2B subunits (Figure 4).



## Concluding remarks

The function of APP is unknown, although new insights into its functional significance have recently been gained through the discovery that it contributes to the regulation of surface expression of NMDA receptors (Hoe *et al.*, 2009; Cousins *et al.*, 2009). Since APP is a member of a gene family that contains two other members, it is important to ascertain if the related APP proteins possess the same properties as APP with respect to their interactions with NMDA receptors. Any observed differences or similarities would thus yield further insight into APP/NMDA receptor protein/protein interactions. In summary, both APLP1 and APLP2 behaved similarly to APP in that they both co-immunoprecipitated with the two major NMDA receptor subtypes, GluN1/GluN2A and GluN1/GluN2B, via interaction with the obligatory GluN1 subunit. Furthermore, APLP1 and APLP2 both enhanced GluN1/GluN2A and GluN1/GluN2B cell surface expression to fold-increases that are similar to those induced by APP.

Thus no differences were apparent in the interactions between NMDA receptors and APP, APLP1 and APLP2. A caveat to this however, is that it is known that all three members can form both homo- and hetero-oligomers (Kaden *et al.*, 2012). Since HEK 293 cells express endogenous APP, it cannot be excluded that either APLP1 and/or APLP2 form heterooligomers with APP and that APLP1 and/or APLP2 are found in immune pellets by virtue of the interaction of APP with GluN1. However, in the immunoprecipitations from native brain tissue, single immunoreactive species are detected by anti-APP, anti-APLP1 and anti-APLP2 antibodies. Because each has a distinct molecular weight (Figure 4), co-immunoprecipitation due to hetero-oligomerisation is unlikely. It is still unclear if the association between APP and NMDA receptors is direct or mediated via an intermediary protein. Whichever is the case, the findings reported here show that the protein binding domain responsible for the association must be conserved between the three family members. It is also unclear why the APP/NMDA receptor interaction, be it direct or indirect, results in an enhanced surface expression. Hoe *et al.* (2009) suggested that APP enhanced GluN2B-containing NMDA receptors by decreasing

receptor internalization, but the mechanism by which this occurs remains elusive. Our findings differ in that APP does not distinguish between GluN1/GluN2A and GluN1/GluN2B since the surface expression of both is enhanced by APP, but, again, the mechanism is yet to be resolved. Further studies are necessary to gain more insight into the role of the APP family in the regulation of cell surface NMDA receptor homeostasis and the possible relationship to Alzheimer's disease.

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## FIGURE LEGENDS

### **Figure 1: APLP1 and APLP2, similarly to APP, co-immunoprecipitate with GluN1 but not GluN2 NMDA receptor subunits**

HEK 293 cells were co-transfected with the clones:- APLP1 + GluN1; APLP1 + GluN2A; APLP1 + GluN2B; APLP2 + GluN1; APLP2 + GluN2A and APLP2 + GluN2B, transfected cell homogenates harvested, detergent solubilized, the soluble extracts collected by centrifugation at 100,000g and immunoprecipitated (IP) with non-immune Ig or the appropriate anti-NMDA receptor antibody as shown and immune pellets were analyzed by immunoblotting all as described under Experimental Procedures. The gel lane layout for the immunoblots is identical where lane 1 = detergent soluble extract; lane 2 = non-immune pellet and lane 3 = immune pellet respectively. GluN1 C2, GluN2A, GluN2B and c-Myc are the antibody specificities used to probe the immunoblots. → denotes GluN1, GluN2A, GluN2B, APLP1 ( $M_r = 87 \pm 1$  kDa) and APLP2 ( $M_r = 141 \pm 3$  kDa) where appropriate. The positions of molecular weight standards ( $\times 10^3$  Da) are shown on the right. The immunoblots are representative of at least  $n = 3$  independent immunoprecipitations from  $n = 3$  independent transfections.

