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ORIGINAL ARTICLE

Neto1 associates with the NMDA receptor/amyloid precursor protein complex

Sarah L. Cousins, Neal Innocent and F. Anne Stephenson

University College London School of Pharmacy, London, UK

Abstract

Neuropilin tolloid-like 1 (Neto1), is a CUB domain-containing transmembrane protein that was recently identified as a novel component of the NMDA receptor complex. Here, we have investigated the possible association of Neto1 with the amyloid precursor protein (APP)695/GluN1/GluN2A and APP695/GluN1/GluN2B NMDA receptor trafficking complexes that we have previously identified. Neto1^{HA} was shown to co-immunoprecipitate with assembled NMDA receptors via GluN2A or GluN2B subunits; Neto1^{HA} did not co-immunoprecipitate APP695^{FLAG}. Co-immunoprecipitations from mammalian cells co-transfected with APP695^{FLAG}, Neto1^{HA} and GluN1/GluN2A or GluN1/GluN2B revealed that all four proteins co-exist within one macromolecular complex. Immunoprecipitations from native brain tissue similarly revealed the existence of a GluN1/GluN2A or GluN2B/APP/Neto1 complex. Neto1^{HA} caused a reduction in the surface expression of both NMDA receptor subtypes, but had no effect on APP695^{FLAG}- or PSD-95$\alpha^{c\text{-}Myc}$ enhanced surface receptor expression. The Neto1 binding domain of GluN2A was mapped using GluN1/GluN2A chimeras and GluN2A truncation constructs. The extracellular GluN2A domain does not contribute to association with Neto1^{HA} but deletion of the intracellular tail resulted in a loss of Neto-1^{HA} co-immunoprecipitation which was paralleled by a loss of association between GluN2A and SAP102. Thus, Neto1 is concluded to be a component of APP/NMDA receptor trafficking complexes.

Keywords: Alzheimer's disease, amyloid precursor protein, Neto1, NMDA receptors, post-synaptic density 95, SAP102.

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*Read the **Editorial Highlight** for this article on page 551.*

Neuropilin tolloid-like 1 (Neto1) and Neuropilin tolloid-like 2 (Neto2) are complement C1r/C1s, Uegf, Bmp1 (CUB) domain-containing transmembrane proteins that were recently identified as novel components of glutamatergic neurotransmitter receptor complexes. Neto1 and Neto2 have both been described as auxiliary subunits of kainate receptors since they were shown to alter the trafficking, channel kinetics and pharmacology of this glutamate receptor subtype in a subunit-dependent manner (Zhang *et al.* 2009; Copits *et al.* 2011; Straub *et al.* 2011a, b; Tang *et al.* 2011; Fisher and Mott 2012; Straub *et al.*, 2011b). In *C.elegans*, SOL-1 and SOL-2, species orthologues of Neto, were shown to be auxiliary subunits of GLR-1, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) glutamatergic receptors (Wang *et al.* 2012). In addition, Neto1 was shown to be a novel component of the *N*-methyl-D-aspartate (NMDA) receptor complex that was critical for maintaining the abundance of GluN2A receptors within the post-synaptic density (Ng *et al.* 2009). Neto1 was enriched in the post-synaptic density where it was shown to co-distribute with the scaffold protein, post-synaptic density-95 (PSD-95), and GluN1 (Ng *et al.* 2009). Neto1 (−/−) mice were found to have deficits in long-term potentiation and NMDA receptor-dependent spatial learning and memory (Ng *et al.* 2009).

We have previously shown (Cousins *et al.* 2009), in agreement with Hoe *et al.* (2009), that amyloid precursor protein (APP) co-immunoprecipitates with assembled GluN1/GluN2 NMDA receptors. Over-expression of the neuronal splice variant of APP, APP695, results in an increase in NMDA receptor cell surface expression in cell lines (Cousins *et al.* 2009). Similarly, over-expression of APP770 resulted in an enhanced NMDA receptor expression in primary cultures

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Address correspondence and reprint requests to F. Anne Stephenson, University College London School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX, UK. E-mail: anne.stephenson@ucl.ac.uk

Abbreviations used: AMPA, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid; APP, amyloid precursor protein; CUB, complement C1r/C1s, Uegf, Bmp1; 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; PSD-95, post-synaptic density 95; SAP102, synapse associated protein 102.

of neuronal cells (Hoe *et al.* 2009). Both these findings are compatible with an ascribed role for APP as a mediator of neuronal trafficking mechanisms possibly functioning as an NMDA receptor auxiliary protein. Since Neto1 was reported to be associated with NMDA receptors and crucially, to play a role in NMDA receptor-mediated learning and memory, it was of interest to determine if Neto1 is a constituent of NMDA receptor/APP macromolecular complexes. We have carried out an investigation to determine if this is the case and we report the findings in this article.

Experimental procedures

Constructs and antibodies

The constructs, pCISGluN1-1a, pCISGluN2A, pCISGluN2B, pCISGluN2B^{FLAG} and pCI-neoAPP695^{FLAG} were as previously described (Cik *et al.* 1993; Hawkins *et al.* 1999; Cousins *et al.* 2009). DNA encoding GluN2A 838-1464 was cloned into the BamHI/EcoRI restriction enzyme sites of pcDNA4 to generate pcDNA4GluN2A 838-1464 yielding the GluN2A C-terminal domain with an N-terminal His tag. 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. DNA encoding Neto1 and Neto 1-372 were cloned via EcoRV/Sal1 restriction enzyme sites of pCMV-4a to generate pCMVNeto1 and pCMVNeto1 1-372 yielding full length Neto1 and Neto1 1-372 with a C-terminal FLAG tag. pGW1PSD-95$\alpha^{c\text{-}Myc}$ and pCMVneo-SAP102$^{c\text{-}Myc}$ were kind gifts of Dr M. Sheng (Genentech Inc., San Francisco, CA, USA). pcDNA3.1Neto1^{HA} was a kind gift from Drs R.R. McInnes (McGill University, Montreal, Canada) and M.W. Salter (University of Toronto, Toronto, Canada) and pCISGluN2A 1-877 a kind gift of Professor G Hardingham (University of Edinburgh).

Anti-GluN1 C2, anti-GluN2A (44–58), anti-GluN2A (1381–1394), anti-GluN2B (46–60) and anti-APP (679–695) antibodies 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-Neto1 (447-533) antibodies were as in Chow *et al.* (2004) and were a kind gift from Drs R.R. McInnes (McGill University) and M. W. Salter (University of Toronto). Anti-HA and anti-synapse-associated protein 102 (SAP102) antibodies were from Abcam (Cambridge, UK); anti-FLAG M2 antibodies were from Sigma-Aldrich (Dorset, UK). Anti-GluN2B antibodies which were raised from a 30 kDa GluN2B C-terminal fusion protein were from Millipore (Billerica, MA, USA). Anti-His antibodies were from Life Technologies Ltd (Paisley, Scotland).

Generation of GluN1/GluN2A NMDA receptor chimeric subunits

Two sets of GluN1/GluN2A chimeras were generated by overlap extension PCR. For the first set, the respective N-terminal regions were swapped such that (GluN1)/GluN2A contained GluN1 1-561 followed by GluN2A 557-1464 and (GluN2A)GluN1 contained GluN2A 1-556 followed by GluN1 562-938. For the second set, the extracellular S2 domains of GluN1 and GluN2A were exchanged. Thus GluN1 (GluN2A S2)/GluN1 contained GluN2A 648-816 and GluN2A (GluN1 S2)/GluN2A contained GluN1 650-812.

