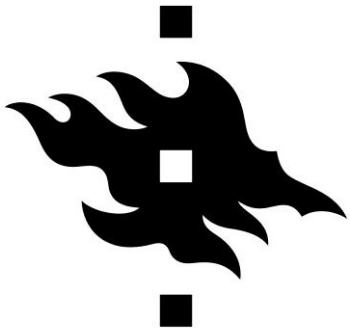

UNIVERSITY OF HELSINKI

Faculty of Biological and Environmental Sciences
Master's Programme in Neuroscience

Antidepressant drugs and nitric oxide synthase inhibitors facilitate neuronal plasticity by preventing nitration of the receptor tropomyosin-related kinase B

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Abstract

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Keywords
Tropomyosin-related kinase B (TRKB), nitric oxide (NO), nitric oxide synthase (NOS), plasticity, antidepressant drugs

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Tiivistelmä

Typpioksidi toimii viestinvälittäjämolekyylinä aivoissa säädellen monien proteiinien toimintaa saaden esimerkiksi aikaan translaation jälkeisiä muutoksia vaikuttamalla proteiinien tyrosiini- ja kysteiinitähteisiin. Typpioksidituotannon estämisellä on havaittu olevan eläinmalleissa samanlaisia vaikutuksia kuin masennuslääkkeillä. Reseptori tropomyosiinireseptorikinaasi B:n (TRKB) on todettu toimivan tärkeänä tekijänä masennuslääkkeiden aikaansaamissa vaikutuksissa ja reseptori itsessään sisältää mahdollisia typpioksidin aiheuttaman nitraation kohteita. Ryhmämme alustavat tulokset osoittavat, että TRKB-reseptorin nitraatio heikentää sen viestinvälitysmekanismeja. Masennuslääkkeet uudelleenjärjestävät ja irrottavat monia proteiineja TRKB-kompleksista. Tutkimme miten valikoiva typpioksidisyntaasin estäjä Nω-propyyli-L-arginiini (NPA) vaikuttaa hiiriin yhteydestä riippuvassa pelkoehdollistamisessa (engl. *contextual fear conditioning*) ja havaitsimme, että typpioksidin tuotannon estäminen NPA:n avulla sai aikaan masennuslääkemäisen vaikutuksen (engl. *antidepressant-like effect*). Havaitsimme myös masennuslääke fluoksetiinin estävän TRKB-reseptorin nitraatiota *in vivo*. Havaitsimme masennuslääkkeinä käytettävien fluoksetiinin, feneltsiinin ja imipramiinin hajottavan TRKB-reseptorin, typpioksidisyntaasin ja typpioksidisyntaasin avustajaproteiinin (CAPON) muodostaman proteiinikompleksin vuorovaikutuksia. Ymmärtääksemme TRKB-reseptorin ja typpioksidisyntaasi-proteiinin vuorovaikutuksia tutkimme niiden rakenteellisia vuorovaikutusalueita hyödyntäen Uniprot-tietokantaa löytämättä välitöntä kontaktipintaa proteiinien välillä, mutta kirjallisuuskatsauksen ja Uniprot-tietokannan tarkastelun jälkeen typpioksidisyntaasin avustajaproteiini (CAPON) havaittiin mahdolliseksi välikappaleeksi typpioksidisyntaasin ja TRKB-reseptorin välille. Saamamme tulokset osoittavat ensimmäistä kertaa masennuslääkkeiden hajottavan TRKB:CAPON:NOS-proteiinikompleksin vuorovaikutuksia suojaten TRKB-reseptoria typpioksidisyntaasin aiheuttamalta nitraatiolta. Masennuslääkkeiden aikaansaama käytösvaikutus saattaa johtua niiden kyvystä estää nitraation aiheuttamaa heikennystä TRKB-reseptorin viestinvälitysmekanismeissa.

