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A peptide from the extracellular region of the synaptic protein α Neurexin stimulates angiogenesis and the vascular specific tyrosine kinase Tie2

Stefania Graziano[1] , Serena Marchiò[2], Federico Bussolino[3]* Marco Arese [1]*

Department of Oncology, University of Torino Medical School, and laboratory of Neurovascular Biology[1], Tumor Microenvironment [2], Vascular Oncology[3] , Institute for Cancer Research @ Candiolo, Candiolo (TO), Italy

Corresponding author: Marco Arese, marco.arese@unito.it

*F.B. and M.A. equally contributed to this report

Fax 011-9933524

Ph 011 -9933508

Abstract

Neurexin (NRXN) and Neuroligin (NLGN) are trans-synaptic proteins involved in vascular biology. NRXN is encoded in long (α) and short (β) isoforms. We have shown that βNRXN modulates blood vessel development in synergy with VEGFA and associates with NLGN. On the other hand αNRXN is also expressed in blood vessels but does not interact with NLGN or act in synergy with VEGFA, thus demonstrating a differential role.

To find clues into the vascular functions of αNRXN, we chose a 7 aa motif that is part of its extracellular region and was formerly selected through a proteomic search for interactors of the vascular receptor Tie2. Next we a) synthetized and modeled such peptide in order to determine its biological activity towards Tie2 in *in vitro* and *in vivo* angiogenesis assays and b) evaluated if αNRXN and Tie2 physically associate in situ during vascular development.

We used biochemical and cellular assays to prove that the synthetic αNRXN peptide a) modulates the angiogenic phenotype of cultured endothelial cells and angiogenesis in vivo and b) efficiently stimulates Tie2 phosphorylation and downstream mediators in endothelial cells. Moreover, we show that αNRXN and Tie2 can be reciprocally immunoprecipated from chicken blood vessels at late stages of vascular development.

These data have a double significance, i.e. provide a novel tool to modulate Tie2 and further suggest the involvement of the NRXN family of synaptic protein in the vascular system through their interaction with a fundamental vascular player.

keywords: Tie2, Neurexin, peptide, structure

1. INTRODUCTION

Neurexins (NRXNs)[1] are a family of pre-synaptic transmembrane proteins involved in the modulation of synaptic activity in partnership with the post synaptic Neuroligins (NLGNs). In mammals, there are 3 different NRXN genes, each presenting 2 alternative promoters which drive the synthesis of 2 major isoforms per gene (a long αNRXN, and a short C- terminal βNRXN). The extracellular region of αNRXN is constituted by three LNS-EGF-LNS repeats while the short βNRXN is made only of the last LNS (6^{th}) domain (Figure S1).

We recently discovered that a) α and βNRXN as well as NLGN are expressed by blood vessels b) βNRXN and NLGN modulate angiogenesis in the chick embryo chorioallantoic membrane assay; c) while both αNRXN and βNRXN co-precipitate with NLGN in brain, only βNRXN does so in blood vessels [1]d) in zebrafish βNRXN but not αNRXN knock-down causes vascular defect in synergy with VEGFA [2]. All these findings raise questions about αNRXN function and protein partners in the vascular system. In a parallel project we screened a phage-displayed random peptide library on the vascular specific receptor Tie2 in order to find either new small modulators of its activity or discover new physiological partners that could participate in the regulation of the complex family of Angiopoietin/Ties. Among the candidate Tie2 ligands there was a peptide corresponding to a portion of αNRXN. This report analyzes the possibility that Tie2 is linked to the vascular αNRXN and demonstrates that a) a cyclic peptide that mimics a region of the second EGF like domain of αNRXN has angiogenesis-promoting activities and specifically displays agonistic activity towards Tie2 and its downstream signaling and b) that Tie-2 and αNRXN co-precipitate from blood vessels in a developmentally regulated fashion.

2. Methods

2.1 **Synthesis and validation of Peptides**

Peptide A2 (**GTGYLGR**) and its cyclic form BbcA2 as well as the control peptide (ADSILRS) were synthetized by New England Peptide (Gardner, MA, USA). The binding of A2 and BbcA2 to Tie2 was verified through ELISA on recombinant Tie2 or by immunofluorescence /FACS on Tie2 overexpressing cells using FITC-conjugated peptides. The control peptide did not bind to Tie-2 in any of these assays (data not shown).

2.2 Proliferation assay

96 well plates were coated with 1% gelatin in PBS. HUVEC cells, pre-incubated for 24 hours with 15 μM peptides or control medium, were seeded in the presence or absence of peptides. After 24 or 48 hours cells were fixed and colored with crystal violet. Absorbance was read at 595 nm with BioAssay Reader HTS 7000 Perkin Elmer.

2.3 Apoptosis-TUNEL assay

HUVEC cells were incubated with 15 μM peptides. TUNEL assay (In Situ Cell Death Detection Kit; Roche, #12-156-792-910) was performed on the slides followed by image analysis using fluorescent microscopy (DM IRB; Leica). DAPI stain (Molecular probes, Invitrogen, Carlsbad, CA) was used to assess total cell number.

2.4 Protein immunoprecipitation from cultured cells and tissues

Confluent HeLa cells were washed three times with cold PBS containing 1mM Na orthovanadate and lysed in lysis buffer (Tris HCl pH 7,5 50mM, NaCl 150mM, SDS 0,1%, 1% TRITON X-100) plus protease and phosphatase inhibitors (50μg/ml pepstatin, 50μg/ml leupeptin, 10μg/ml aprotinin, 1mM PMSF, 100μM

1) **Abbreviations:** αNRXN *(αNeurexin); β*NRXN *(βNeurexin); A2: 7 aa peptide from the second EGF like domain of alpha Nrxn; BbcA2: backbone cyclic form of A2; Bbc control: control peptide in the cyclic form; EC: endothelial cells; LNS domain: laminin A/ neurexin/ sex hormone-binding globulin repeats; NLGN: Neuroligin*

$ZnCl_2$, 1mM Na orthovanadate, and 10mM NaF) for 30min. After centrifugation (30min at 11,000g), supernatant was quantified with the BCA Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL).