Mammalian cell transfections

Human embryonic kidney (HEK) 293 cells were transfected using the calcium phosphate method as described (Cik *et al.* 1993). For double transfections where one clone was Neto1, 10 μg DNA were used at a 1 : 1 ratio, for example, 5 μg Neto1^{HA}: 5 μg GluN1. For triple transfections a total of 20 μg DNA was used with a ratio of 1 : 3 GluN1 : GluN2A (10 μg total) with 10 μg Neto1^{HA}, 10 μg APP695^{FLAG} or 10 μg PSD-95$\alpha^{c\text{-}Myc}$. For quadruple transfections, a total of 30 μg DNA was used with a ratio of 1 : 3 GluN1 : GluN2A (10 μg total) with 10 μg Neto1^{HA} and 10 μg APP695^{FLAG}. 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 either 1% (v/v) Triton X-100 100 000 g detergent extracts of HEK 293 transfected cells or 1% (w/v) sodium deoxycholate 100 000 g extracts of adult rat brain (Cousins *et al.* 2009). Immunoprecipitating antibodies used were anti-GluN1 C2, anti-GluN2A (1381–1394), anti-GluN2B (46–60), anti-APP (679–695), anti-His, anti-HA, anti-FLAG or non-immune Ig antibodies. The resulting immune pellets were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis followed by immunoblotting.

Immunoblotting

Immunoblotting was carried out using 7.5% (w/v) polyacrylamide slab mini-gels all as described previously (Papadakis *et al.* 2004). Primary antibodies used were as follows: anti-GluN1 C2; anti-GluN2A (1381–1394); anti-APP (679–695); anti-Neto1 (447-533), anti-GluN2B, anti-SAP102, anti-FLAG and anti-HA. 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 the 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 and for quadruple transfections and in brain where four different antibodies were used for immunoblotting of immune pellets, 12% of the immune pellets were analysed by the precipitating primary antibody, 25% of the pellets for two additional specificity antibodies and always 38% for anti-Neto1 activity.

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 NMDA receptors are only expressed at the cell surface when they are co-assembled as

GluN1/GluN2A or GluN1/GluN2B receptors (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 ± SEM and were analysed by the Student's two-tailed paired *t*-test.

Results and Discussion

Neto1^{HA} co-immunoprecipitates with assembled heteromeric NMDA receptors via GluN2

The association of NMDA receptors with Neto1 is controversial. Ng *et al.* (2009) reported that anti-Neto1 antibodies immunoprecipitated GluN1, GluN2A and GluN2B from radio-immunoprecipitation assay buffer (a buffer containing the detergents, 1% Non-idet P-40, 0.1% sodium dodecyl sulfate and 0.5% deoxycholate)-solubilized extracts of synaptosomes. Co-immunoprecipitation was independent of PSD-95 since all three GluNs co-immunoprecipitated with a Neto1 construct, Neto1-Δ20HA, which lacked an intracellular domain and also did not co-immunoprecipitate with PSD-95 (Ng *et al.* 2009). In contrast, Straub *et al.* (2011a, b) using 1% Triton to solubilize rat brain lysates could not detect GluN1 or indeed PSD-95 in anti-Neto1 or anti-Neto2 immune pellets thus inferring that since all NMDA receptors contain GluN1, Neto1 and Neto2 do not associate with NMDA receptors. To resolve these different observations, we carried out immunoprecipitations from detergent extracts of HEK 293 cells transfected with the single clones GluN1, GluN2A, GluN2B; GluN1/GluN2A and GluN1/GluN2B binary combinations and 1% sodium deoxycholate extracts of rat brain. The results are shown in Figs 1–3.

First, for single subunit transfections, Neto1^{HA} was co-immunoprecipitated by anti-GluN2A and anti-GluN2B but not anti-GluN1 antibodies indicating, in agreement with Ng *et al.* (2009) that Neto1^{HA} associates with GluN2A and GluN2B subunits (Fig. 1). Since Neto1^{HA} does not associate with GluN1, in binary transfections, immunoprecipitations were carried out with anti-GluN1 antibodies thus if Neto1

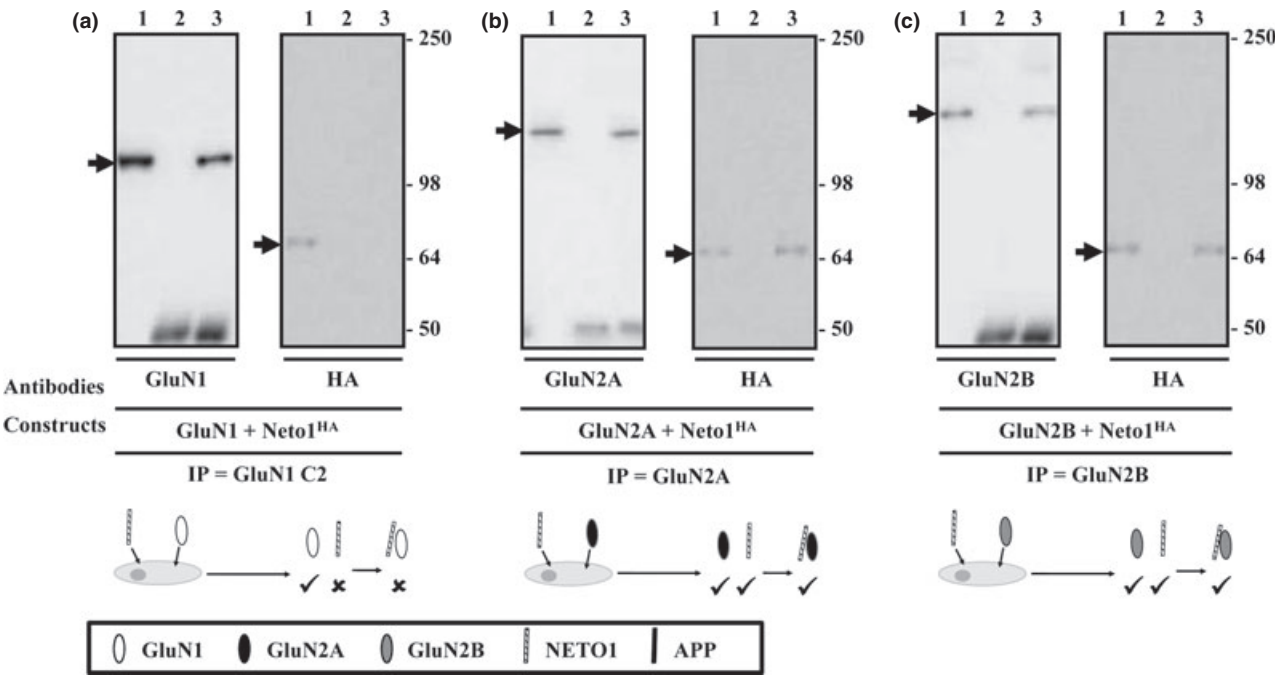

Fig. 1 Neto1^{HA} co-associates with GluN2A and GluN2B but not GluN1 in transfected human embryonic kidney (HEK) 293 cells: demonstration by co-immunoprecipitation. HEK 293 cells were co-transfected with the clones:- GluN1 + Neto1^{HA} (a); GluN2A + Neto1^{HA} (b) and GluN2B + Neto1^{HA} (c). Transfected cell homogenates were harvested, detergent solubilized, the soluble extracts collected by centrifugation at 100 000 *g*, and immunoprecipitated with non-immune Ig, anti-GluN1 C2 (a), anti-GluN2A (b) or anti-GluN2B (c) antibodies and immune pellets were analysed 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 = anti-NR1 C2, anti-GluN2A or anti-GluN2B immune pellet respectively. GluN1, GluN2A, GluN2B and HA are the antibody specificities used to probe the immunoblots. → denotes GluN1, GluN2A, GluN2B and Neto1^{HA} where appropriate. The positions of molecular weight standards (× 10^3 Da) are shown on the right. The schematic diagrams illustrate the experimental protocol and the results such that √ = detection in immune pellets and X = no detection in immune pellets. This key applies for all subsequent figures. The immunoblots are representative of at least $n = 3$ independent immunoprecipitations from $n = 3$ independent transfections.