Avainsanat
Tropomyosiinireseptorikinaasi B (TRKB), typpioksidi, typpioksidisyntaasi, muovautuvuus, masennuslääkkeet

Ohjaajat
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Muita tietoja
Tutkimusprojekti suoritettiin Eero Castrénin tutkimusryhmässä Helsingin Neurotiedekeskuksessa

Table of contents

Abstract

Nitric oxide (NO) is an important signalling molecule in the brain. NO regulates the function of many proteins by interacting with tyrosine and cysteine residues, thus inducing post-translational modifications. In animal models, inhibition of NO production triggers behavioural effects similarly to those induced by antidepressant drugs. Receptor tropomyosin-related kinase B (TRKB) has been identified as a key player mediating antidepressant drug (AD) induced effects, and it's a potential target for NO since it displays multiple potential sites for nitration. Preliminary results from our group indicate that TRKB nitration impairs its signalling, and AD uncouple many proteins from TRKB and reorganizes TRKB protein complex. We examined the effect of selective nitric oxide synthase (NOS) inhibitor N^{ω}-propyl-L-arginine (NPA) in mice submitted to the contextual fear conditioning and found out that inhibiting NO production with NPA has an antidepressant-like effect on mice. We also found out that AD fluoxetine prevents nitration of TRKB receptors *in vivo* and antidepressant drugs fluoxetine, phenelzine and imipramine disrupt the interactions of TRKB, NOS1 and NOS1 adaptor protein (CAPON) in co-immunoprecipitation assay. To understand the nature of TRKB and NOS1 interaction, we thus examined the protein domains in NOS1 and TRKB using Uniprot database, and we were unable to identify sites that could interact directly. Literature search for NOS1 adapting proteins followed by Uniprot data mining indicated CAPON as a potential candidate to mediate NOS1:TRKB interaction. Our data shows for the first time that antidepressant drugs disrupt TRKB:CAPON:NOS1 interaction, thus protecting TRKB from NOS1-induced nitration. ADs might induce their behavioural effects by preventing NO-induced impair in TRKB signalling.

List of abbreviations

NPA	N^{ω}-propyl-L-arginine
7-NI	7-nitroindazol
AD	Antidepressant drug
ANOVA	Analysis of variance
AP2	Adaptor protein
BDNF	Brain-derived neurotrophic factor
$BDNF^{+/-}$	Brain-derived neurotrophic factor deficient
BSA	Bovine albumine serum
CAMK	Calmodulin kinase
CAPON	Carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase protein
cGMP	Cyclic guanosine monophosphate
CREB	Cyclic AMP response element binding protein
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
E	Embryonic day
ERK	Extracellular signal-regulated kinase
FLX	Fluoxetine
GTP	Guanosine-5’-triphosphate
HRP	Horseradish peroxidase
IP3	Inositol tri-phosphate
MAOi	Monoamine oxidase inhibitor
MAPK	Mitogen-activated protein kinase
NA_3VO_4	Sodium orthovanadate
NB	Neurobasal medium
NFDM	Non-fat dried milk
NMDA	N-methyl-D-aspartate receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
NOS1AP	Nitric oxide synthase adaptor protein
NOSi	Nitric oxide synthase inhibitor
NRI	Norepinephrine reuptake inhibitors
PBS	Phosphote-buffered saline
PID	Phosphotyrosine interaction domain
PKC	Protein kinase C
PLCγ1	Phospholipase gamma
PVDF	Polyvinylidene difluoride
SEM	Standard error of mean
sGC	Soluble guanylyl cyclase
SSRI	Selective serotonin reuptake inhibitors
TBS	Tris-buffered saline
TCA	Tricyclic antidepressant

Introduction

Nitric oxide (NO) is an important signalling molecule in the brain (Moroz, 2001), playing a role in many neurophysiological and pathophysiological phenomena (Garthwaite, 2008). NO is synthetized from L-arginine in a reaction catalysed by neuronal nitric oxide synthase (NOS1), which in turn is regulated by calcium dynamics within the cell (Courtney and Nicholls, 1992; Alderton et al., 2001; Reichardt, 2006).