1mg of proteins were pre-cleared by incubation for 90 minutes with protein A-Sepharose (Amersham Biosciences, Piscataway, NJ) and then incubated with rabbit anti-NRXN (1,5µg/mg) or rabbit anti-Tie2 C-20 (2,5µg/mg) for 1 h. The immune complexes were recovered on protein A-Sepharose for 90 minutes, beads were washed four times and detected by immunoblot. Proteins were separated by 8% SDS-PAGE electrophoresis gel, transferred to PVDF membrane (Millipore, Billerica, MA), incubated with mouse monoclonal anti-NRXN antibody (BD, Franklin Lakes, NJ), or anti-Tie2 C-20 antibody (Sigma-Aldrich, St. Louis, MO). Horseradish-peroxidase conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA)was used and visualized by ECL system (Amersham Biosciences, Piscataway, NJ).

Five Chicken Chorioallantoic Membranes were used for each experimental point. Frozen tissues were disaggregated and lysed 40min in ice with a lysis buffer previously set-up (Tris HCl pH 7,5 20mM; aCl 100mM, NaHC03 20mM, KCl 4mM, MgCl2 2,5mM; 10% glycerol; 1% triton X-100; 1% CHAPS and protease and phosphatase inhibitors. After centrifugation (30min at 11,000g), supernatant was quantified. 5mg of proteins were pre-cleared by incubation for 90 min with protein A-Sepharose (Amersham Biosciences, Piscataway, NJ) and then incubated with rabbit anti-NRXN (1,5µg/mg) or rabbit anti-Tie2 C-20 (2,5µg/mg) overnight. The immune complexes were recovered on protein A-Sepharose for 90 minutes, beads were washed four times and detected by immunoblot. Proteins were separated by 4-15% gradient SDS-PAGE mini-protean TGX(Biorad, Hercules, CA), transferred to PVDF membrane (Millipore, Billerica, MA), incubated with mouse monoclonal anti-NRXN antibody (BD, Franklin Lakes, NJ), or with rabbit anti-Tie2 C-20 antibody (Sigma-Aldrich, St. Louis, MO).

2.5 Migration assay

Subconfluent HUVEC were starved overnight in M199 2%FBS and 1%BSA. The assay was done with Boyden chamber with a membrane soaked in 1% gelatin. The lower chamber was filled with serum free medium+ stimuli (10 µg/µl VEGF or 15 µM peptide), while the upper was filled with a HUVEC cells suspension (125.000 CELLS/WELL). After 5 hours the chamber was opened and the cells on the membrane were fixed in methanol and colored with Eosin/Hematoxylin.

2.6 Morphogenesis assay in matrigel

48 wells microtiter plates were covered with 150 µl of matrigel (Sigma-Aldrich, St. Louis, MO). 13000 Huvec/well were prepared in M199 10%FBS with or without 15µM of peptides. Photographs were taken after 5 hours. Total length of network of tubuli was calculated through an analysis performed by WinRHIZO program (Regent Instruments).

2.7 Sprouting assay

HUVEC cells suspended at density of 4cells/µl in M199 20% methocel and 200µl for each spheroid was seeded into non adhesive round-bottom 96-well plate. After 12h at 37°C (5% CO_2, 100% humidity) spheroids were harvested, centrifuged (15min at 300g) and resuspended in collagen solution (Roche, Indianapolis, IN) with 15 µM of peptide. Photographs were taken after 24 hours. Total length of the sprouts was calculated through an analysis performed by WinRHIZO program (Regent Instruments).

2.8 CAM assay

Fertilized chick embryos were incubated for 3 days at 37°C at 70% humidity. A small hole was made over the air sac at the end of the egg and a second hole was made directly over the embryonic blood vessels. After 7 days, cortisone acetate–treated filter disks (5 mm) were saturated with 5µl of 100 ng/ml Ang1 (R&D Systems) and peptides at concentration of 15 µM. After 3 days CAMs were fixed with PBS-4%

paraformaldehyde for 10min at room temperature, filter disks were excised and pictures were taken with a QIcam FAST1394 digital color camera (QImaging) connected to the stereomicroscope (model SZX9; Olympus).

2.9 Analysis of the Tie2 phosphorylation pathway

HUVEC cells were incubated with M199 10%FCS with 15 μM peptides for one hour. The cells were then lysed with hot Laemli Buffer and after centrifugation the protein were quantified. 30μg proteins were separates by 7,5% SDS-PAGE mini-protean TGX (Biorad, Hercules, CA), transferred to PVDF membrane (Millipore, Billerica, MA), incubated with mouse monoclonal Anti-Phosphotyrosine, 4G10® Platinum(Millipore, Billerica, MA), with rabbit anti-pAkt (S473)(D9E) antibody, with mouse monoclonal p-p44/42 MAPK (T202/Y204)(E10) antibody (both Cells Signalling, Danvers, MA). For total protein normalization purposes we used rabbit anti-Tie2 C-20 antibody (Sigma-Aldrich, St. Louis, MO), rabbit anti-Akt (C67E7) and rabbit p44/42 MAPK (both Cells Signalling, Danvers, MA). Horseradish-peroxidase conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) was used and visualized by ECL system (Amersham Biosciences, Piscataway, NJ).

3. RESULTS

3.1 A NRXN-EGF domain peptide affects the angiogenic properties of endothelial cells and in vivo angiogenesis.

A phage display screening on Tie2 provided a large set of 7 aa target peptides of which one (A2- NH2-GTGYPGR-COOH) was highly homologous to a sequence (NH2-GTGYLGR-COOH) within the second EGF-like domain of αNRXN (Figure S1A). Analysis of this sequence on the αNRXN crystallography (PDB 3POY [3], PDB 3QCW [4]) showed that the peptide is exposed on the external surface of the protein where it is found in a coiled conformation (Figure S1B). Studies were pursued further with the A2 homologous sequence synthesized in a linear (A2) and cyclic (backbone cyclic, named BbcA2) forms and specific assays were performed.

3.1.1 In vitro assays for proliferation, apoptosis and migration

To verify a possible effect of the peptides on proliferation and cell death, EC were treated with A2 and BbcA2 in proliferation and TUNEL assays. Neither had a significant effect even after a 24 hours incubation (Figure S2). On the other hand, migration assays with Boyden chamber showed that BBcA2 promotes the migration of EC cells compared to all controls (Figure 1A).

