Title: Pharmacologically diverse antidepressant drugs disrupt the interaction of BDNF receptor TRKB and the endocytic adaptor AP-2

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Abstract

The activation of TRKB (tropomyosin-related kinase B), the receptor for brain-derived neurotrophic factor (BDNF), is a crucial step in brain plasticity. Several antidepressant drugs are reported to activate TRKB, and animals with compromised BDNF or TRKB system are refractory to these drugs' effects. However, it remains unclear whether these compounds employ a common mechanism to activate BDNF-TRKB signaling.

We have used mass spectrometry to investigate proteins that are co-immunoprecipitated with TRKB from the hippocampus of mice treated with the antidepressant fluoxetine or vehicle. We found that the interaction of several proteins with TRKB was disrupted in fluoxetine-treated brain samples, including several components of the AP-2 complex involved in vesicular endocytosis. Silencing of AP2M (mu subunit) expression in MG87.TRKB fibroblast cell line that overexpresses TRKB led to an increase in the surface positioning of TRKB as well as to a higher response to BDNF, observed as the levels of active TRKB (pTRKB). Consistent with these findings, animals lacking one of the *Ap2m1* gene copies also displayed increased levels of active TRKB in the hippocampus.

We also addressed the effect of multiple antidepressant drugs on the interaction of TRKB and AP2M by ELISA. Pharmacologically diverse drugs: imipramine, fluoxetine, phenelzine and rolipram, decreased the interaction between TRKB:AP2M, and increased TRKB surface expression levels. However, while the interaction between full length TRKB and AP2M was disrupted by fluoxetine, the interaction between AP2M and the TRKB C-terminal peptide was found resistant to this drug suggesting additional binding sites targeted by fluoxetine or an indirect mechanism of action of the antidepressants.

Taken together, our data suggest that the TRKB:AP2M interaction is a potential target for several antidepressants with diverse chemical structures and modes of action, leading to increased exposure of TRKB at the cell surface and facilitated activation by BDNF.

Keywords: antidepressants; AP-2 complex; TRKB; BDNF

Introduction

Compromised plasticity has been suggested as one of the major causes of depression (1). Supporting this idea, morphological and functional deficits have been observed in the brains of patients with mood disorders (2, 3, 4). Reduced neurotrophic support, such as by BDNF signaling via TRKB, has been linked to the atrophy observed in these patients in the neuronal networks regulating mood and cognition (5, 6).

Antidepressant drugs, mainly targeting monoamine neurotransmission, such as serotonin (5-HT) and noradrenaline, have been suggested to act by improving neuronal connectivity, plasticity, and information processing (4). In line with this, an intact BDNF-TRKB signaling system is crucial for the efficacy of antidepressant compounds, as mice overexpressing a dominant negative TRKB isoform (TRKB.T1) or heterozygous BDNF null (*Bdnf+/−*) are not responsive to antidepressant drugs (7). Furthermore, the activation of TRKB receptor signaling, and putatively the reinstatement of plasticity, is associated to the response to antidepressants (7–9).

The binding of BDNF to TRKB initiates phosphorylation of tyrosine residues in the intracellular portion of the receptor (10) which allows docking of adaptor proteins to these sites. Phosphorylated tyrosine 515, for example, serves as a docking site for SHC adaptor protein, while phospholipase C gamma (PLCg1) binds to the phosphorylated Y816 residue (11). Interestingly, the phosphorylation of TRKB receptors at Y706/7 and Y816 residues are enhanced by several antidepressants in adult mouse hippocampus while no change was detected at the Y515 residue (7, 9). Despite the evidence on the necessity of TRKB receptor activation for antidepressant responses, the mechanism by which these drugs might trigger TRKB activation remains unclear.

In this scenario, proteins able to modulate TRKB transit to and from the cell surface, therefore modulating its exposure to BDNF, posed as an interesting possibility to understand the antidepressant-induced effects. In line with this idea, our mass spectrometry-based screen for TRKB interactors identified the endocytic adaptor protein complex-2 (AP-2). Components of AP complexes 1, 2 and 3, are crucial for clathrin-dependent endocytosis, and participate in the removal of several transmembrane receptors, and their ligands (12). AP-2 is a heterotetrameric complex which consists of two large chains (alpha- and beta-adaptin), one medium chain (mu, AP2M), and one small chain (sigma) (13).TRK receptors have been identified among the targets for rapid clathrin-mediated endocytosis upon binding to their ligands (14). Earlier studies demonstrated that BDNF-induced phosphorylation of TRKB receptors recruits AP-2 complex and clathrin to the plasma membrane in cultured hippocampal neurons (15). More recently, a novel relationship has been established between TRKB receptors and AP-2, suggesting that autophagosomes containing activated TRKB are transported to the cell soma in an AP-2-dependent manner (16).

In the present study, we hypothesized that an interaction between TRKB and AP-2 might be a possible site of action for antidepressant drugs. Specifically, we identified a putative binding site on the TRKB receptor for AP2M, which provides a known interface for cargo-binding, and examined the impact of AP2M downregulation on the activation and surface exposure of TRKB. Finally, we tested the effects of several antidepressant drugs such as fluoxetine, imipramine, phenelzine, and rolipram on the interaction of TRKB receptors with AP2M.

Methods

Animals:

Adult (7 weeks old) male C57BL/6 mice from Harlan, kept with free access to food and water, were used for TRKB immunoprecipitation and mass spectrometry analysis. Briefly, the animals received a single intraperitoneal injection of fluoxetine (30 mg/kg) and were euthanized by CO_2. The hippocampi were collected and processed as described below. All procedures were in accordance with international guidelines for animal experimentation and the County Administrative Board of Southern Finland (ESLH-2007-09085/Ym-23, ESAVI/7551/04.10.07/2013).