### **Figure 2: Assembled GluN1/GluN2A and GluN1/GluN2B co-immunoprecipitate with APLP1 and APLP2**

HEK 293 cells were co-transfected with the clones:- APLP1 + GluN1+ GluN2A or GluN2B; APLP2 + GluN1 + GluN2A or GluN2B, transfected cell homogenates harvested, detergent solubilized, the soluble extracts collected by centrifugation at 100,000g and immunoprecipitated (IP) with non-immune Ig or anti-GluN2A or anti-GluN2A/B antibodies as shown and immune pellets were analyzed by immunoblotting all as described under Experimental Procedures. The gel lane layout for the immunoblots is identical where lane 1 = detergent soluble extract; lane 2 = non-immune pellet and lane 3 = immune pellet respectively. GluN1 C2, GluN2A, GluN2B and c-Myc are the antibody specificities used to probe the immunoblots. → denotes GluN1, GluN2A, GluN2B, APLP1 and APLP2 where appropriate. The positions of molecular weight standards ( $\times 10^3$  Da) are shown on the

right. The immunoblots are representative of at least  $n = 3$  independent immunoprecipitations from  $n = 3$  independent transfections.

**Figure 3: APLP1 and APLP2 associate with GluN1/GluN2A and GluN1/GluN2B NMDA receptors in the brain**

Detergent extracts (100,000g) were prepared from adult rat brain, immunoprecipitations carried out using anti-GluN1 C2 antibodies or non-immune Ig and immune pellets were analysed by immunoblotting using anti-GluN1 C2; anti-GluN2A; anti-GluN2B, anti-APLP1 and anti-APLP2 antibodies. → denotes GluN1, GluN2A, GluN2B, APP, APLP1 and APLP2. The positions of molecular weight standards ( $\times 103$  Da) are shown on the right. The immunoblots are representative of at least  $n = 3$  independent immunoprecipitations each carried out in triplicate from at least three separate detergent extract preparations.

**Figure 4: APLP1 and APLP2, similarly to APP, enhance GluN1/GluN2A and GluN1/GluN2B NMDA receptor cell surface expression with no concomitant change in receptor subunits**

A and B, HEK 293 cells were co-transfected in triplicate in parallel with the clones shown and cell surface receptor expression was measured 20 h post-transfection using anti-GluN2A 44-58 Cys or anti-GluN2B 46-60 Cys antibodies as appropriate. The results are expressed as the fold increase in cell surface expression where the expression of control transfections i.e. GluNR1/GluN2A/pCIS and GluNR1/GluN2B/pCIS = 1. Values are the means  $\pm$  SEM for at least the following independent transfections,  $n = 6$  (GluN1/GluN2A; GluN1/GluN2A/APP; GluN1/GluN2A/APLP1; GluN1/GluN2A/APLP2);  $n = 4$  (GluN1/GluN2B; GluN1/GluN2B/APP; GluN1/GluN2B/APLP1; GluN1/GluN2B/APLP2); C and D, HEK 293 cells were co-transfected with GluN1/GluN2A or GluN2B/APP; GluN1/GluN2A or GluN2B/APLP1 or, GluN1/GluN2A or GluN2B/APLP2 cell homogenates collected and analysed by quantitative immunoblotting all as described in Experimental Procedures. The results are expressed as the fold change in subunit expression i.e. in the presence divided by in the absence of exogenous APP; APLP1 or APLP2. Results are the mean  $\pm$  SEM for at

least n = 3 immunoblots from 3 independent transfections for all transfections. \* p < 0.025\*\*; p < 0.01; \*\*\* p < 0.005; \*\*\*\* p < 0.001; \*\*\*\*\* p < 0.0005.