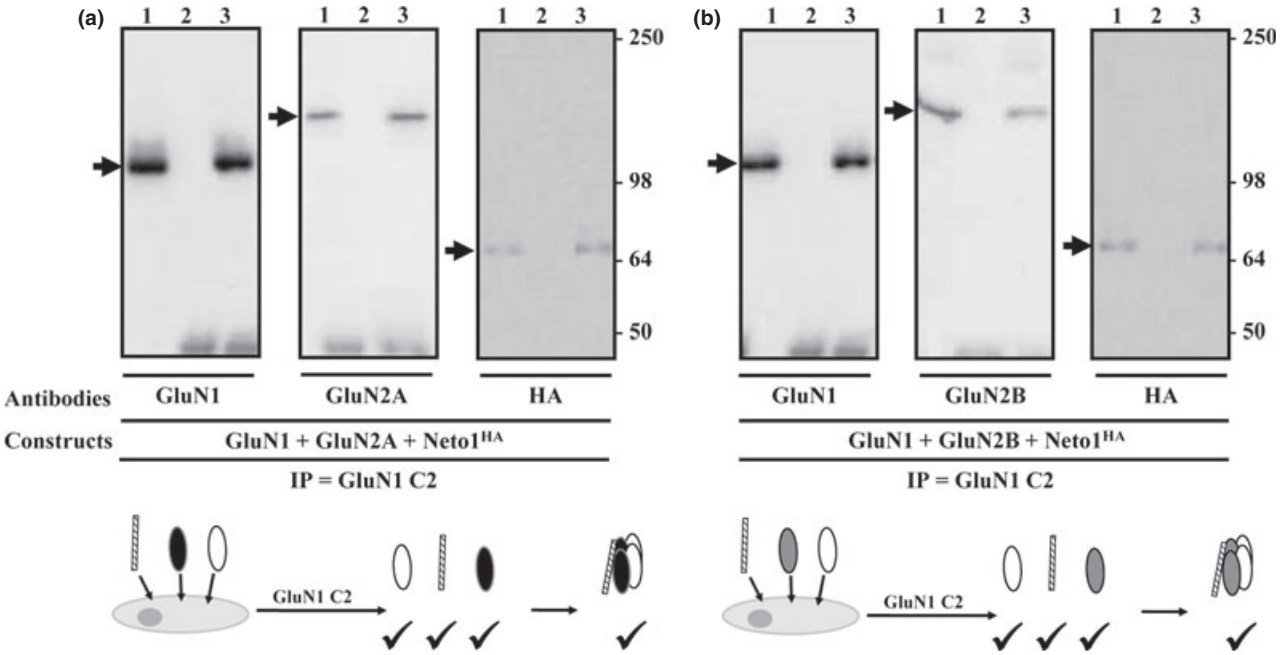

Fig. 2 Neto1^{HA} co-associates with assembled GluN1/GluN2A and GluR1/GluN2B NMDA receptors in transfected human embryonic kidney (HEK) 293 cells: demonstration by immunoprecipitation with anti-NR1 C2 antibodies. HEK 293 cells were co-transfected with the clones:- GluN1 + GluN2A + Neto1^{HA} (a) or GluN1 + GluN2B + Neto1^{HA} (b). Transfected cell homogenates were harvested, detergent solubilized, the soluble extracts collected by centrifugation at 100 000 *g*, and immunoprecipitated with non-immune Ig or anti-NR1 C2 antibodies and immune pellets were analysed 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 = anti-GluN1 C2 immune pellet respectively. GluN1, GluN2A, GluN2B and HA are the antibody specificities used to probe the immunoblots. → denotes GluN1, GluN2A, GluN2B and Neto1^{HA} where appropriate. The positions of molecular weight standards (× 10^3 Da) are shown on the right. The immunoblots are representative of at least $n = 3$ independent immunoprecipitations from $n = 3$ independent transfections.

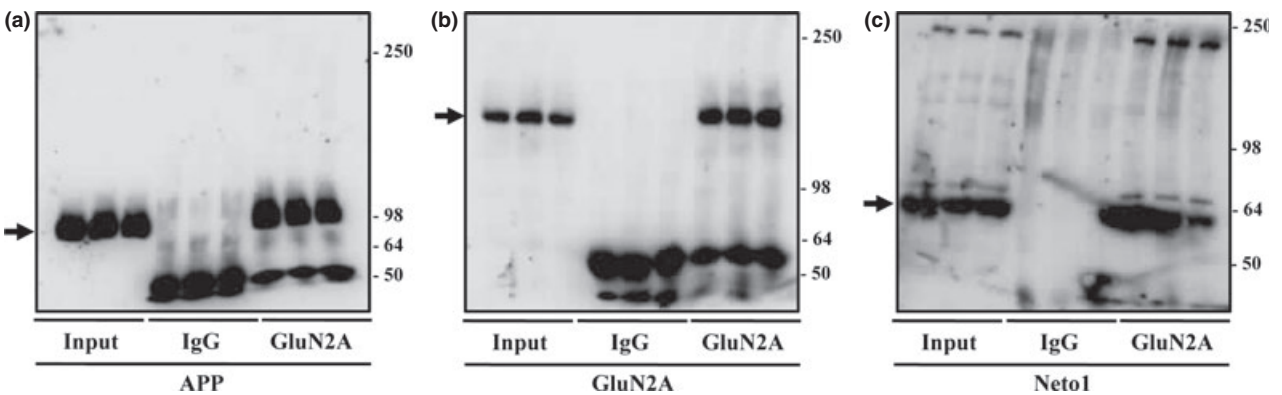

Fig. 3 GluN2A co-immunoprecipitates both amyloid precursor protein (APP) and Neto1 from detergent extracts of adult mammalian brain. Detergent extracts (100 000 g) were prepared from adult rat brain, immunoprecipitations in triplicate carried out using anti-NR2A (1381-1394) antibodies or non-immune Ig and immune pellets were analysed by immunoblotting. (a) immunoblotting with anti-GluN2A antibodies; (b) immunoblotting with anti-APP antibodies and (c) immunoblotting with anti-Neto1 antibodies. The gel lane layout is identical for each immunoblot where Input = triplicate samples of detergent soluble extract; Ig = triplicate non-immune pellets and GluN2A = triplicate anti-GluN2A (1381-1394) immune pellets. → denotes GluN2A, APP and Neto1 where appropriate. The positions of molecular weight standards (× 10^3 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.