The generation of floxed AP2M mice carrying loxP sites in front of exon 2 and behind exon 3 of the AP2M encoding gene *Ap2m1* is described in literature (17). These conditional knockout (KO) mice were crossed with CMV-Cre deleter mice to achieve ubiquitous excision of exon 2 and 3. The resulting AP2M.het mice were crossed with AP2M.wt mice, thus excluding the generation of AP2M homozygous KO animals which are embryonically lethal (18). All animals were genotyped prior to experiments using PCR on genomic DNA extracted from tissue biopsies, and confirmed by western blotting analysis of hippocampus. The KO allele was detected using the forward primer GCTCTAAAGGTTATGCCTGGTGG and the reverse primer CCAAGGGACCTACAGGACTTC that generate a fragment of 404 bp (PCR at 58°C, 25 cycles). All experiments involving AP2M.het mice were reviewed and approved by the ethics committee of the “Landesamt für Gesundheit und Soziales” (LAGeSo) Berlin and were conducted according to the committee’s guidelines. For brain dissection, animals were anesthetized with isoflurane prior to cervical dislocation. The hippocampus was dissected on ice, and processed as described below.

Drugs:

Acetylsalicylic acid (aspirin, Sigma Aldrich, #A2093), fluoxetine (Bosche Scientific, #H6995), imipramine (Sigma Aldrich, #I7379-5G), phenelzine (Sigma Aldrich, #P6777), rolipram (Sigma Aldrich, #R6520), recombinant human BDNF (Peprotech, #450-02), and

k252a (TRK inhibitor, Sigma-Aldrich, #K2015) were used (19, 20). All compounds were dissolved in DMSO, except BDNF which was diluted in PBS.

Immunoprecipitation and mass spectrometry:

For TRKB proteome qMS analysis, adult mouse hippocampal samples (n=3) were mechanically homogenized in NP lysis buffer [20 mM Tris-HCl; 137 mM NaCl; 10% glycerol; 0.05 M NaF; 1% NP-40; 0.05 mM Na3VO4; containing a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich, #P2714 and #P0044, respectively)]. The samples were centrifuged (16000xg) at 4ºC for 15 min, and the supernatant was subjected to pre-clearing (control) and TRKB immunoprecipitation using Protein-G sepharose containing spin columns (Pierce Spin Columns Snap Cap #69725). Briefly, 30 µl of Protein-G sepharose were added to the columns and washed twice with 200 µl NP buffer. Samples (400 µg of total protein) were cleared through the columns without antibody (1 h, under rotation at 4°C). Unbound samples were collected (1000xg, 1 min at 4ºC) pre-incubated with TRKB antibody (Anti-mouse TRKB Antibody, R&D Systems, #AF1494, 5 µl) and cleared through new Protein-G sepharose tubes. After 1 h rotation in the cold, the columns were washed twice with NP buffer and twice with TBS buffer (1000xg, 1 min at 4°C). The proteins were then eluted from the columns with 0.1 M glycine (3x, 1000xg, 1 min, 4°C), or with Laemmli buffer for samples submitted to western blotting; and stored at -80°C until further analysis. Cysteine bonds of the proteins were reduced with TCEP [Tris(2-carboxyethyl)-phosphine hydrochloride salt, #C4706 Sigma-Aldrich, USA] and alkylated with iodoacetamide (#57670 Fluka, Sigma-Aldrich, USA). Samples were digested with trypsin (Sequencing Grade Modified Trypsin, #V5111, Promega), and digested peptides were purified with C18 microspin columns (Harvard Apparatus, USA) (21).

Mass spectrometry analysis was performed on an Orbitrap Elite hybrid mass spectrometer (Thermo Scientific) coupled to EASY-nLC II system (Xcalibur version 2.7.0 SP1, Thermo Scientific). The peptides were separated with a 60 min linear gradient from 5 to 35% of buffer B (98% acetonitrile and 0.1% formic acid in MS grade water). The analysis was performed in data-dependent acquisition: a high resolution (60 000) FTMS full scan (m/z 300-1700) was followed by top20 CID-MS2 scans in ion trap (Energy 35). The maximum fill time was 200 ms for both FTMS (Full AGC target 1000000) and the ion trap (MSn AGC target of 50000). The MS/MS spectra were searched against the mouse component of the UniProtKB- database (release 2012_08, X entries) using SEQUEST search engine in Proteome Discoverer™ software (version 1.4, Thermo Scientific). Carbamidomethylation (+57.021464 Da) of cysteine residues was used as static modification, and oxidation (+15.994491 Da) of methionine was used as dynamic modification. Precursor mass tolerance and fragment mass tolerance were set to <15 ppm and <0.8 Da, respectively. A

maximum of two missed cleavages was allowed, and results were filtered to a maximum false discovery rate (FDR) of 0.05. The final hit table was rendered based on the following criteria: the average signal from three controls from Sepharose columns only ≤0.33, specific signal minus signal from Sepharose column ≥2.

Cell culture and silencing of AP2M expression:

The cell line 3T3 (mouse fibroblasts) stably transfected to express TRKB (MG87.TRKB) were used for *in vitro* assays (20). The cells were maintained at 5% CO2, 37°C in Dulbecco's Modified Eagle's Medium (DMEM, containing 10% fetal calf serum, 1% penicillin/streptomycin, 1% L-glutamine and 400 mg/ml G418).

For silencing of AP2M expression, MG87.TRKB cells were transfected with a mix of 4 AP2M specific shRNA sequences (Origene, USA, #TG712191 or scrambled sequence as control) using Lipofectamine 2000 as vehicle. The expression of GFP reporter was confirmed by microscopy 24 h after transfection, and AP2M expression levels were determined by western blotting. The cells were challenged with BDNF (0 or 25 ng/ml/15min) and lysed for ELISA or western blotting as described below.