NO has signalling roles in both peripheral and central nervous systems. For instance, NO regulates gastrointestinal functions (Toda and Herman, 2005; Bartho et al., 2008) and vasodilation in peripheral nervous system (Toda and Okamura, 2003). In central nervous system, NO has both neuroprotective and neurotoxic properties, depending on its concentration (Calabrese et al., 2007) and it's involved in neurodegenerative diseases (Steinert et al., 2010). It is also an important modulator of neuronal plasticity; post-synaptically generated NO can modulate both pre- and post-synaptic targets. On the pre-synaptic side, NO has many effects on synaptic function and plasticity, affecting both glutamatergic and GABAergic synapses and functions (Hardingham et al., 2013). Nitric oxide is also involved in long-term potentiation in cerebellar (Jacoby et al., 2001), neocortical (Hardingham and Fox, 2006) and hippocampal areas (Bon and Garthwaite, 2001; Bellamy and Garthwaite, 2002; Bon and Garthwaite, 2003; Garthwaite, 2008). Even though NO itself is rapidly inactivated in brain tissue (Garthwaite, 2018), its signalling pathways can have long lasting effects on cells by S-nitrosylation of histone deacetylase 2 (Nott et al., 2008) and other transcriptional factors (Contestabile, 2008), eventually leading to regulation of gene expression via cyclic AMP response element binding protein (CREB) (Riccio et al., 2006). Currently, the most well understood NO target is guanylyl cyclase (sGC). NO targets haem group in sGC, an enzyme-coupled receptor (Bellamy and Garthwaite, 2002) that converts GTP into cGMP. Cyclic-GMP regulates the activity of protein kinases, phosphodiesterases and cyclic nucleotide-gated channels (Lucas et al., 2000; Garthwaite, 2010). However, NO and derived factors can modulate cellular targets also via post-translational modification of target proteins, such as S-nitrosylation or nitration. S-nitrosylation makes a reversible addition of NO group to cysteine residue thiol/sulfhydryl group forming a S-nitrosothiol derivative, which has an effect on properties of protein interactions and activity (Shahani and Sawa, 2012; Koriyama and Furukawa, 2018). NO can also directly modulate tyrosine residues in proteins through nitration. Nitrated proteins may be driven towards degradation and nitration seems to compete with phosphorylation in tyrosine residues (Shi et al., 2007; Abello et al., 2009).

Conversion of L-arginine to nitric oxide is dependent on calcium influx via N-methyl-D-aspartate receptor (NMDAr) channels or calcium release from internal storage (Brenman et al., 1996; Rose et al., 2003). N-terminal region of neuronal nitric oxide synthase (NOS1) contains PDZ domain that binds to postsynaptic density-95 (PSD-95) protein, which in turn anchors GluN2 subunit of NMDAr. PDZ domain of NOS1 can also interact with NOS1 adaptor protein (NOS1AP, also known as CAPON) (Jaffrey et al., 1998). CAPON regulates development of dendritic spines (Richier et al., 2010), dendritic patterning (Carrel et al., 2009) and it has been associated with

psychiatric disorders (Candemir et al., 2016; Wang et al., 2016). Previously, CAPON was considered solely an inhibitor of NOS1-activity (Jaffrey et al., 1998), but more recently this view has shifted towards CAPON's role as a functional mediator between NMDAr and NOS1 (Courtney et al., 2014).

New findings from our group indicate that NO might impair the signalling of tropomyosin-related receptor kinase B (TRKB). *In silico* analysis of full-length TRKB sequence identified potential nitration sites in cytoplasmic tyrosine residues (Biojone et al., 2015). Further characterization confirmed TRKB nitration *in vitro* and *in vivo*, and revealed that nitration induces TRKB internalization, via an endocytic adaptor protein (AP2) clathrin-dependent mechanism, and subsequent degradation (Biojone et al., *in preparation*).