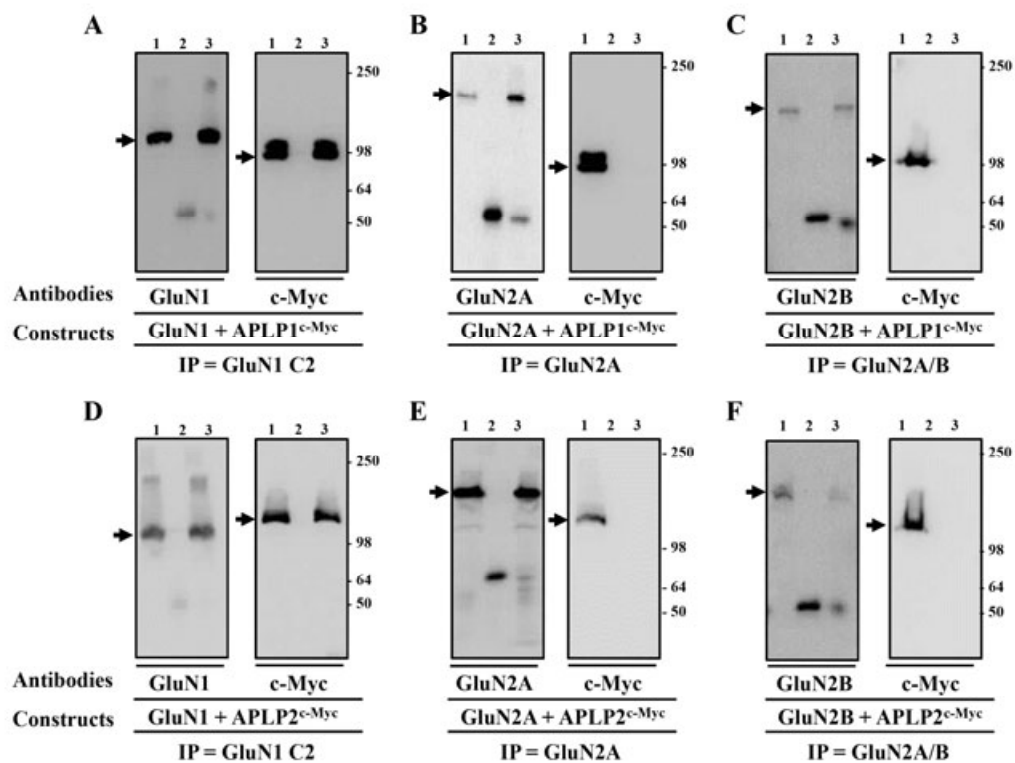


Figure 1

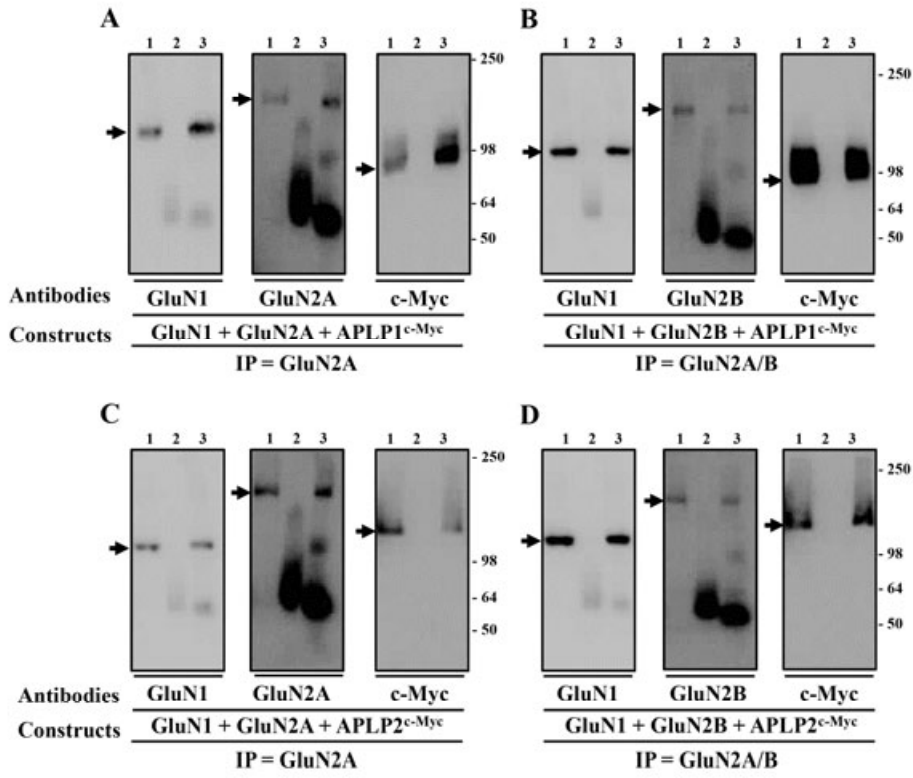


Figure 2