3.1.2 In vitro morphogenesis

As two of the most relevant assays for angiogenic potential, we measured the ability of EC to form networks on matrigel or to sprout from collagen spheroids upon A2 and BbcA2 treatment. In the case of network formation on matrigel (Figure 1B), as with the migration assay, only BBcA2 induced a statistically significant increase in the tube network length. In the case of spheroid sprouting both A2 and BbcA2 induced positive responses, with BbcA2 inducing a response similar in extent to Ang1 (Figure 2A).

3.1.3 In vivo angiogenesis

We finally tested angiogenic activities of the peptides *in vivo*. We chose the chick chorioallantoic membrane (CAM) assay, which is used as an *in vivo* model both to study physiological angiogenesis and to test pro- and anti-angiogenic compounds. The peptides A2 and BbcA2 applied at day 10, induced the formation of capillary bifurcations, with BbcA2 being significantly more effective than its linear counterpart A2 (Figure 2B). Globally, these results indicate that the αNRXN EGF II peptide induces angiogenesis and that mimicking its natural conformation though cyclization positively influences its biological activities.

3.1.4 BbcA2 induces Tie2 phosphorylation

As a complement to the phenotypic data shown above we next tested whether A2 could modulate Tie2 signal transduction in EC . Ang1 has been demonstrated to induce activation via phosphorylation of pathways downstream to Tie2 [5]. To determine the activity of our peptides, they were incubated with EC and the phosphorylation of Tie2, Akt and Erk was measured. Figure 3 shows that BbcA2 and A2 enhance the phosphorylation of Tie-2 and of the downstream mediators AKT and ERK over their respective controls. This attests that the effect on EC migration and morphogenesis is complemented by a specific activation of Tie2 and of its main downstream mediators.

3.2 αNRXN and Tie2 physically associate in vivo and their interaction is modulated during development

As an extension to the above data, the physical interaction between Tie2 and αNRXN was tested. Preliminary sets of experiments were dedicated to identifying complexes of these two proteins in overexpressing Hela Cells. αNRXN was immunoprecipitated and the resulting precipitate was immunoblotted with the antibody against Tie2. Figure S3 shows that Neurexin and Tie-2 can indeed be co-immunoprecipitated in these conditions.

To evaluate Nrxn-Tie2 interaction in a physiologically significant environment, we used the chicken chorioallantoic membrane. Figure 4 shows that at the stage E10 αNRXN and Tie2 can be efficiently co-immunoprecipitated from the CAMs but not from the brain, where the two proteins are also present. . To evaluate a possible modulation of the interaction between αNRXN and Tie2 during vascular development, we performed co-immunoprecipitation experiments using CAMs at days 5, 10 or 15 days. Interestingly, even though the two proteins are expressed at significant levels at all stages, they interact only from day 10 onward and with increasing intensity (Figure 4B), when blood vessels are mature and well structured.

4. DISCUSSION

Prior to this report, we discovered that the synaptic protein αNRXN was expressed in the vascular system, but unlike the related βNRXN and NLGN, does not interact with VEGFA. Here, starting from the data collected through a parallel project, the αNRXN link to another pivotal family of vascular mediators, the Ang/Tie, was considered.

The data describe above data have two implications:

First, they provide the basis for the construction of a novel targeting tools for Tie2 starting from BbcA2 as lead compound. This receptor and its ligands are involved in disparate activities and its pharmacological modulation may have an impact in wide array of clinical settings, ranging from angiogenesis to inflammation to cancer [6] . BbcA2 is a small peptide activator of the Tie2. Although other instances of peptide activators of signaling pathways are known, in the case of Tie2, another 7aa ligand has been identified with inhibitory effects [7]. One hypothesis is that the latter peptide may function by inhibiting Tie2 dimerization [8] while BbcA2 could relieve the receptor from some inhibitory interactions, of which the most plausible is the one with the strictly related, orphan receptor Tie1[9], or that with αNRXN, shown above. The idea that the interaction between Tie2 and αNRXN is inhibitory (i.e. antiangiogenic) is supported by the fact that they associate only when blood vessels are mature and stabilized. Indeed, the CAM is a well characterized developmental model in which different stages of vascular remodeling, including endothelial “coverage” by developing smooth muscle cells, have been recognized at late stages of development [10]. In addition, it is

well known that Ang1 and Tie2 null mice display defects in the maturation and maintenance of the integrity of blood vessels [11].
Second, our data emphasize the involvement of NRXNs in the vascular system by linking them to a key family of vascular modulators such as the Ang/Tie system. In particular, along with our previous data, they suggest that Tie2 may be a ligand for αNRXN alternative to NLGN, and that αNRXN and βNRXN may have differential activities in blood vessels, i.e. pro-angiogenic for βNRXN and vessel stabilizing for αNRXN.

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LEGEND TO THE FIGURES

Fig.1: BbcA2 induces migration and tube formation of HUVEC cells.

A) Boyden chamber assay shows that only BbcA2 (15 uM) displays a statistically significant effect on migration.
The graph displays the number of migrated cells from 3 different experiments (n=9 for each experimental point).
Data are expressed as mean ±SEM. ANOVA gave F= 8.771 .** $P < 0.01$ for BbcA2 vs Bbc Control by Bonferroni posttest.
B) Upper panel: tube morphogenesis assay: representative images showing tube formation on matrigel 5 hours after plating. Lower panel: quantification of total tube length from 3 different experiments (n=9 for each experimental point). Data are expressed as mean ±SEM. ANOVA gave F= 30.88 .** $P < 0.01$ for BbcA2 vs Bbc Control by Bonferroni postTest
Control: linear control peptide; Bbc control: Backbone cyclic control peptide

Fig. 2: A2 and BbcA2 induce HUVEC sprouting and in vivo angiogenesis.