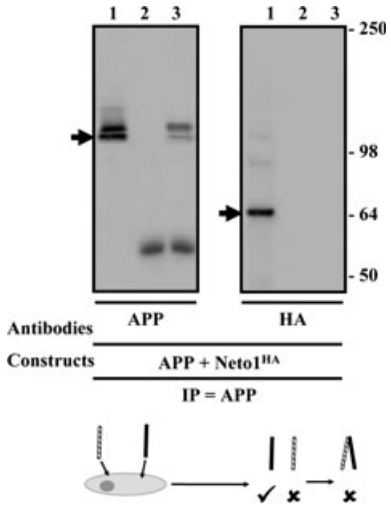

Fig. 4 Amyloid precursor protein (APP)695^{FLAG} does not co-immunoprecipitate Neto1^{HA} from transfected human embryonic kidney (HEK) 293 cells. HEK 293 cells were co-transfected with the clones: APP695^{FLAG} + Neto1^{HA}, transfected cell homogenates harvested, detergent solubilized, the soluble extracts collected by centrifugation at 100 000 *g* and immunoprecipitated with non-immune Ig or anti-APP antibodies and immune pellets were analysed 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 = anti-APP immune pellet respectively. APP and HA are the antibody specificities used to probe the immunoblots. → denotes APP and Neto1^{HA} where appropriate. The positions of molecular weight standards (× 10^3 Da) are shown on the right. The immunoblots are representative of at least $n = 3$ independent immunoprecipitations from $n = 3$ independent transfections.

and GluN2A or GluN2B were detected in immune pellets, it must mean that Neto1^{HA} associates with assembled GluN1/GluN2A and GluN1/GluN2B NMDA receptors. The results in Fig. 2 show that this is indeed the case with GluN1, GluN2A or GluN2B and Neto1^{HA} being found in all immune but not in control, non-immune pellets. Finally, anti-GluN2A antibodies immunoprecipitated Neto1 from detergent extracts of rat brain (Fig. 3). Thus, these results concur with those of Ng *et al.* (2009) in that at least Neto1 associates with GluN2-containing NMDA receptors. The differences between the two original reports may be explained by the detergent extraction conditions. NMDA receptors are notoriously difficult to solubilize from native tissue because of their embedding within the post-synaptic density. It was previously shown that 1% Triton, the conditions employed by Straub *et al.* (2011a, b), solubilizes a pool of unassembled GluN1 subunits (Chazot and Stephenson 1997) hence the detection of GluN1 in brain lysates but not in immune pellets because of the lack of soluble GluN2 subunits. However, harsher detergent conditions, that is, 1% sodium deoxycholate as used here or radio-immunoprecipiation assay buffer as in Ng *et al.* (2009) are required to solubilize GluN1/GluN2 heteromers. Since association with Neto1 is via GluN2, co-association is only detected in immune pellets from brain extracts solubilized with these detergents.

It should also be noted that here, immunoprecipitations were carried out from 100 000 g detergent extracts. Ng *et al.* (2009) used a 14 000 g supernatant which is not a *bona fide* soluble preparation, nevertheless, our findings concur.

GluN1/GluN2 assembled NMDA receptors form ternary complexes with APP and Neto1

To study the possible association of Neto1 with NMDA receptor/APP complexes first, it was investigated if APP695^{FLAG} could associate with Neto1^{HA}. Both were co-expressed in HEK 293 cells and immunoprecipitations carried out using anti-APP antibodies. APP695^{FLAG} was found in immune pellets but Neto1 was not detected thus Neto1^{HA} does not co-immunoprecipitate with APP695^{FLAG} (Fig. 4). To determine if Neto1^{HA} associates with NMDA receptor/APP695^{FLAG} complexes, GluN1, GluN2A or GluN2B, APP695^{FLAG} and Neto1^{HA} were co-expressed in HEK 293 cells and immunoprecipitations carried out with anti-APP antibodies. Immunoreactivities for all four expressed proteins were detected in immune but not in non-immune pellets (Fig. 5). Since APP interacts with only GluN1; APP does not interact with GluN2 or Neto1 alone; GluN1 interacts with GluN2 and all four proteins are found in the immune pellet, it must mean that they are all co-associated in one macromolecular complex, that is, APP695^{FLAG}/GluN1/GluN2/Neto1^{HA} (Fig. 5).

To determine if the quaternary complex is found in native tissue, anti-APP immunoprecipitations were carried out from detergent extracts of adult rat brain (Fig. 6). GluN2A, GluN2B and Neto1 immunoreactivities were all detected in immune but not in control, non-immune pellets thus showing that all four proteins can be found in one complex. Further, this is the case for both GluN2A- and GluN2B-containing NMDA receptors (Fig. 6). Note that the existence of the APP/GluN1/GluN2/Neto1 quaternary complex is demonstrated by co-immunoprecipitation, although these experiments show that the four proteins are associated they do not show that they each interact directly. Association between different pairs may be because of an intermediary accessory protein as illustrated in Fig. 5.

The effect of Neto1^{HA} on the surface expression of NMDA receptors

We previously showed that APP695^{FLAG} enhanced the surface expression of both GluN1/GluN2A and GluN1/GluN2B NMDA receptors. Since we showed that APP and Neto1 are both part of the NMDA receptor macromolecular complex, it was of interest to determine the effect of Neto1 on NMDA receptor surface expression and APP695^{FLAG}-enhanced NMDA receptor surface expression. Thus in parallel, GluN1/GluN2A and GluN1/GluN2B were both co-expressed with