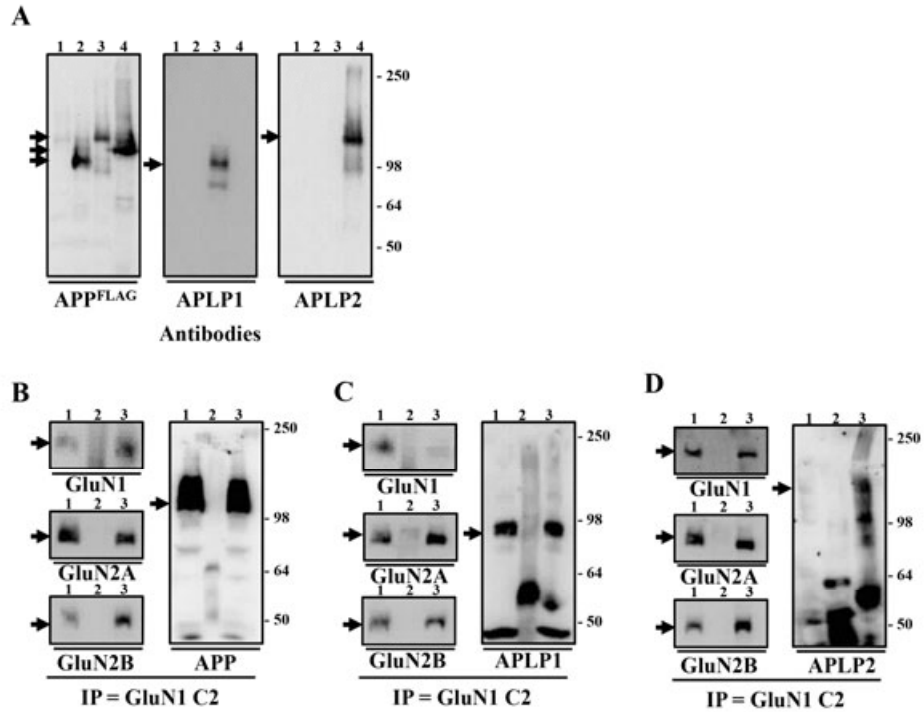


Figure 3

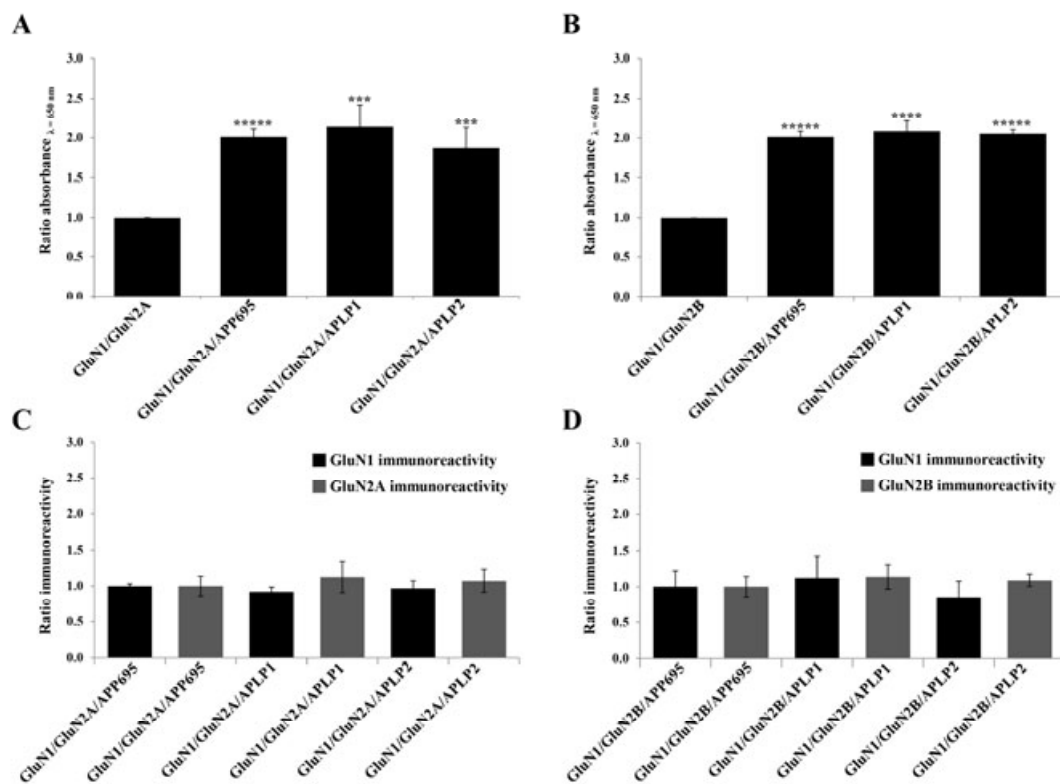


Figure 4