A) Sprouting assays. Upper panel : representative images of collagen sprouting upon different treatments. Lower panel: quantification of total sprout length from 4 different experiments (n=15 for each experimental point). Data are expressed as mean ±SEM. ANOVA gave F= 14.53 .*** $P < 0.001$ for BbcA2 vs Bbc Control ** $P < 0.01$ for A2 vs Control by Bonferroni posttest.
B) CAM angiogenesis assay: upper panel: representative images of CAMs upon different treatments. Lower panel: quantification graph from 5 different experrints. (n=25 per each experimental point). Bars represent the number of vessel bifurcations per disc counted after the different treatments Data are expressed as mean ±SEM. ANOVA gave F= 45.73 .** * $P < 0.001$ for BbcA2 vs Bbc ** $P < 0.01$ for A2 vs Control by Bonferroni posttest.
Control: linear control peptide; Bbc control: Backbone cyclic control peptide

Fig 3: A2 and BbcA2 induce the the activation of the Tie2 downstream pathway.

A) HUVEC cells were incubated with the different peptides for 1 hour and the phosphorylation status of Tie2, AKT and ERK was revealed by western blot.
B) Quantification analysis: bars represent the ratio between the intensity of the phosphorylated / total protein bands as quantified by image analysis.
Data are expressed as mean ±SEM from four different experiments

Control: linear control peptide; Bbc control: Backbone cyclic control peptide

Fig 4: Tie2 and alpha Nrxn can be reciprocally immunoprecipitated from the chicken Cam.

A) Reciprocal immunoprecipitation of Tie 2 and aNrxn from chicken CAMs.
Tie2 or Nrxn were immunoprecipitated from E10 chicken embryo CAMs or brain and the precipitates were blotted for the respective partner. Nrx and Tie2 are espressed by both CAMs and brain, but are co-immunoprecipitated with high efficiency only from the CAMs.

B) Developmental modulation of the Tie2/alphaNrxn interaction.Co-immunoprecipitation experiments performed as in panel A on CAMs at 5, 10 or 15 days of development show that the Tie2/alpha Nrxn interaction occurs only at day 10 and 15 when blood vessels are mature and well structured.