Sample processing and western blotting:

Samples from hippocampi of male and female AP2M.wt or AP2M.het mice were sonicated in NP lysis buffer, centrifuged (16000xg at 4°C for 15 min), and the supernatants were submitted to SDS-PAGE. Samples from hippocampi of 7 weeks-old male mouse was used to confirm the TRKB:AP2M co-immunoprecipitation (as described above) by western blotting. Samples from MG87.TRKB cells were homogenized in NP lysis buffer and submitted to SDS-PAGE or AP2M:TRKB ELISA. For cell-free ELISA assays described below, the samples were acidified with 1M HCl (pH ~2) at room temperature for 15min, and the pH was restored to 7.4 with 1M NaOH. This procedure aims to disrupt non-covalent interactions between proteins in a given complex.

The resolved proteins were transferred to PVDF membranes, blocked with 5% BSA in TBST [20 mM Tris-HCl; 150 mM NaCl; 0.1% Tween-20; pH 7.6] for 2 h at room temperature and incubated overnight at 4°C with antibodies against TRKB (R&D Systems; #AF1494, 1:1000); AP2M (Santa Cruz; #sc-515920, 1:1000), pTRKB.Y515 (Cell Signaling, #9141, 1:1000), pTRKB.Y706/7 (Cell Signaling, #4621, 1:1000), pTRKB.Y816 (Cell Signaling, #4168, 1:1000) or GAPDH (Cell Signaling, #2118 or Abcam, #ab8245, 1:5000). The membranes were then incubated with HRP-conjugated secondary antibodies (anti-Gt, Invitrogen, #61-1620; anti-Rb, Bio-Rad, #170-5046; or mouse IgG kappa binding protein, Santa Cruz, #516102; 1:5000), and the HRP activity was detected by incubation with ECL

and exposure to a CCD camera. The signal from each target was background-subtracted, normalized by GAPDH or TRKB and expressed as percentage of AP2M.wt, scrambled or vehicle-treated groups.

AP2M:TRKB interaction and surface TRKB ELISA assays:

MG87.TRKB cells were incubated with fluoxetine, imipramine, rolipram, aspirin, which was used as negative control (at 0, 1, 10 or 100 µM) or phenelzine (at 0, 0.01, 0.1, 1, 10 or 100 µM). An independent group received k252a (20 mM), followed 10min later by fluoxetine (0 or 10 µM) or BDNF (0 or 25ng/ml). The cells were lysed 15 min after the last drug administration. For surface TRKB analysis, the cells were incubated with fluoxetine or imipramine (0 or 10 µM) for 15 min or 2 h. The concentration ranges were chosen based on literature (20).

The AP2M:TRKB interaction was determined by ELISA, based on the general method described in literature (20), with minor adjustments to detect TRK:AP2M interaction. Briefly, white 96-well plates (OptiPlate 96F-HB, Perkin Elmer) were coated with capturing anti-TRK antibody (Cell Signal; #92991S, 1:1000) in carbonate buffer (pH= 9.8) overnight (ON) at 4ºC. Following a blocking step with 2%BSA in TBS-T (2 h, RT), samples were incubated ON at 4ºC. The incubation with antibody against AP2M (1:2000, ON, 4ºC) was followed by HRP-conjugated mouse IgG kappa binding protein (1:5000, 2h, RT). Finally, the chemiluminescent signal generated by the reaction with ECL was analyzed in a plate reader (Varioskan Flash, Thermo Scientific).

The surface levels of TRKB were also determined by ELISA (22). Briefly, following the treatment with fluoxetine or imipramine (10 µM; 15 min or 2 h), the MG87.TRKB cells, cultivated in clear bottom 96-well plates (ViewPlate 96, Perkin Elmer), were washed with ice-cold PBS and fixed with 100 µl of 4% PFA per well. After washing with PBS and blocking with PBS containing 5% nonfat dry milk and 5% BSA, the samples were incubated with primary anti-TRKB antibody (R&D Systems, #AF1494, 1:1000 in blocking buffer) ON at 4ºC. Following washing, the samples were incubated with HRP-conjugated antibody (1:5000 in blocking buffer) for 1 h at RT. The cells were washed 4x with 200 µl of PBS for 10 min each. Finally, the chemiluminescent signal generated by reaction with ECL was analyzed in a plate reader.

The cell-free assays were performed in white 96-well plates (OptiPlate 96F-HB, Perkin Elmer). The plates were precoated with anti TRKB antibody (1:1000, #AF1494, R&D Systems) in carbonate buffer (pH 9.8), ON at 4ºC. Following blocking with 3% BSA in PBST buffer (2 h at RT), 120 µg of total protein from each sample (processed as described above) were added and incubated overnight at 4ºC under agitation. The plates were then washed 3x with PBST buffer, and fluoxetine (0-100 µM) was added for 15 min. Following the

washing protocol, the secondary antibody against AP2M was added (1:2000) ON at 4ºC under agitation. The plates were then incubated with HRP-conjugated mouse IgG kappa binding protein (1:5000) for 2 h at RT. The HRP activity was detected by luminescence following incubation with ECL in a plate reader.

For the cell-free AP2M:TRKB C-terminal interaction, the plates were precoated with antibody against AP2M (1:500) in carbonate buffer, then samples (processed as described above) from MG87.TRKB cells were added ON at 4ºC. N-biotinylated synthetic peptides (WT, pY, or Y/A; Genscript, USA, sequences described in figure 1) of the last 26 aa of rat TRKB (0.1 mg/ml= 34 mM, in PBST) were added for 1 h at RT; followed by HRP-conjugated streptavidin (Thermo Fisher, #21126, 1:10000) for 1 h at RT. The luminescence was determined upon incubation with ECL as described. In an independent experiment, immobilized AP2M from MG87.TRKB lysates was incubated with pY TRKB C-terminal peptide and fluoxetine (0 or 10 µM) for 1 h at RT; and the luminescence was determined via HRP-conjugated streptavidin activity reaction with ECL by a plate reader.