TRKB signalling plays an important role in the central nervous system, ranging from neuronal survival to synaptic plasticity (Huang and Reichardt, 2001). Binding of brain-derived neurotrophic factor (BDNF) to its main target, TRKB, induces the receptor dimerization and activates autoregulatory phosphorylation in cytoplasmic tyrosine residues Y705/706 (Huang and Reichardt, 2001). Phosphorylation of the autoregulatory loop renders the possibility to elicit signalling in different downstream pathways via further phosphorylation of the residues Y515 and Y816. Phosphorylation of Y515 recruits Src-homology containing transforming protein (Shc), and Y816 recruits phospholipase gamma (PLC-γ1). Shc-mediated downstream pathways include PI3-kinase and mitogen-activated protein kinase(MAPK)/extracellular signal-regulated kinase (ERK) pathways, which are involved in local axonal growth and neuronal survival (Atwal et al., 2000). PLC-γ1 hydrolyses phosphatidylinositol 4,5,biphosphate (PIP_2) to generate inositol tri-phosphate (IP_3) and diacylglycerol (DAG) (Huang and Reichardt, 2003). IP_3 and DAG together regulate different protein kinases and Ca^{2+}-calmodulin dependent targets; protein kinase C (PKC) and calmodulin kinases (CaMKs) (Reichardt, 2006), which in turn phosphorylate CREB required for associative learning and hippocampal long-term potentiation in mice (Korte et al., 1995; Minichiello et al., 2002; Gruart et al., 2007). TRKB is also required for adult neurogenesis in hippocampus (Li et al., 2008).

TRKB has been identified as a key player mediating the effects of antidepressant drugs (AD) (Castren and Rantamaki, 2010). TRKB can be activated by AD (Saarelainen et al., 2003) through phosphorylation of Y816 residue and activation of PLC-γ1 pathway without altering phosphorylation of site Y515 (Rantamäki et al., 2007). AD-induced TRKB activation can happen independently of BDNF binding to TRKB, or monoamine neurotransmitter blockade (Rantamäki et al., 2011). AD-induced plasticity covers several aspects from single synapse to network level rearrangements - they increase synaptic plasticity by enhancing long-term potentiation (Wang et al., 2008), neuronal complexity by increasing branching dynamics (Bessa et al., 2009; Chen et al., 2011) and restore juvenile-like plasticity in the adult brain (Vetencourt et al., 2008; reviewed in Umemori et al., 2018). ADs also increase adult neurogenesis (Malberg et al., 2000; Sairanen et al., 2005), however, it is controversial whether their behavioural effects require neurogenesis (Santarelli et al., 2003; Bessa et al., 2009; reviewed in Castrén and Hen, 2013). Hence, antidepressants augment neural plasticity

(Castrén and Hen, 2013; Umemori et al., 2018) and their therapeutic effect is proposed to result, at least partly, from modulation of neuronal networks (Castrén, 2005; Rantamäki and Yalcin, 2016). However, the exact molecular mechanisms mediating AD-induced TRKB signalling are still unclear.

Inhibition of NO production induces behavioural effects similarly to antidepressant drugs in animal models. For example, NOS1-inhibition *in vivo* by 7-nitroindazol (7-NI) has been shown to facilitate fear extinction in contextual fear conditioning paradigm (Lisboa et al., 2015) and another NOS1-inhibitor aminoguanidine has been shown to have panicolytic-like properties in rats through TRKB signalling (Ribeiro et al., 2019).

Having in mind the similarities between the effects of NOS inhibitors and AD, and the recent findings from our group indicating that AD reorganize TRKB protein complex (Fred et al., 2019), we hypothesized that AD induce their effects by dissociating NOS1 from TRKB protein complex, thus protecting it from nitration. In this thesis we investigate how AD fluoxetine, imipramine and phenelzine affect the interactions of TRKB:CAPON:NOS1 protein complex *in* vitro, how fluoxetine affects TRKB nitration *in vivo* and if NOS1 inhibition *in vivo* by N^{ω}-propyl-L-arginine (NPA) induces an antidepressant-like effect in contextual fear conditioning model.

Materials and methods

Ethical statement

All experiments were performed according to institutional guidelines and were approved by the County Administrative Board of Southern Finland (ESAVI/10300/04.10.07/2016). All the efforts were made to minimize animals suffering and number of animals used in experiments.

Animals

Animals were kept in standard housing conditions (temperature 22°C, 12-hr light/dark cycle) and were provided with food and water *ad libitum*. 16-week old female mice (C57BL6/j-RccHsd background) were used for *in vivo* nitration of TRKB receptor experiment. 16-week old male mice (C57Bl/6J000664 background) haploinsufficient to BDNF and their wildtype littermates were used for fear conditioning experiment. Animals were euthanised with carbon dioxide prior to sample collection.