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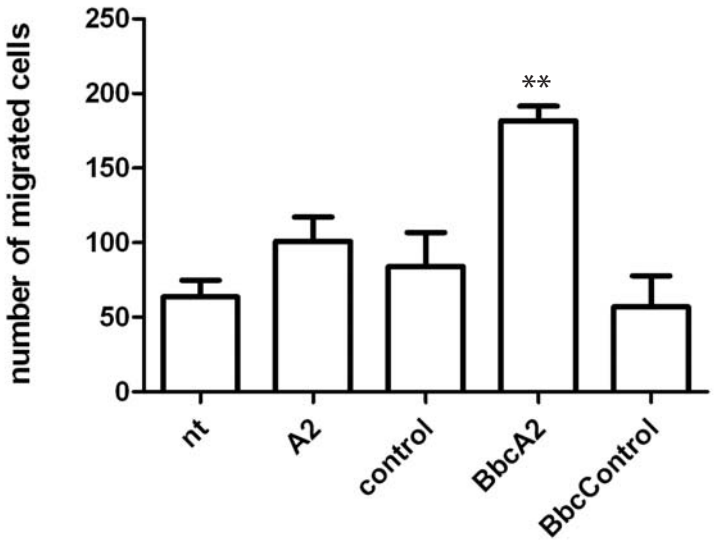
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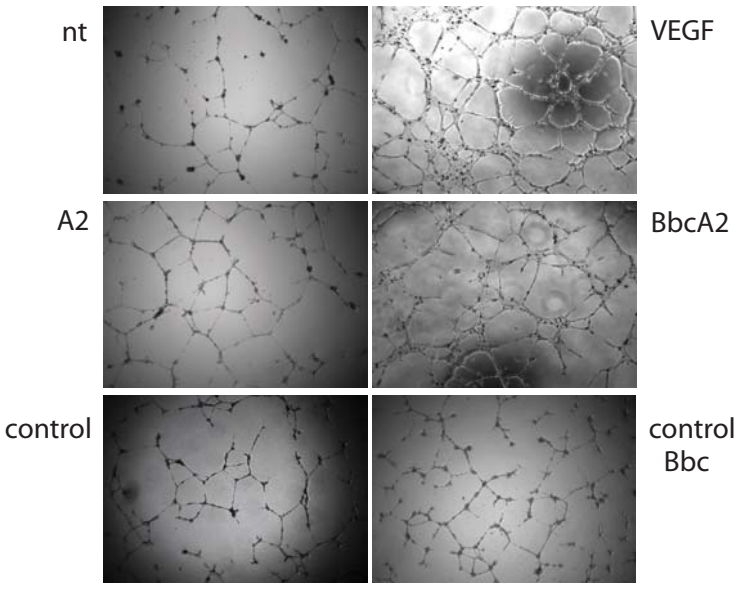
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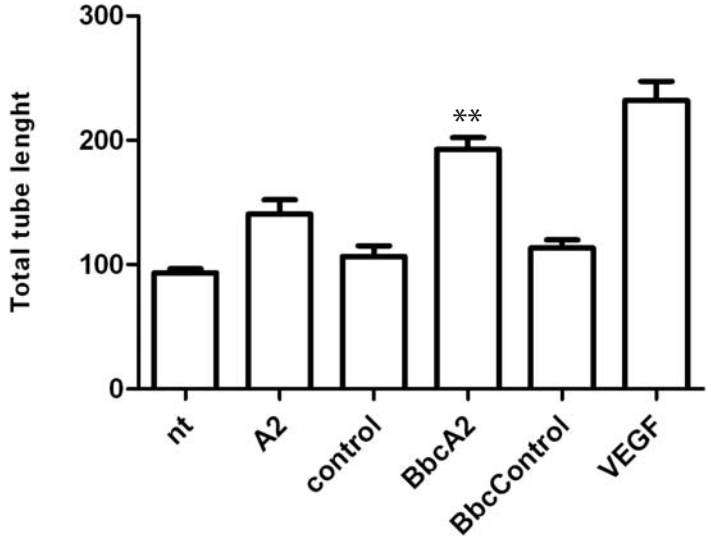
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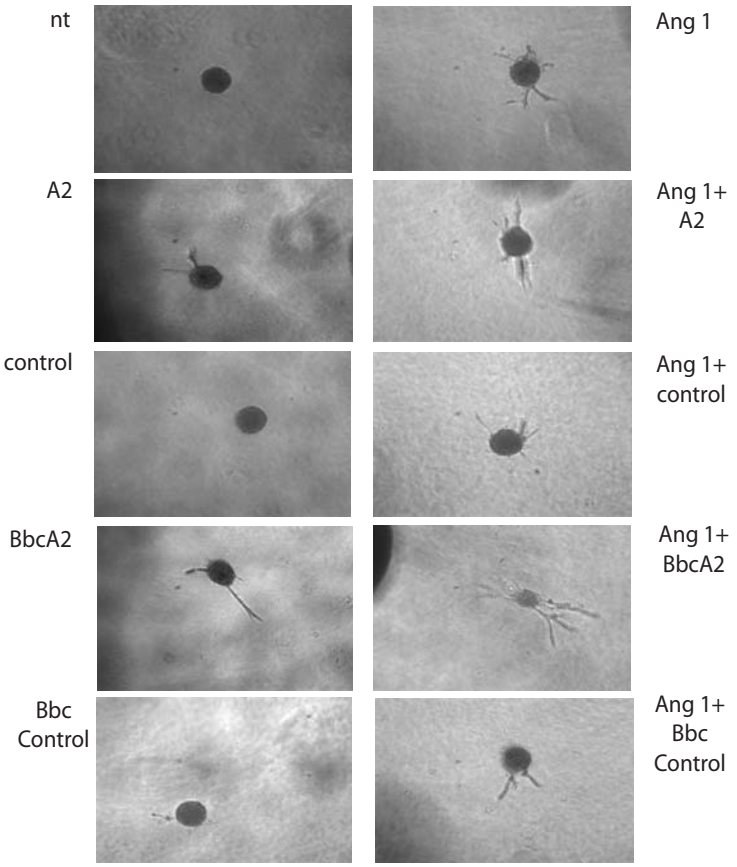
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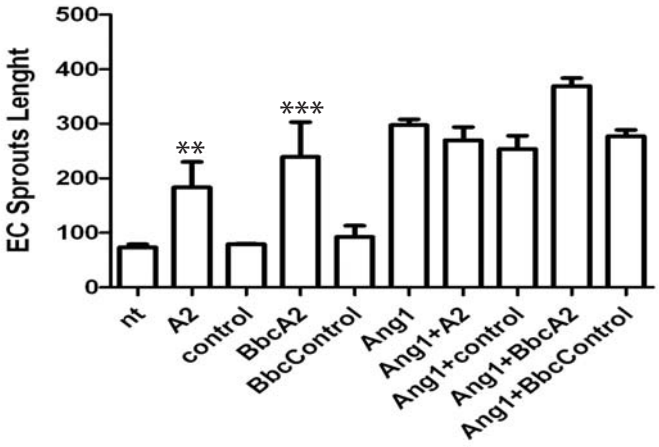
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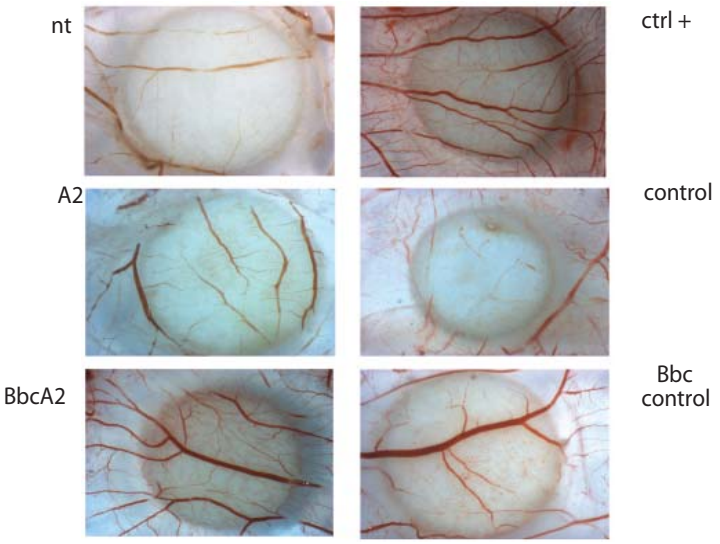