The background-subtracted signal from each sample in each of the described assays was expressed as percentage of control group (vehicle-treated or WT peptide).

Structural bioinformatics data mining and docking assay:

In order to address a putative direct interaction between AP2M and TRKB, we submitted the full length, canonical sequence of rat and mouse TRKB (Uniprot: Q63604 or P15209, respectively) to Eukaryotic Linear Motif library (23). Several TRG_ENDOCYTIC_2 motifs (TRG2; a tyrosine-based sorting signal responsible for the interaction with AP2M subunit) were found for both mouse and rat TRKB. Of particular interest, the Y816 residue is also activated by BDNF and antidepressants. Thus, the sequence of the last 26aa residues of rat TRKB (TRKB.ctrl, TRKB.Y816E, and TRKB.Y816A) and full length AP2M (model generated through RaptorX server and aligned with the C-terminal structure available in RCSB databank: 2PR9) were submitted to docking simulations using CABS-docking server (24).

Statistical analysis

Data was analyzed by Student's t test, one- or two-way ANOVA (or their non-parametric equivalents) followed by Fisher's LSD *post hoc* test, when appropriate. Values of $p<0.05$ were considered significant.

Results

AP2M is an interaction partner of TRKB receptors

Samples from adult mouse hippocampus were processed for co-immunoprecipitation with TRKB-specific antibody and analyzed by mass spectrometry to investigate the TRKB interactome. The list of identified interactors comprises several components of the synaptic and endocytic machinery (Fig. 1a) such as synaptic vesicle glycoproteins, syntaxins, clathrin and AP-2 along with other transport-related proteins. The complete list of interactors can be found in the supplement table 1. In the present study, we decided to focus on the interaction with AP-2 as a putative modulator of TRKB surface localization and activation. We confirmed the interaction between TRKB and AP-2 by performing further co-immunoprecipitation and western blotting experiment using samples from mouse hippocampus and the TRKB overexpressing fibroblast cell line MG87.TRKB, as found in figure 1b.

Many of AP-2´s cargo proteins are bound via recognition of single or multiple tyrosine-based motifs by AP2M subunit. These motifs in the cytosolic tails of transmembrane receptors consist of four amino acid residues (25). The initial tyrosine residue of the motif is followed by two random amino acids, and the signal ends with one of the hydrophobic residues; leucine, isoleucine, phenylalanine, valine or methionine (26). We searched for such tyrosine-based motifs in mature full length TRKB. The Eukaryotic Linear Motif library (elm.eu.org) in fact indicated the presence of multiple putative tyrosine-based sorting signals [classified as TRG_ENDOCYTIC_2 motifs (TRG2)] in the cytosolic domain of the rat and mouse canonical TRKB sequence, as seen in figure 1d. Interestingly, the presumed AP2M binding site YLDI, which is located within the C-terminal portion of TRK, is phosphorylated following antidepressant administration (9).

In order to assess the supposed interaction mechanism between AP2M and TRKB, we generated a model of AP2M in the RaptorX server (27, 28) and aligned it with AP2M C-terminal chain crystal structure from RCSB databank (2PR9) (Fig. 1e). Since the alignment indicated a low root-mean squared deviation (RMSD) from the crystal structure (2Å), whereby proposing a reliable structure for docking, we proceeded to the simulations. To this aim, the AP2M model and the last 26aa residues from the C-terminal portion of TRKB; including the mutations: Y816A and Y816E (phosphomimetic), were uploaded to CABS-dock server (24). We obtained the Gibbs' free energy for the AP2M:TRKB C-terminus peptide interaction, and the lowest 10 values from each group was used in statistical analysis. The interaction energy of AP2M:TRKB.Y816E complex was lower, suggesting a more stable interaction, while it was significantly higher for AP2M:TRKB.Y816A, compared to AP2M:TRKB.ctrl [Kruskal-Wallis= 25.83; $p<0.0001$] (Fig. 1c). Based on these *in silico* results, we proceeded to analyze the AP2M:TRKB C-terminus interaction *in vitro* by ELISA. For this purpose, the N-biotinylated synthetic peptides (ctrl, pY or Y816A) of the last 26 aa of

rat TRKB were used. The interaction between AP2M and the phosphorylated TRKB peptide (TRKB.pY) was stronger compared to control peptide. In line with our earlier evidence, the AP2M:TRKB.Y816A interaction was weaker in comparison with the control peptide [Kruskal-Wallis= 20.48; p<0.0001] (Fig. 1g).