Cell cultures

Cortical and hippocampal cells were cultured as described earlier (Sahu et al., 2019). Procedures done on rats followed institutional guidelines by the University of Helsinki internal license number KEK17-016. For hippocampal cell cultures, E17-E18 embryos collected from pregnant female Wistar rats [Envigo (Harlan labs, U.K.)] and transferred to Falcon tube with phosphate-buffered saline (PBS) on ice. Hippocampi was dissected and transferred to preparation medium [HBBS (Hank's balanced salt solution), 1 mM sodium pyruvate and 10 mM HEPES, pH 7.2] and then transferred into new tube with prewarmed (37°C) papain buffer solution [1 mg DL-Cysteine HCl, 1 mg bovine serum albumin (BSA) and 25 mg glucose in 5 ml PBS] with papain solution (0.5 mg papain, 10 µg DNase I in 5 ml PBS). Cells were triturated (trituration medium: 10 µg DNase I in 10 ml preparation medium) and resuspended in 1 ml of fresh growing medium (NB^{++}: Neurobasal medium with 2 % B27 supplement, 1 % L-glutamine and 1 % penicillin-streptomycin) at room temperature. Cells were plated at density of 125 000 cells per well on coverslips on 4-well plate and grown in at 37°C 5 % CO_2 humidified incubator (Heracell, Heraeus).
For cortical cell cultures, E17-E18 foetuses were collected from pregnant female Wistar rats. Cortices were dissected and let to recover in Dulbecco's modified Eagle's medium (DMEM) (+10 % fetal calf serum, 1 % L-glutamine, 1 % penicillin-streptomycin). The tissue was triturated in DMEM++ and re-suspended in DMEM++. Glial cells and other debris were removed, and, in the end, cells were re-suspended in growing medium (NB^{++}). Cells were plated at the density of 250 000 cells per well onto 24-well plates (well diameter 1.9 cm^2).

Drug treatment of cell cultures

Cortical cell cultures were treated with fluoxetine (#H6995, Bosche Scientific), imipramine (#I7379- 5G, Sigma–Aldrich) or phenelzine (#P6777, Sigma–Aldrich) in final concentration of 10 µM, and PBS (1X) was used as vehicle. Cells were incubated in 37°C for 10, 15 or 30 minutes depending on the experiment. For sample collection, the plates were transferred to ice and the medium removed. Cells were washed with 1X PBS and then lysed in a shaker at +4°C with 100 µL lysate buffer [lysis buffer without phosphatase-inhibitors for 100 ml: 670 µl 3 M Tris-Hcl, pH 8.0, 2740 µl 5 M NaCl-solution, 9600 µl 0.5 M NaF-solution, 1000 µl NP-40, 10 000 µl glycerol and Milli-Q H_2O up to 100 µl, supplemented with phosphatase inhibitors added before use: 100 µl 100 mM NA_3VO_4 (natrium-VO_4) stock and 200 µl 25X complete inhibitor mix (Roche) and NP-lysis buffer up to 5 ml] for 30 minutes.

Immunoassay protocol for ELISA

The plates [96-well Optiplate HB (PerkinElmer Life Sciences)] were incubated overnight at +4°C on shaker with antibody against TRKB (100 µl per well) [goat anti-TRKB, R&D systems AF1494. 1:500 in carbonate buffer (0.0025 M sodium bicarbonate, 0.025 M sodium carbonate. pH adjusted to 9.7)]. Wells were blocked using 150 µl 3 % BSA/TBS-T (Tween 1:1000 in TBS) solution for 2 hours in room temperature on shaker. Blocking buffer was removed and drug treated samples were added (100 µl per well). The plates were incubated overnight at +4°C on shaker. Samples were removed from the plates, and following wash, the secondary antibody (mouse anti-CAPON sc-374504 or mouse anti-NOS1 sc-5302 1:1000 in 3 % BSA/PBS-T, 100 µl per well), was incubated overnight at +4°C on shaker. Following wash, the tertiary antibody [horseradish peroxidase (HRP) conjugated goat anti-mouse, Bio-Rad #1721011 (1:5000 in 3 % BSA/PBS-T)] was added (100 µl per well) and the plates were covered in foil and incubated for 2 hours at room temperature. Following another washing step, ECL mix (1:1, 100 µl per well. Pierce™ ECL Western Blotting substrate #32109, Thermo Scientific) was added and the luminescence detected in a plate reader (Varioskan Flash, Thermo Scientific).