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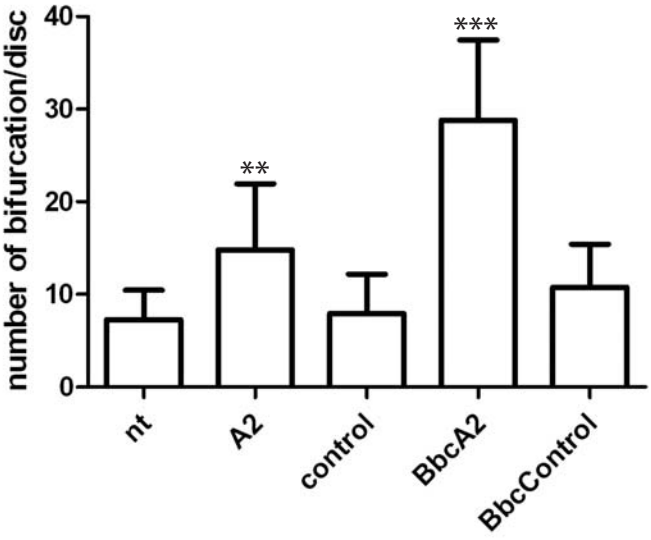
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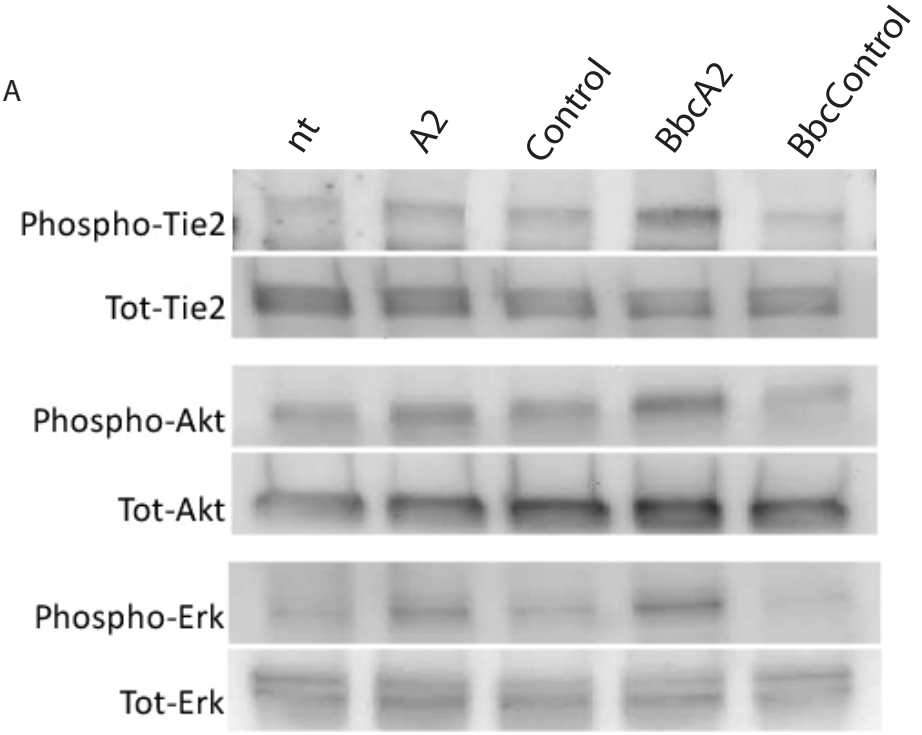
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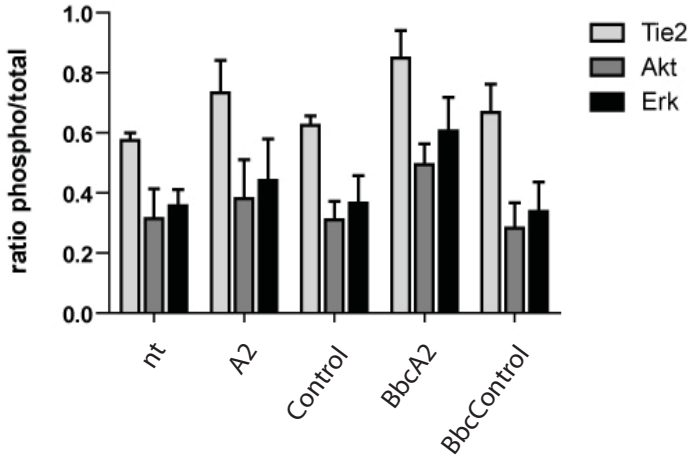
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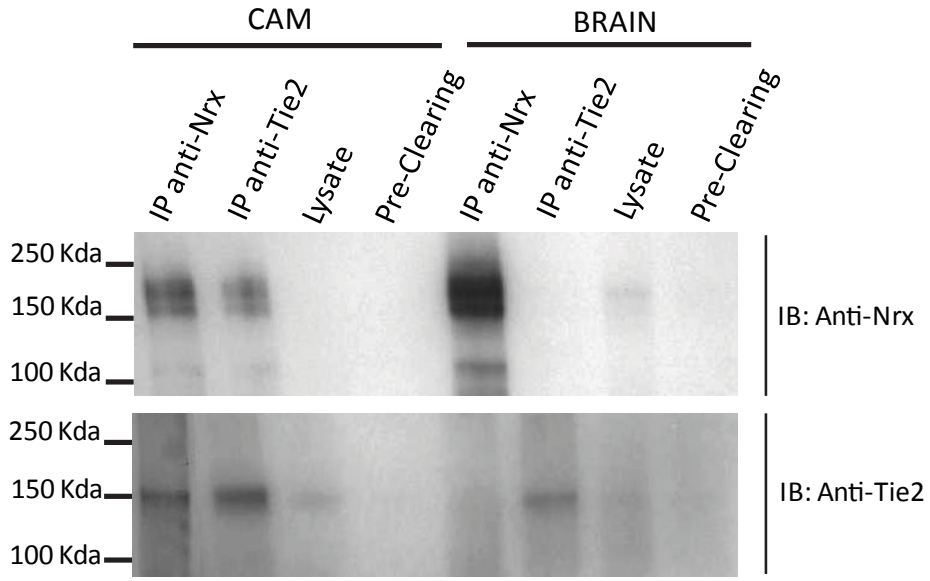
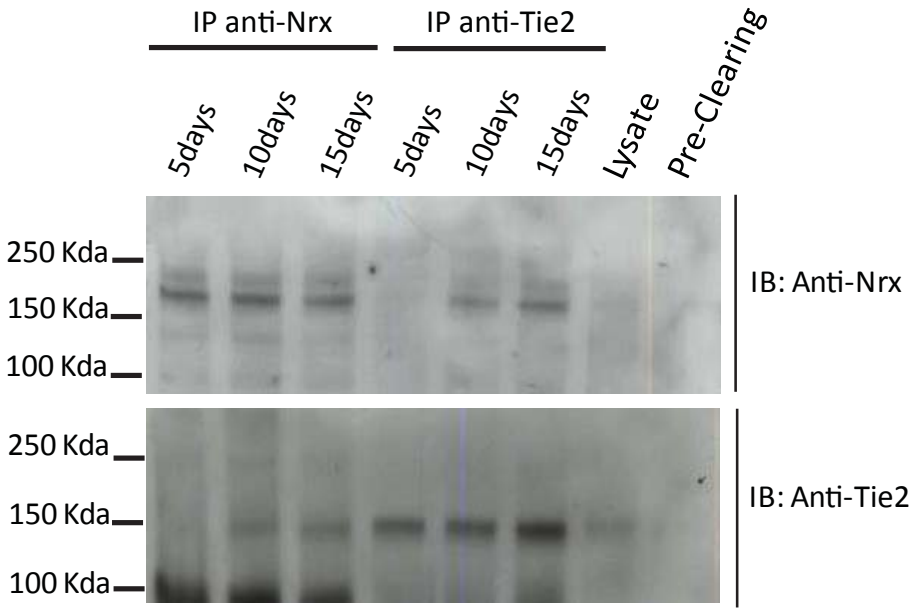
0.8
0.6
0.4
0.2
0.0
Tie2
Akt
Erk
nt
A2
Control
BbcA2
BbcControl