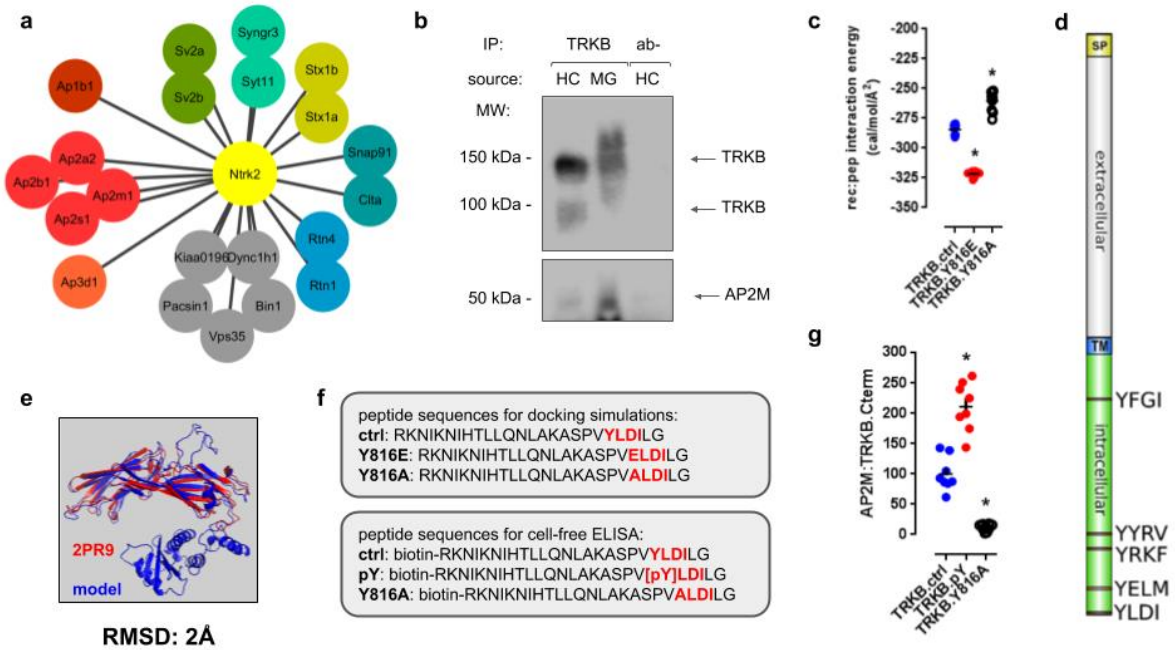

Figure 1. Interaction between TRKB and AP-2 complex. (a) Putative TRKB (*Ntrk2*) interactors (*Sv2a, Sv2b, Syngr3, Syt11, Stx1a, Stx1b, Snap91, Clta, Rtn4, Rtn1, Kiaa0196, Dync1h1, Pacsin1, Vps35, Bin1, Ap3d1, Ap2a2, Ap2b1, Ap2m1, Ap2s1, Ap1b1*) involved in vesicle trafficking were grouped according to function. Red/orange: members of the AP-1/2/3 complexes; for a full list of interacting proteins, please refer to supplement table 1. **(b)** Co-immunoprecipitation of TRKB and AP-2 complex in samples from MG87.TRKB cells and hippocampal neurons. **(c)** Simulation of AP2M:TRKB peptide interaction. The phosphomimetic peptide (TRKB.Y816E, red) shows lower free energy for the receptor:peptide docking than wild-type peptide (TRKB.ctrl, blue), while TRKB.Y816A mutant (black circles) displayed the highest free energy values (n=10/group). **(d)** The very C-terminal region of TRKB exhibits an AP2M-binding domain (TRG_ENDOCYTIC_2, red), identified by Eukaryotic Linear Motif library. Yellow: SP, signal peptide; grey: extracellular portion; blue: TM, transmembrane; green: intracellular. **(e)** Alignment of the full length AP2M model (from RaptorX server) and C-terminal chain from RCSB databank (2PR9); root-mean squared deviation (RMSD)= 2Å. **(f)** The sequence of the TRKB C-terminus peptides used for *in silico* and *in vitro* peptide-AP2M docking and binding assays, respectively; red: TRG_ENDOCYTIC_2-positive motif and mutated sites. **(g)** *In vitro* interaction of AP2M:TRKB.C-terminus peptides (0.1mg/ml= 34uM) measured by ELISA. Phosphorylated peptide (TRKB.pY, red) showed the higher interaction with AP2M compared to control peptide (TRKB.ctrl, blue), while TRKB.Y816A mutant (black) displayed the lowest interaction levels (n=8/group).*p<0.05 for comparison to the wild-type peptide TRKB.ctrl.

Role of AP2M in TRKB function

Previous studies indicate that depleting AP2M from mammalian cells prevents the assembly of functional AP-2 complexes (29) and corresponds to an AP-2 loss of function (17). Thus, we investigated the impact of AP2M downregulation on TRKB receptor signaling. The reduction of AP2M in MG.TRKB cells [t(4)=3.00; p=0.0399] (Fig. 2b) did not alter overall TRKB levels [t(4)=0.35; p=0.74] (Fig. 2c). However, the cell surface localization of TRKB

receptors was enhanced [t(28)= 7.71; p<0.0001] as measured by surface ELISA (Fig. 2e). Moreover, silencing of AP2M enhances the effect of BDNF on phosphorylation of Y706/7 [t(4)=2.82; p=0.0477] (Fig. 2a) and Y816 [F(1,20)=12.21; p=0.0023] (Fig. 2d) residues in TRKB as measured by western blotting and ELISA, respectively. Finally, the reduced levels of AP2M in the hippocampus of haploinsufficient AP2M.het mice [t10=4.63; p=0.0009] (Fig. 2f) was associated with increased levels of pTRKB [genotype: F(1,29)=17.59; p=0.0002] at Y706/7 and Y816 residues (Fisher's LSD, p=0.015 and 0.0016, respectively) (Fig. 2h). However, we also observed reduced levels of total TRKB in the hippocampus of these animals as shown by western blotting [t(10)=2.794; p=0.019] (Fig. 2g). This result was unexpected, especially as brain lysates of neuron-specific AP2M null mice were reported to have increased TRKB expression levels (16). However, the complete loss of an essential endocytic protein might cause very different compensatory changes than its partial reduction.