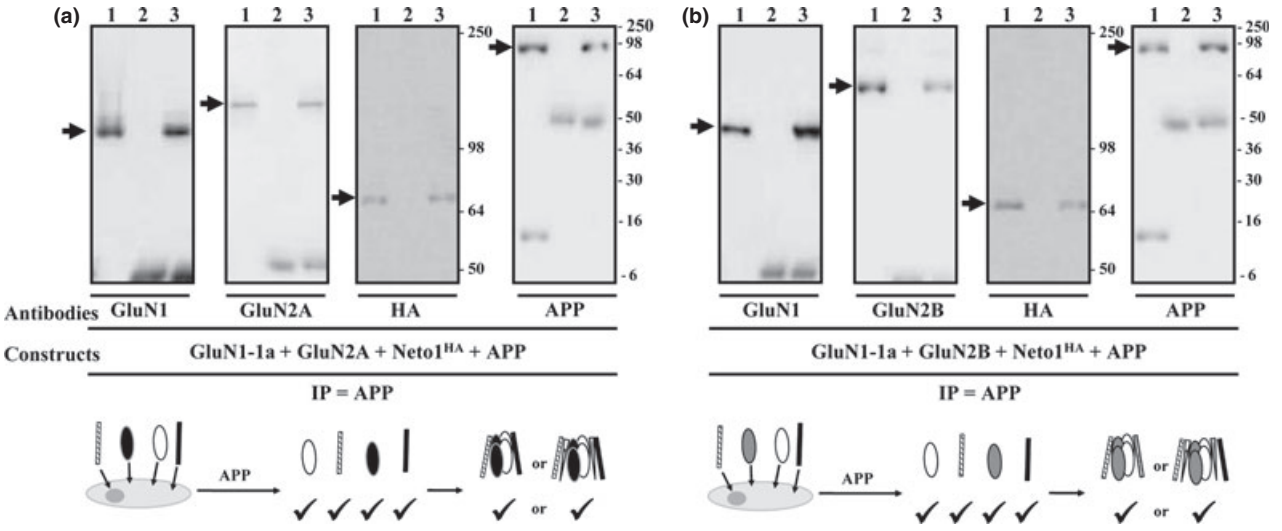

Fig. 5 APP695^{FLAG}/GluN1/GluN2A/Neto1^{HA} and APP695^{FLAG}/GluN1/GluN2B/Neto1^{HA} form ternary complexes in transfected human embryonic kidney (HEK) 293 cells: demonstration by immunoprecipitation with anti-APP antibodies. HEK 293 cells were co-transfected with the clones: GluN1, GluN2A, APP695^{FLAG} and Neto1^{HA} (a) and GluN1, GluN2B, APP695^{FLAG} and Neto1^{HA} (b), transfected cell homogenates harvested, detergent solubilized, the soluble extracts collected by centrifugation at 100 000 *g* and immunoprecipitated with non-immune Ig or anti-APP antibodies and immune pellets were analysed 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 = anti-APP immune pellet respectively. APP, GluN1, GluN2A, GluN2B and HA are the antibody specificities used to probe the immunoblots. → denotes APP, GluN1, GluN2A, GluN2B and Neto1^{HA} where appropriate. The positions of molecular weight standards (× 10^3 Da) are shown on the right. The immunoblots are representative of at least *n* = 3 independent immunoprecipitations from *n* = 3 independent transfections.

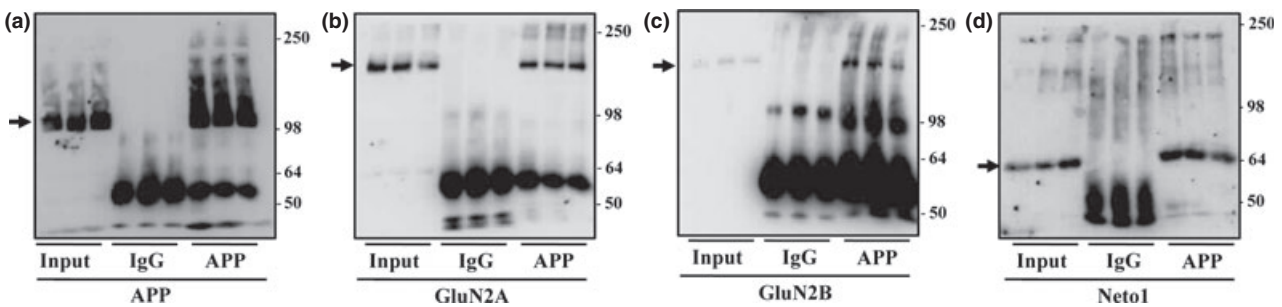

Fig. 6 APP/GluN2A/Neto1 and APP/GluN2B/Neto1 complexes exist in the brain: demonstration by immunoprecipitation with anti-APP antibodies. Detergent extracts (100 000 g) were prepared from adult rat brain, immunoprecipitations in triplicate carried out using anti-APP antibodies or non-immune Ig and immune pellets were analysed by immunoblotting. (a) Immunoblotting with anti-APP antibodies; (b) immunoblotting with anti-GluN2A antibodies, (c) immunoblotting with anti-GluN2B antibodies and (d) with anti-Neto1 antibodies. The gel lane layout is identical for each immunoblot where Input = triplicates samples of detergent soluble extract; Ig = triplicate non-immune pellets and APP (671-695) = triplicate anti-APP (1381-1394) immune pellets. → denotes APP, GluN2A, GluN2B and Neto1 where appropriate. The positions of molecular weight standards (× 10^3 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.

either PSD-95$\alpha^{c\text{-}Myc}$; SAP102$^{c\text{-}Myc}$; APP695^{FLAG}; Neto1^{HA}; PSD-95$\alpha^{c\text{-}Myc}$ + Neto1^{HA} or APP695^{FLAG} + Neto1^{HA} and surface expression was determined 24 h post-transfection. The results are summarized in Fig. 7.

As previously reported, PSD-95$\alpha^{c\text{-}Myc}$ and APP695^{FLAG} both enhanced GluN1/GluN2A and GluN1/GluN2B surface expression; SAP102$^{c\text{-}Myc}$ had no effect (Rutter *et al.* 2002; Cousins *et al.* 2008, 2009). Interestingly, Neto1^{HA} resulted in a significant decrease in surface expression for both GluN1/GluN2A (28 ± 5%, *n* = 13) and GluN1/GluN2B (30 ± 7%, *n* = 6) but with no change in total GluN1, GluN2A or GluN2B expression (Fig. 7). Neto1^{HA} had no effect on PSD-95$\alpha^{c\text{-}Myc}$- or APP695^{FLAG}-enhanced NMDA receptor expression (Fig. 7). Ng *et al.* (2009) reported that a lack of Neto1 as in Neto1 (−/−) mice does not alter the surface expression of GluN2A or GluN2B, rather a significant (~ 30%) decrease in GluN2A in the postsynaptic density was observed suggesting that Neto1 is