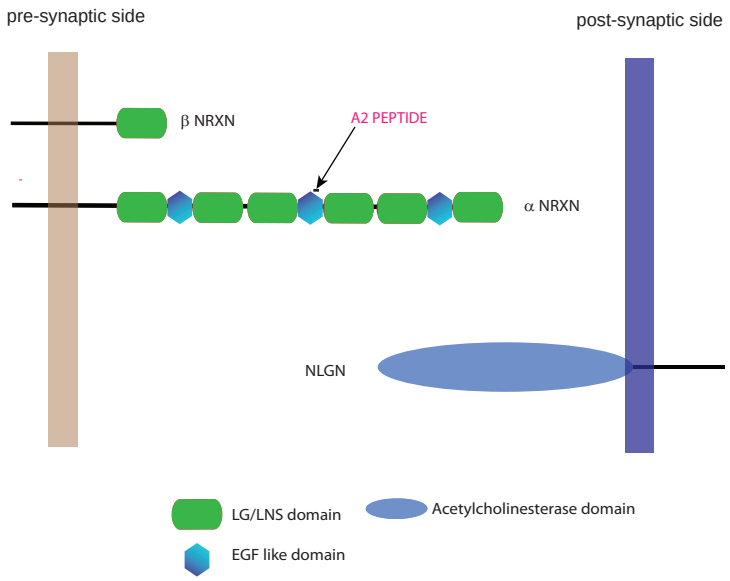
A

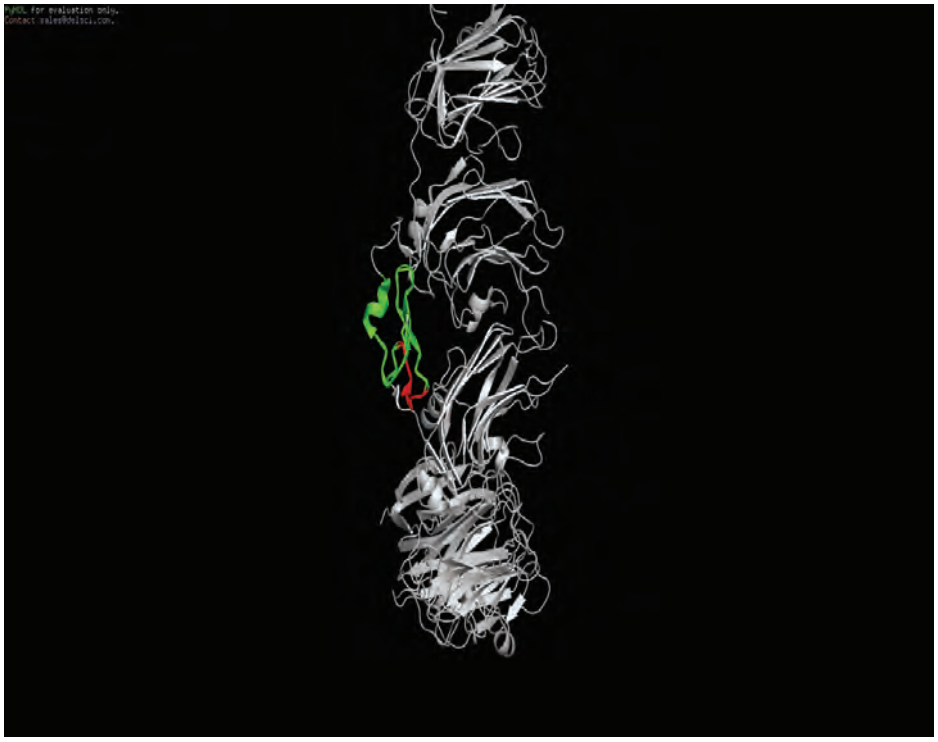

CAM
BRAIN
IP anti-Nrx
IP anti-Tie2
Lysate
Pre-Clearing
IP anti-Nrx
IP anti-Tie2
Lysate
Pre-Clearing
250 Kda
150 Kda
100 Kda
IB: Anti-Nrx
250 Kda
150 Kda
100 Kda
IB: Anti-Tie2

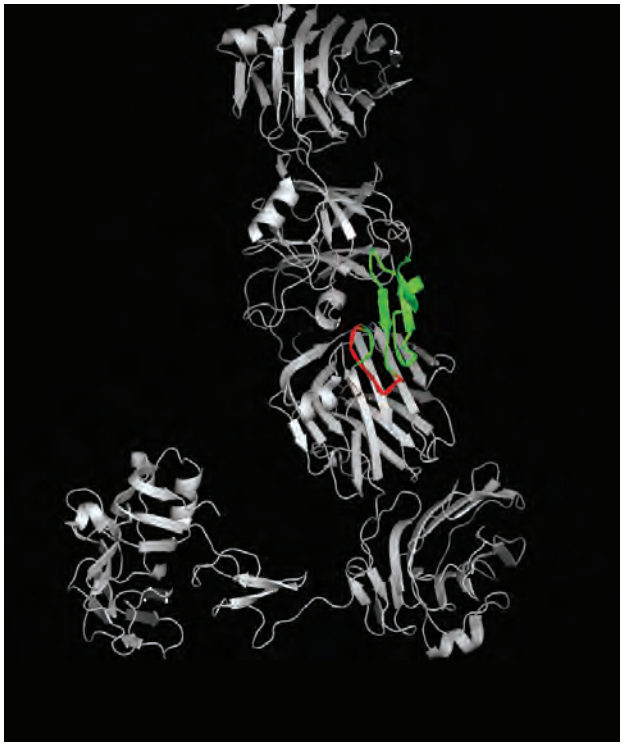
B


IP anti-Nrx
IP anti-Tie2
5days
10days
15days
5days
10days
15days
Lysate
Pre-Clearing
250 Kda
150 Kda
100 Kda
IB: Anti-Nrx
250 Kda
150 Kda
100 Kda
IB: Anti-Tie2

A







FigS1:

A) schematic representation of the Neurexins (alpha and beta) and Neuroligins arrangements and structures at the synapse and the position of the A2 peptide.

B) Display of the three dimensional position of the A2 peptide (sequence : GTGYLGR) on the second EGF domain of aNRXN (modeled on PDB 3POY with the Pymol software, The PyMOL Molecular Graphics System, Schrödinger, LLC.) .
Green : EGF domain, red: A2 peptide.