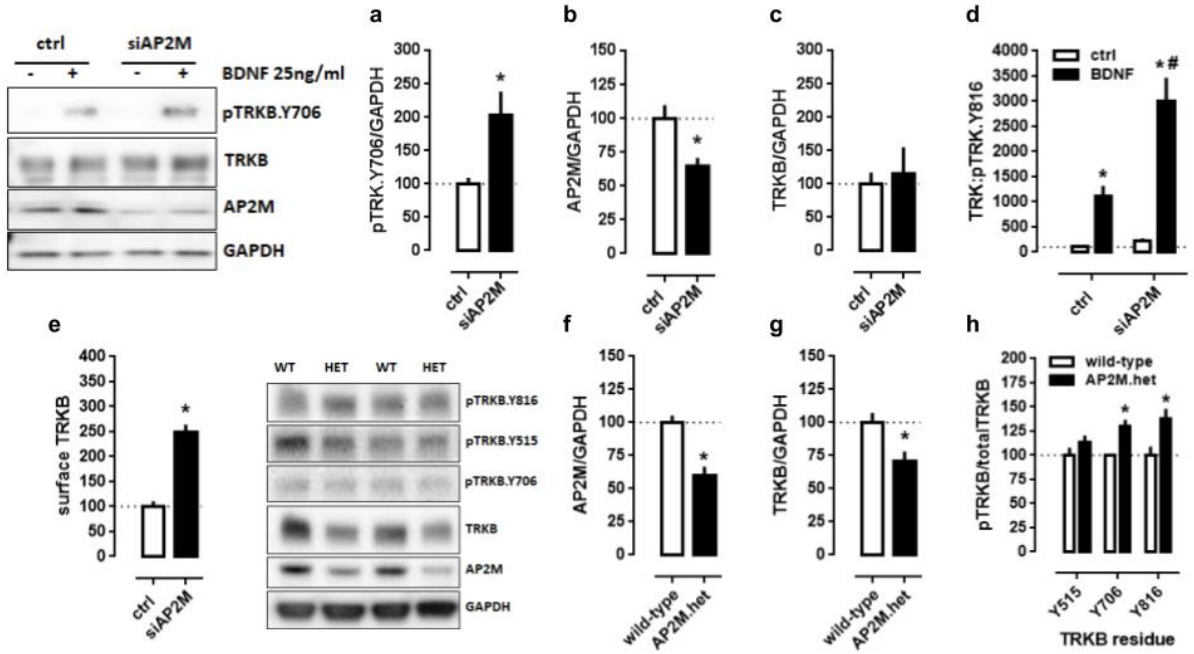

Figure 2. AP2M regulates TRKB cell surface localization and response to BDNF. (a, d) Cultured MG87.TRKB cells transfected with siRNA to silence the expression of AP2M showed increased phosphorylation of TRKB at pY706 and pY816 in response to BDNF (n=3-6/group) as shown by western blotting and ELISA, respectively. **(b)** The partial decrease in AP2M expression was not associated with a decrease in **(c)** total TRKB levels. **(e)** The silencing of AP2M expression led to an increased exposure of TRKB at the cell surface (n=6/group) as measured in surface ELISA. **(f)** AP2M.het animals showed decreased levels of AP2M and **(g)** total TRKB in the hippocampus, associated with **(h)** increased levels of phosphorylated TRKB at Y706 and Y816 residues (n=5-6/group). *p<0.05 for comparison with ctrl/wild-type group; #p<0.05 for comparison with ctrl/siAP2M group.

Antidepressants and BDNF differentially affect the TRKB:AP2M interaction

The systemic administration of fluoxetine (30 mg/kg, ip, 30 min) suggests a disruptive effect on TRKB and AP2M interaction, among a list of proteins affected by the drug administration that can be found in supplement table 2. Classical antidepressant drugs, such

as fluoxetine, imipramine, and rolipram, but not the negative control aspirin were able to uncouple TRKB:AP2M interaction in MG87.TRKB cells as measured by ELISA [fluoxetine: $F(3,26)=4.554$, $p=0.011$; imipramine: $F(3,43)=7.883$, $p=0.0003$; rolipram: $F(3,19)=6.78$, $p=0.0027$; aspirin: $F(3,40)=0.082$, $p=0.96$], with effects ranging from 1 to 100 μM (Fisher's LSD $p<0.05$) (Fig. 3a). Likewise, phenelzine was effective in disrupting the TRKB:AP2M interaction [Kruskal-Wallis=19.34; $p=0.0017$] with effective doses at 1-10 μM (Fisher's LSD $p<0.05$) (Fig. 3b).

The administration of fluoxetine or imipramine also increased the levels of surface exposed TRKB as measured in surface ELISA [$F(1,54)=62.54$; $p<0.0001$] (Fig. 3c, d). Interestingly, the disruptive effect of fluoxetine on the TRKB:AP2M interaction is not dependent on TRKB activation, since pretreatment with the TRK inhibitor k252a was not able to prevent such effect [interaction effect: $F(1,20)=0.3066$; $p=0.586$] (Fig. 3e). Contrary to what was observed for antidepressants, BDNF administration (25 ng/ml) resulted in an increased interaction of TRKB receptors with AP2M, which was dependent on TRKB-activation, since pretreatment with k252a prevented this effect [interaction effect: $F(1,20)=4.528$; $p=0.046$] (Fig. 3f).

Finally, we observed that fluoxetine disrupted the interaction between full length TRKB and AP2M in a cell-free ELISA [$F(5,30)=9.71$; $p<0.0001$] with effective doses ranging from 0.3-100 μM (Fisher's LSD, $p<0.05$ for all) (Fig. 3g). However, no effect of fluoxetine (10 μM) was observed on the interaction between AP2M and the TRKB C-terminal peptide in cell-free ELISA [$t(15)= t=0.35$; $p=0.73$] (Fig. 3h).