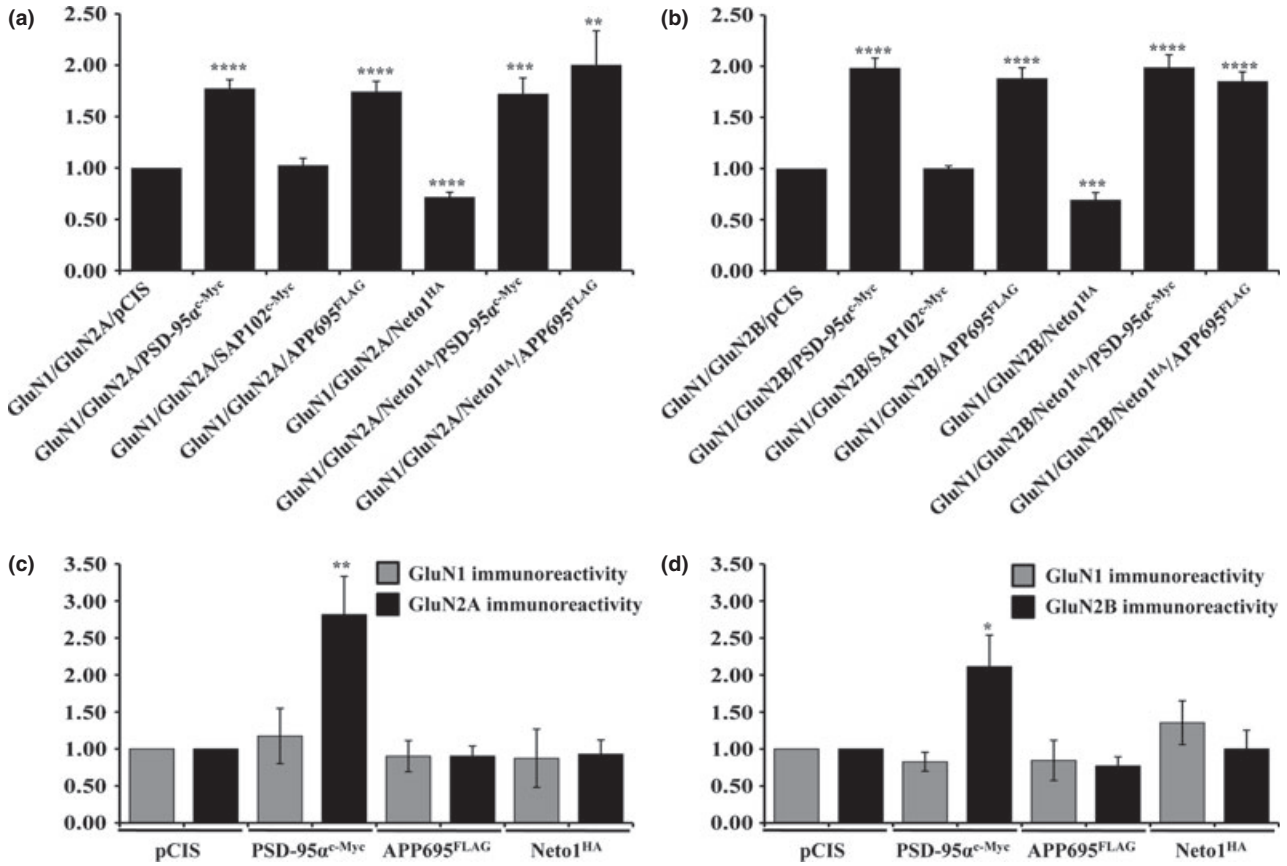

Fig. 7 Neto1^{HA} decreases surface expression of GluN1/GluN2A and GluN1/GluN2B receptors. (a and b) Human embryonic kidney (HEK) 293 cells were co-transfected in triplicate in parallel with the clones shown and cell surface receptor expression was measured 20 h post-transfection using anti-GluN2A 44-58 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, that is, GluNR1/GluN2A/pCIS and GluNR1/GluN2B/pCIS = 1. Values are the means ± SEM for at least the following independent transfections, $n = 13$ (GluN1/GluN2A; GluN1/GluN2A/PSD-95; GluN1/GluN2A/SAP102; GluN1/GluN2A/APP; GluN1/GluN2A/Neto1); $n = 6$ (GluN1/GluN2A/PSD-95/Neto1 and GluN1/GluN2A/APP/Neto1; $n = 6$ (GluN1/GluN2B; GluN1/GluN2B/PSD-95; GluN1/GluN2B/SAP102; GluN1/GluN2B/APP; GluN1/GluN2B/Neto1; GluN1/GluN2A/PSD-95/Neto1 and GluN1/GluN2A/APP/Neto1). (c and d) HEK 293 cells were co-transfected with GluN1/GluN2A and GluN1/GluN2B plus the clone indicated on the abscissa, 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, that is, in the presence divided by in the absence of exogenous APP695^{FLAG}. Results are the mean ± SEM for $n = 3$ immunoblots from three independent transfections. $^{*}p < 0.025$; $^{**}p < 0.01$; $^{***}p < 0.005$; $^{****}p < 0.0005$.

involved in the targeting and/or stability of GluN2A-NMDA receptors at synapses. The findings here show that Neto1 can influence surface NMDA receptor expression although in the presence of PSD-95 as would be the case *in vivo*, the Neto1-induced decrease in surface expression is not apparent. A decrease in receptor cell surface expression with no change in total overall expression levels of GluN1, GluN2A and GluN2B subunits suggests that either Neto1 enhances receptor internalization or it can decrease receptor insertion into the plasma membrane. The presence and thus association of assembled GluN1/GluN2A receptors with PSD-95 may prevent these possibilities.

Intracellular GluN2A C-terminal domains mediate association of Neto1^{HA} with NMDA receptors

Neto1 is a single transmembrane domain protein comprising of two extracellular CUB domains of ~ 110 amino acids, a low-density lipoprotein receptor domain class A motif and a cytoplasmic tail of ~ 167 amino acids that includes a C-terminal TRV class I PDZ binding motif (Fig. 8). Ng *et al.* (2009) showed that removal of the Neto1 cytoplasmic C-terminal tail and the transmembrane domain did not abolish Neto1/NMDA receptor interaction. Further, a Neto1 construct that contained only the signal sequence and the CUB1 domain retained the ability to associate with GluN2A; thus, it was concluded that the