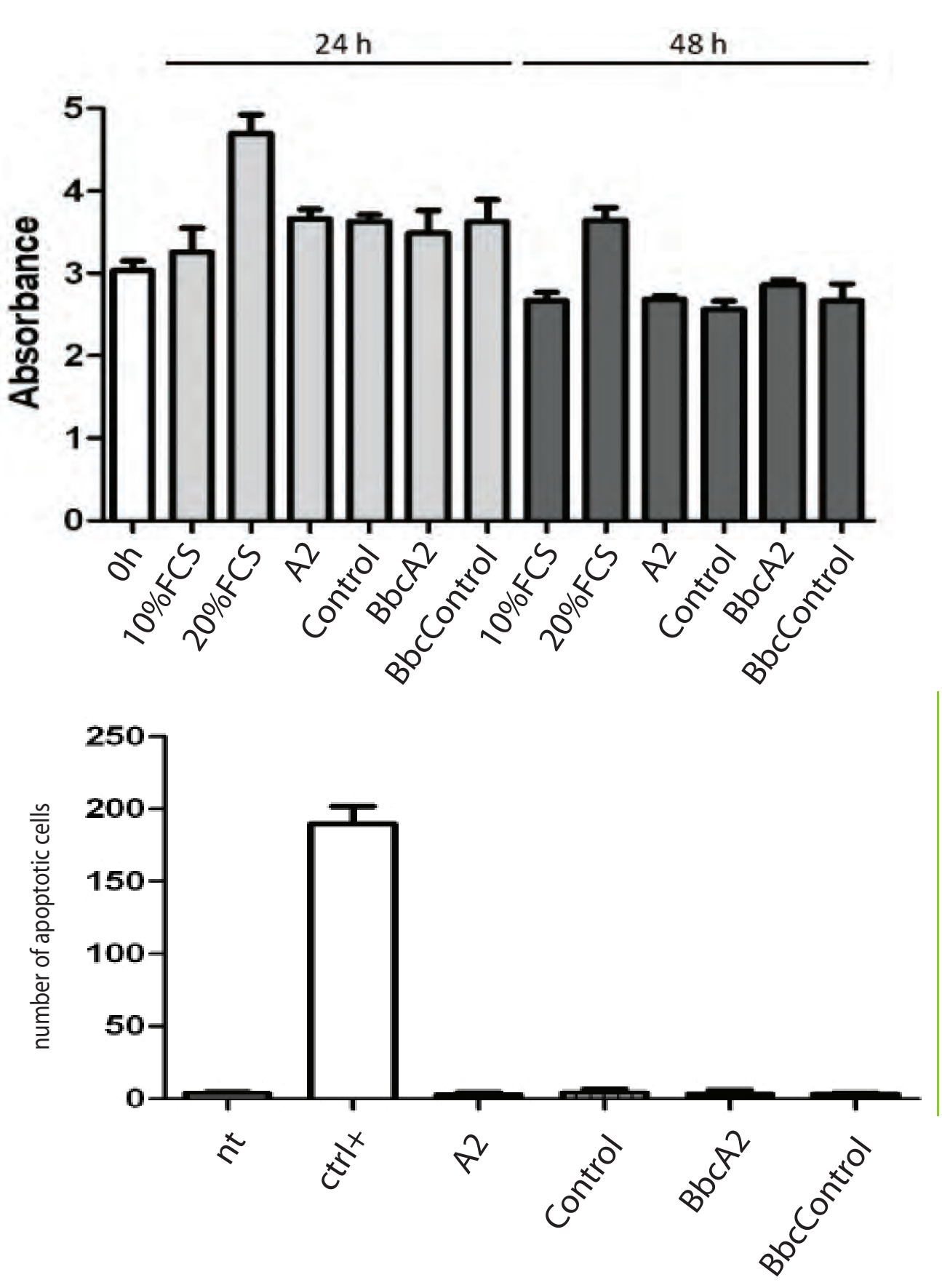


Fig S2: Assays for proliferation and apoptosis of HUVEC cells upon A2 and BBCA2 treatment.
A) treated or untreated HUVEC cells (15µM for 36 hours) were fixed and stained with crystal violet. Proliferation was evaluated by absorbance reading at 595 nm.
B) HUVEC cells were treated as above and apoptosis was measured by a TUNEL assay. Quantification of apoptotic cells was manually performed using fluorescence microscopy

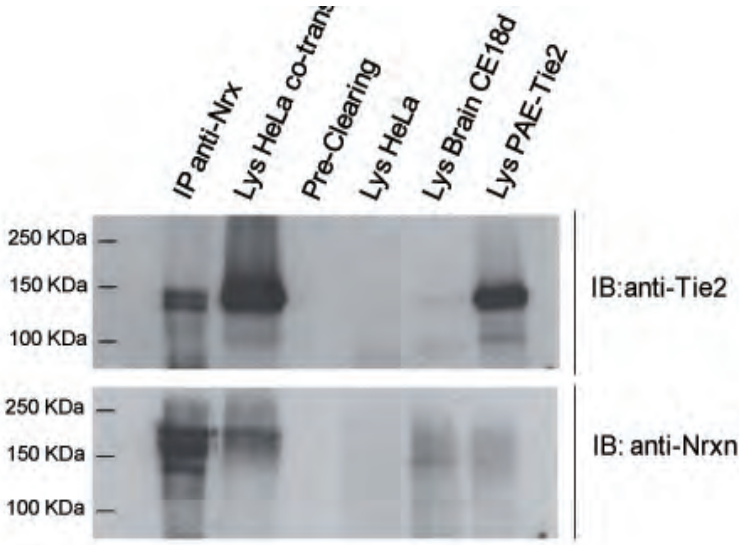


Fig S3: in vitro co-immunprecipitation of Tie2 and alphaNRXN.
alphaNRXN was immunoprecipated from Hela cells overexpressing alphaNRXN and Tie2 and the resulting precipitate was blotted against Tie2 (upper gel) or Nrxn (lower gel).
Lysate obtained from E18 Chick embryo brain was used as a positive control for Neurexin blotting while lysate from Tie2 overexpressing porcine aortic endothelial cells was used as a positive control for Tie2 blotting.