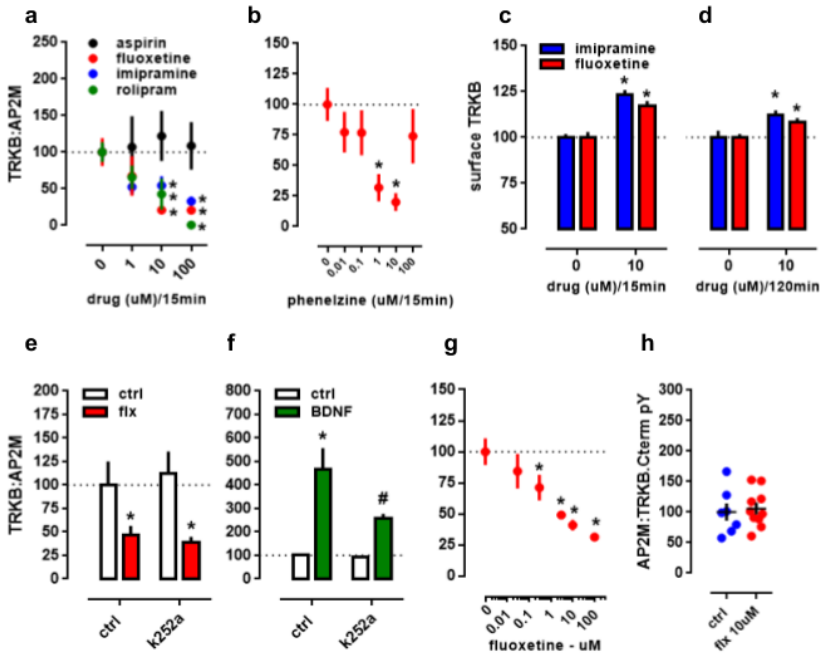

Figure 3. Antidepressant-induced disruption of TRKB and AP2M interaction. (a) MG87.TRKB cell cultures were treated with 1-100 µM of aspirin (black), fluoxetine (red), imipramine (blue) or rolipram (green) for 15 min. The TRKB:AP2M interaction was disrupted by fluoxetine, imipramine and rolipram (at 10 and 100 µM) while no effect of aspirin was observed in ELISA (n=6-12/group). **(b)** MG87.TRKB cells were exposed to different concentrations of phenelzine (0.01-100 µM) for 15 min, and disruption of the TRKB:AP2M interaction was observed at 1 and 10 µM in ELISA (n=6-12/group). **(c, d)** Surface levels of TRKB increased following fluoxetine or imipramine treatment as measured by surface ELISA (10 µM; n=15/group). **(f)** ELISA measurement showed that the treatment of MG87.TRKB cells with k252a (10 µM) prevents the effect of BDNF (25 ng/ml), but not **(e)** fluoxetine (10 µM) on the TRKB:AP2M interaction (n=6-12/group). **(g)** As shown by a cell-free ELISA, fluoxetine was able to disrupt the AP2M interaction with full length, but not with **(h)** the TRKB C-terminal peptide in lysates from MG87.TRKB cells (n=6-10/group). *p<0.05 for comparison with ctrl/0 group.

Discussion

In the last years, several pieces of evidence have indicated that sub-chronic (30) and chronic fluoxetine treatment (31, 32) can induce changes in the proteomics profile of rodent brains. Fluoxetine was found to alter the expression of proteins involved in neuroprotection, serotonin biosynthesis, and axonal transport in hippocampus and frontal cortex (31), as well as in endocytosis and transport in visual cortex (32).

The present study identified the endocytic complex AP-2 as a target whose interaction with TRKB is reduced following acute fluoxetine treatment. Since AP-2 appeared as a promising candidate for the modulation of TRKB surface levels and activity putatively taking part as downstream of antidepressant treatment, we selected it for further investigation. In line with a robust interaction between AP-2 and TRKB we found all subunits of the AP-2 in the same complex with TRKB, while in case of the related proteins, *ie* AP-3 and AP-1, only one subunit was identified for each *Ap3d1* and *Ap1b1*. Furthermore, we

identified tyrosine-based motifs in the TRKB receptor as putative binding sites for the AP2M subunit of the AP-2 adaptor complex. Especially the region following the Y816 residue of TRKB conformed to the classical consensus for an AP2M-interacting motif. In line with its classical role as endocytic adaptor protein, AP-2 appears to regulate the surface localization of its interactor TRKB, and therefore its signaling. Decreased levels of AP2M resulted in increased surface localization of TRKB, facilitating the activation by its ligand BDNF. Consistent with this, we found increased levels of activated TRKB in the hippocampus of AP2M heterozygous mice. Finally, we observed that antidepressant drugs such as fluoxetine, imipramine, rolipram, and phenelzine are able to disrupt the binding between AP2M and TRKB resulting in increased exposure of TRKB at the cell surface.

It has been established that activation, endocytosis, and trafficking of TRKB receptors regulate the propagation and fate of the downstream signaling response (33). The post-endocytic sorting machinery plays a major role during this fate-determination process (34). In this scenario, some of the vesicles carrying the receptors are destined for retrograde transport towards the cell body (35, 36), while some are recycled back to the cell surface (37). For instance, Pincher, a membrane trafficking protein, has been shown to regulate the formation of TRK-carrying endosomes from the plasma membrane ruffles in soma, axon, and dendrite (38). Pincher-mediated retrograde transport of signaling TRK receptors are rescued from lysosomal degradation (39). Moreover, BDNF-induced survival signaling in hippocampal neurons has been shown to be regulated by Endophilin-A, that is involved in the endosomal sorting of active TRKB receptors (40). Evidence suggests that the maintenance of long-term potentiation in hippocampal slices depends on the recycling and re-secretion of BDNF, thus, emphasizing the necessity of TRKB receptor recycling back to the cell surface (41). Another study indicates that Slitrk5, by coupling to TRKB receptors, promotes its recycling to the cell surface in striatal neurons (42).