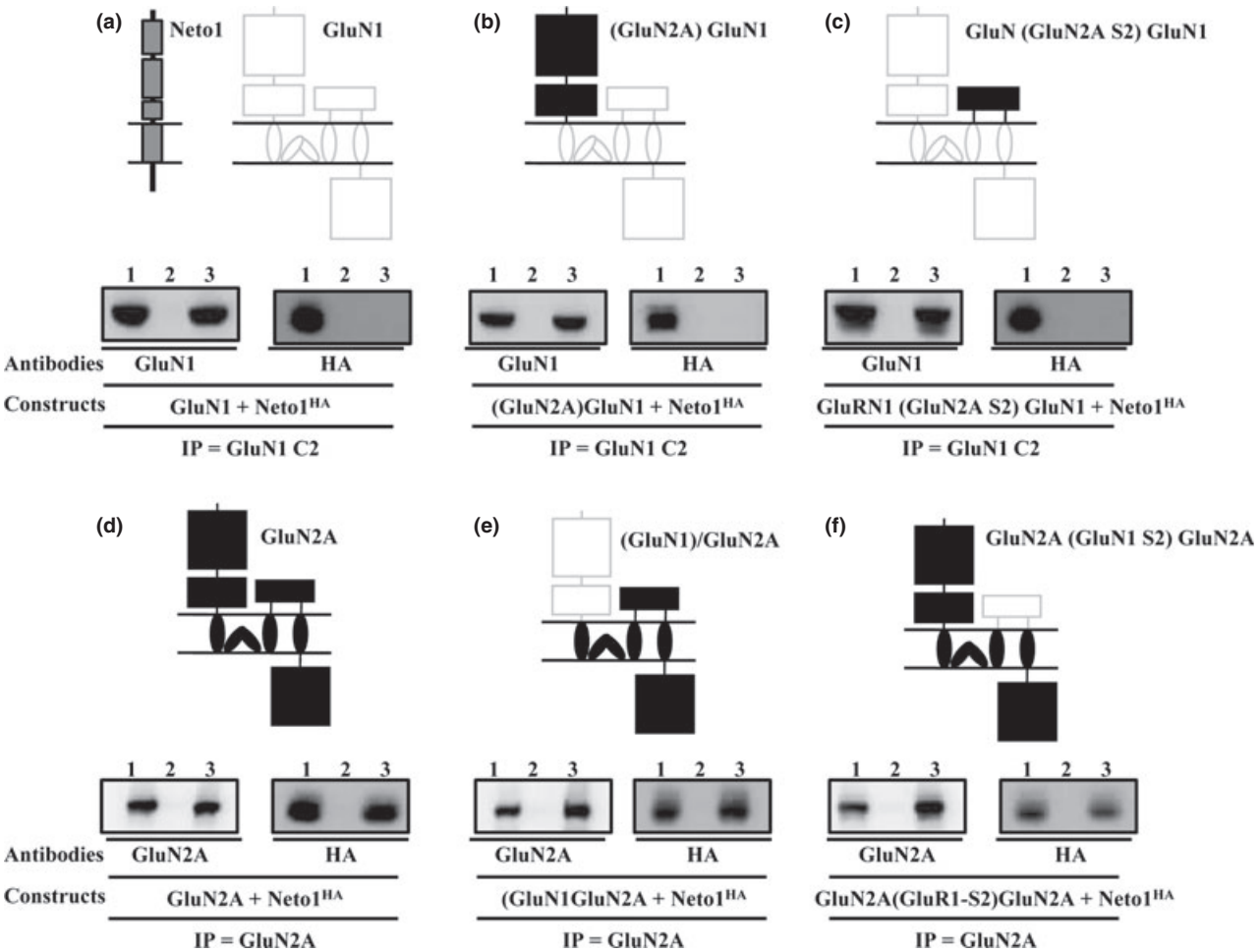

Fig. 8 Extracellular GluN2A domains do not mediate association of Neto1 with NMDA receptors. (a–f) Clones encoding the depicted GluN1/GluN2 chimeras were generated as shown. Each clone was co-transfected with Neto1^{HA}, transfected cell homogenates harvested, detergent solubilized, the soluble extracts collected by centrifugation at 100 000 *g* and immunoprecipitated with non-immune Ig or anti-GluN1 C2 or GluN2A (1381-1394) antibodies and immune pellets were analysed by immunoblotting all as described under Experimental Procedures. GluN1, GluN2A and HA are the antibody specificities used to probe the immunoblots. → denotes the respective chimera and Neto1^{HA} where appropriate. The immunoblots are representative of at least $n = 3$ independent immunoprecipitations from $n = 3$ independent transfections.

Neto1/NMDA receptor interaction is mediated via the extracellular Neto1 CUB1 domain. To determine where within the GluN2A extracellular domain Neto1 binds, a series of GluN1/GluN2A chimeras were generated, each was co-expressed with Neto1^{HA} in HEK 293 cells and immunoprecipitations carried out. For the first generation chimeras, the GluN2A N-terminal LIVBP + the S1 domain were swapped for the corresponding regions of GluN1 and vice versa. For the second generation chimeras, GluN2A S2 was swapped for GluN1S2 domain and vice versa (Fig. 8). Surprisingly, it was found that replacing the GluN2A extracellular regions with GluN1 had no effect on the association with Neto1^{HA}, that is, Neto1^{HA} was always co-immunoprecipitated with constructs containing the GluN2A transmembrane and intracellular domains but not with constructs containing GluN2A extracellular domains (Fig. 8).

To investigate the association further, GluN1 + Neto1^{HA} were co-expressed in parallel with a GluN2A C-terminal truncated construct, that is, GluN2A 1-877; with wild type, GluN2A and additionally, a tagged construct which encoded only the GluN2A intracellular domain, that is, GluN2A 838-1464. The results in Fig. 9 show that deletion of the GluN2A C-terminal domain results in the loss of co-immunoprecipitating Neto1^{HA}. Conversely, Neto1^{HA} is co-immunoprecipitated with GluN2A 838-1464. This implies that it is the C-terminal GluN2A domain that mediates directly or indirectly, the association with Neto1^{HA}. The reciprocal experiment was carried out whereby the Neto1 C-terminal tail was deleted 7 amino acids after the transmembrane domain, that

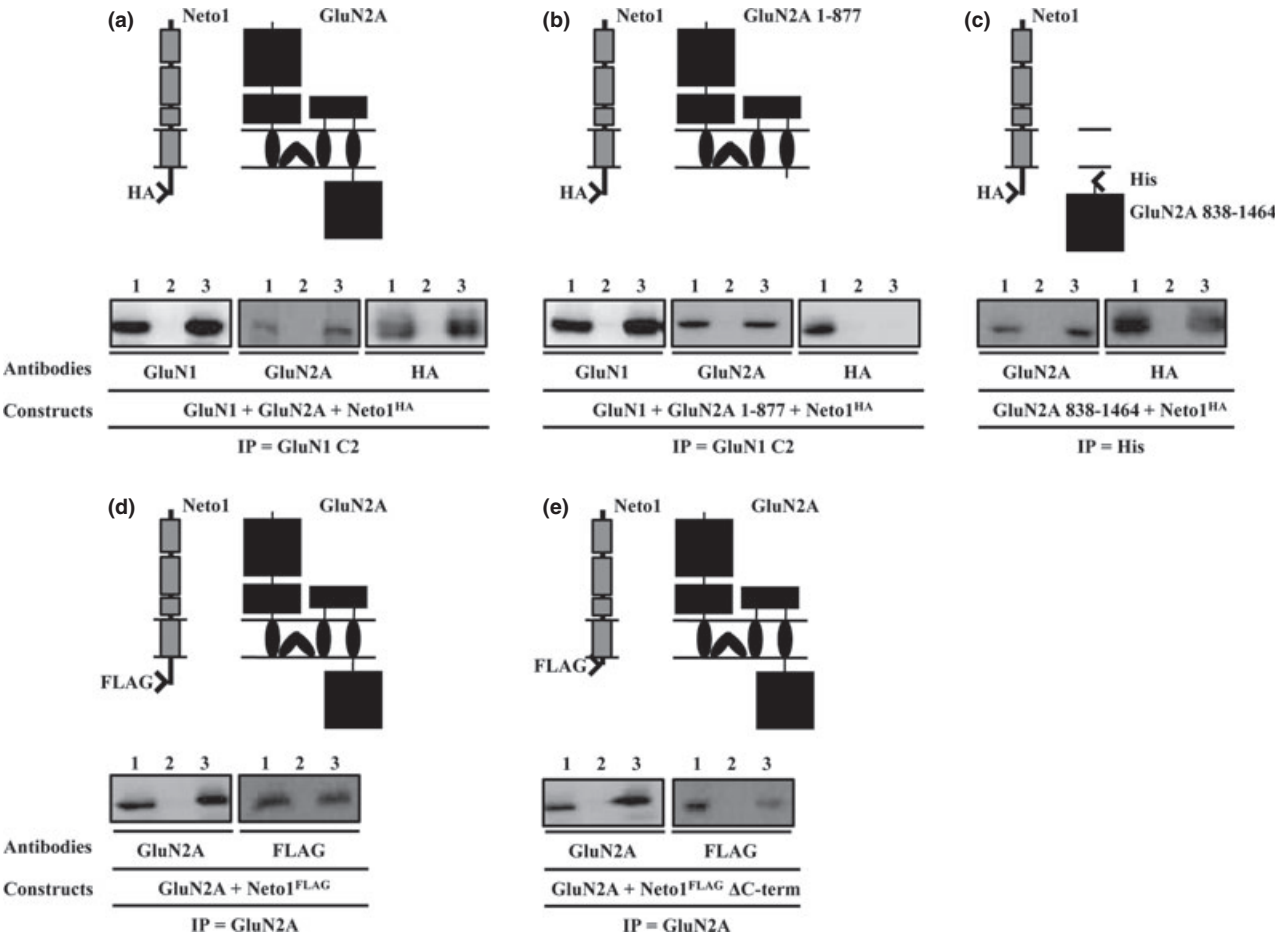

Fig. 9 The GluN2A intracellular C-terminal domain mediates association with Neto1. Transfections were carried out using the clones encoding Neto1^{HA} alone or, GluN1/GluN2, GluN1/GluN2A 1-877 and GluN2A 838-1464 with Neto1^{HA}, or, with GluN2A/Neto1^{FLAG} and GluN2A/Neto1^{FLAG} 1-372 clones as depicted (a–e). Cell homogenates were harvested, detergent solubilized, the soluble extracts collected by centrifugation at 100 000 *g* and immunoprecipitated with non-immune Ig, anti-GluN1 C2, anti-His or anti-GluN2A antibodies and immune pellets were analysed by immunoblotting all as described under Experimental Procedures. GluN1, GluN2A, His and FLAG are the antibody specificities used to probe the immunoblots The immunoblots are representative of at least $n = 3$ independent immunoprecipitations from $n = 3$ independent transfections.

is, Neto1 1 -372. Surprisingly, anti-FLAG immunoreactivity, that is, Neto1, was found in immune pellets for Neto1 1-372 co-expressed with full length GluN2A (Fig. 9).

As described above, Ng *et al.* (2009) found that Neto1 immunoprecipitates with PSD-95. SAP102 is a closely related scaffold protein that is endogenously expressed in HEK 293 cells (Sans *et al.* 2003; Lin *et al.* 2004). To investigate the possibility that it is SAP102 that mediates association with Neto1^{HA}, Neto1^{HA} was expressed alone in HEK 293 cells, immunoprecipitated and the immune pellet screened for Neto1^{HA} and SAP102 immunoreactivity (Fig. 10). For these experiments, the sensitivity of the immunoblots was increased by using the SuperSignal West Femto ECL substrate and also analysing 80% of the immune pellet for SAP102. Under these conditions, SAP102 was detected in immune but not in control non-immune pellets (Fig. 10). Simultaneously, the GluN2A 1-877 truncation construct was co-expressed with GluN1 and SAP102; it was found that GluN2A 1-877 did not co-immunoprecipitate SAP102. Thus, there is a correlation between the capability of GluN2A to co-immunoprecipitate SAP102 and Neto1^{HA}. However, SAP102 does not co-immunoprecipitate with Neto1^{FLAG} 1-372 suggesting that GluN2A/Neto1 association is not mediated via SAP102.

Concluding comments

In this article, we have identified a new player in the NMDA receptor/APP trafficking complexes. We have shown that Neto1 co-immunopreciptates with NMDA receptor/APP trafficking complexes via association with GluN2, in heterologous expression and in native brain tissue. Furthermore, we have shown that it is the GluN2A C-terminal domain that mediates Neto1 association. The

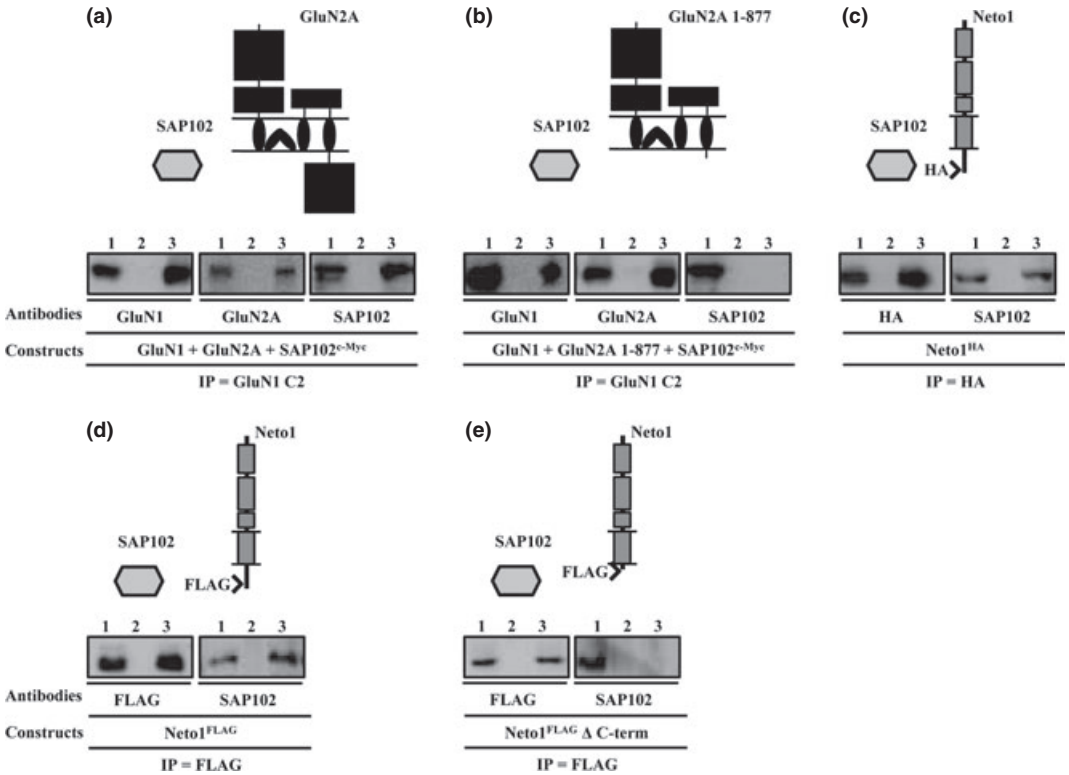

Fig. 10 Synapse associated protein 102 (SAP102) does not mediate the co-immunoprecipitation of Neto1 and GluN2A. Transfections were carried out using the clones depicted encoding SAP102 with GluN1/GluN2A, GluN1/GluN2A 1-877, Neto1^{HA}, Neto1^{FLAG} or Neto1^{FLAG} 1-372 (a–e), cell homogenates harvested, detergent solubilized, the soluble extracts collected by centrifugation at 100 000 g and immunoprecipitated with non-immune Ig, anti-GluN1 C2, anti-HA or anti-FLAG antibodies and immune pellets were analysed by immunoblotting all as described under Experimental Procedures. GluN1, GluN2A, HA, SAP102 and FLAG are the antibody specificities used to probe the immunoblots. The immunoblots are representative of at least $n = 3$ independent immunoprecipitations from $n = 3$ independent transfections.

original work of Ng *et al.* (2009) showed that Neto1 associated with assembled NMDA receptors via GluN2A and the Neto1 extracellular N-terminal CUB1 domain. We conclude that Neto1 associates with NMDA receptors via GluN2; however, our findings are difficult to reconcile with a direct association between GluN2 and Neto1 since the removal of the GluN2A extracellular domain has no effect on GluN2A/Neto1 co-immunoprecipitation. Rather, we demonstrate that it is the intracellular region of GluN2A that mediates association. Further, since Ng *et al.* (2009) showed that deletion of the C-terminal 20 amino acids of Neto1 and the results presented here which deleted a further 141 amino acids from the Neto1 intracellular domain showed that these truncated constructs retained the ability to co-immunoprecipitate with GluN2A, it is hard to visualize how association between the two proteins could be direct. Since it is endogenously expressed in HEK cells, SAP102 is a potential candidate for an intermediary protein since it co-immunoprecipitates with both Neto1 and GluN2A. However, SAP102 does not co-immunoprecipitate with Neto1^{FLAG} 1-372 and thus such a possibility is excluded. Overall, these findings suggest that Neto1 is not an NMDA receptor auxillary subunit since the protein–protein interactions that we have determined imply that their association is indirect.

Nevertheless, a new player in NMDA receptor/APP trafficking complexes has been identified. Future studies will ascertain how APP and Neto1 interactions regulate the appropriate surface expression of this key neurotransmitter receptor.

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