AP-2, as a member of the endocytic machinery (43), contributes to the formation of clathrin-coated vesicles around cargo proteins for endocytosis (44). In line with this idea, our results indicate a BDNF-induced enhancement of TRKB and AP2M interaction, suggesting that the receptor is endocytosed upon activation. Although acute treatment (e.g. 30 min) with antidepressant drugs can induce a rapid phosphorylation of TRKB receptor in the mouse brain (7), these drugs disrupted the interaction of TRKB:AP2M. Thus, we suggest that the differential regulation of TRKB:AP2M by BDNF and antidepressant drugs may underlie the differential surface expression of the receptor. Zheng and colleagues (22) have shown that BDNF quickly reduces the TRKB receptor surface level, while our data indicates that fluoxetine and imipramine enhance cell surface expression of TRKB. Interestingly, AMPA receptors, also committed to AP-2 complex-dependent endocytosis in the brain (45, 46), are internalized upon activation by glutamate. However, while the activation of NMDA receptors

results in rapid reinsertion of AMPAR subunits to the cell surface, AMPARs activated in the absence of NMDAR activation are destined for degradation in lysosomes (47). Thus, the source of the activation signal recruits alternative machineries to differentially regulate the fate of the target receptor.

Competitive binding can also be a crucial factor for determining the fate of a receptor as it was shown by Lavezzari and colleagues (48). These authors found that the interaction of postsynaptic density (PSD) proteins: PSD-95, PSD-93 and SAP-97 with NMDA receptor prevents endocytosis via blocking the binding of AP-2 to the designated motif on the C-terminus of the NR2B subunit. According to these authors, due to binding to PSD proteins, the surface expression of NR2B subunits was significantly upregulated. Furthermore, the competitive binding of N-ethylmaleimide-sensitive fusion protein (NSF) and AP-2 to GluR2 subunit of AMPA receptors regulates the cell surface level of this subunit, thus NSF acts as a steric blocker for the AP-2 complex (45). Based on this evidence, it is plausible to suggest that the antidepressant drug-induced prevention of AP-2 complex recruitment to TRKB receptors (or the disruption of formed complexes) allows binding of other proteins such as PLCg1 upon activation by BDNF, resulting in enhanced TRKB signaling. This argument is supported with our finding that the AP2M binding site on TRKB receptors, Y816, is the same one which is also targeted by PLCg binding and favored by antidepressants (9).

Although the AP-2 complex is best known for its major role in endocytosis (49), it remains doubtful whether the main purpose of the identified AP-2:TRKB interaction in the present study is the facilitated endocytosis of TRKB. A recent study performing endocytosis assays with WT and AP2M null neurons overexpressing EGFP-TRKB and treated with BDNF could not detect a significant defect in the uptake of TRKB upon loss of AP-2. Instead, the authors showed that the retrograde axonal transport of autophagosomes containing TRKB receptor is dependent on AP-2 linking the autophagy adaptor LC3 to $p150^{Glued}$, an activator of the dynein motor required for autophagosome transport (16). Interesting to highlight, LC3 (UniProt: Q91VR7) was co-precipitated with TRKB, and fluoxetine treatment decreased (in fact abolished) such interaction as shown by our mass spectrometry data. This led us to conclude that the action of the AP-2 complex is not necessarily restricted to the neuronal surface, but can take place in different neuronal compartments. Therefore, there is not only the possibility that the antidepressant drugs disrupt the interaction of TRKB receptors with AP2M at the cell surface, but they might also uncouple the interaction while both TRKB and AP2M are present on these autophagosomes which are transported towards the cell soma. Disruption of the AP2M:TRKB interaction either at the surface or on internal vesicles could prevent the interaction of the complex with the members of the autophagy-lysosome pathway that can lead to degradation of TRKB receptors (16). The delayed delivery of the signaling endosomes to soma may locally enhance the specific signaling

pathways that can recruit more plasticity-related proteins to the synapses. However, the two above mentioned scenarios are complementary rather than mutually exclusive. Recently, a study led by Eran Perlson described that BDNF-induced activation of TRKB leads to the internalization and dimerization of this receptor (50). These authors argue that TRKB is expressed in the cell surface as a monomer and only after BDNF-binding and internalization, in a process dependent on dynamin, the dimerization and consequent signaling is triggered.

In the present study we combined *in vivo*, *in vitro* and *in silico* methods to gain insight into putative interaction domains between TRKB and AP-2. The *in silico* techniques allowed us to obtain *a priori* knowledge about possible interactions, especially involving the Y816 residue and the AP2M subunit. In fact, in the present study, the *in silico* results on the AP2M interaction with the TRKB C-terminus matched the *in vitro* observations very well. As predicted by the CABS-docking server, AP2M displayed an increased interaction with phosphorylated TRKB (simulated using the phosphomimetic mutation Y816E). Interestingly, the Y816A mutation led to a decrease below the levels of control peptide (ctrl, non-phosphorylated) both observed in *in silico* (interpreted as a less stable complex) and *in vitro* assays. This suggests a putative interaction between AP2M and non-activated TRKB. The role of such interaction, if any, or its occurrence *in vivo*, remains to be investigated.

At this point, we speculated that the interaction of AP2M and TRKB could be a putative target for antidepressant drugs. Supporting this idea, cell-free assays indicated that the disruption of the TRKB:AP2M by fluoxetine happens in a dose-dependent manner. However, the same effect was not replicated in the AP2M:TRKB.peptide interaction assay. Therefore, although our data supports a putative binding site for antidepressant drugs in the TRKB complex, it is unlikely to be located between the receptor C-terminal portion and AP2M subunit. Moreover, such elusive site would present a low affinity for fluoxetine, given the effective doses are found above 0.3 µM.

Altogether, in the present study we suggest a putative novel mechanism where the drug-induced disruption of the TRKB:AP2M regulates TRKB receptor signaling. However, further investigation will be necessary to address the precise mechanism and the long-term consequences of such a disruption.

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