In vivo nitration of TRKB receptor

Sixteen-week old female mice (C57BL6/j-RccHsd background) received fluoxetine (15 mg/kg) or vehicle (1x PBS) intraperitoneally and were euthanised with carbon dioxide for sample collection 30 minutes after injection. Another cohort of mice received fluoxetine or vehicle diluted in drinking water and were euthanised with carbon dioxide after 7 days for sample collection. Samples were mechanically homogenised using NP lysis buffer containing phosphatase inhibitors and then centrifuged (16,000 x *g*) at 4°C for 15 min. Supernatant was immunoprecipitated with antibody against mouse nitrotyrosine (1 µg (5 µl) per sample, sc-32757, Santa Cruz) overnight at +4°C after which co-immunoprecipitated TRKB levels were detected by Western Blotting. Thirty microlitres of Recombinant Protein G - Sepharose™ 4B (catalogue #101241) was used to collect protein from overnight incubated supernatant.

Samples were incubated with Protein G-Sepharose for 2 hours at +4°C and then washed thrice with TBS buffer (1000 x *g*, 1 min at +4°C). Twenty-five microlitres of 2x Laemmli buffer (0.02% bromophenol blue, 4% SDS, 10% B-mercaptoethanol, 0.125M Tris pH 6.8, 20% glycerol, ad milliQ water) was added to tubes containing the pellet and boiled at 95°C for 3 minutes. Samples were loaded on NuPAGE gel (Invitrogen™ NuPAGE™ 4-12% Bis-Tris Protein Gels, #NP0323BOX), 20 µl per well and the gel ran under 100 V for the first 30 minutes and then under 150 V until the blue front disappeared from the gel. The resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Amershan Biosciences) and incubated with anti-TRKB antibody (goat anti-mouse TRKB antibody, #AF1494, R&D Systems, 1:1000 in 3 % BSA/TBST) overnight at +4°C. Membrane was subsequently incubated with HRP-coupled antibody (rabbit anti-goat, 1:5000 in NFDM, #61-1620, Invitrogen) for 1 hour at room temperature. After HRP-coupled antibody incubation, membrane was briefly washed with TBST thrice and with TBS once. For detection, ECL^{++} mix (40:1) (Pierce™ ECL Plus Western Blotting substrate #32132, Thermo Scientific) was added on the membrane and bands were detected in cooled camera system (LAS-3000 Imaging System, Fuji). The signal from the pictures were analyzed with ImageJ (version 1.52p) and expressed as percentage from control group.

Fear conditioning experiment

Sixteen-week old male mice (C57BL6 background) haploinsufficient to BDNF ($BDNF^{+/-}$) and wild-type littermates were submitted to contextual fear conditioning protocol (Curzon P, Rustay NR, 2009). Briefly, initial basal freezing (freezing response determined as immobility over 3 seconds) was measured by infrared beam detection system within the fear conditioning apparatus. Mice were conditioned with 5 foot shocks (0.6 mA, 1 sec, 30-120 s intervals) after which conditioned basal freezing level was measured. On the following day, 30 minutes before the first extinction training, the mice received a single i.p. injection of either 1x PBS (vehicle) or N^{ω}-propyl-L-arginine (NPA, 1 mg/kg). In fear extinction training mice were re-exposed to same context for 5 minutes on three consecutive days. Mice were euthanised with carbon dioxide after the third re-exposure trial.

In silico analysis of TRKB, NOS1, CAPON interaction domains

Uniprot database (https://www.uniprot.org/) was used to search for information about TRKB, NOS1 and CAPON (P15209, Q9Z0J4 and Q9D3A8 in Uniprot database, respectively) interaction domains.

Statistical analysis

Data are expressed as mean and standard error of mean (SEM). One- or two-way ANOVA were used, followed by Dunnett's, Tukey's or Sidak's multiple comparisons tests when applicable. F- and p-values, as well as degrees of freedom, are indicated in results text section. P-values less than 0.05 were considered statistically significant. GraphPad Prism (version 8.0.1) was used for statistical analyses.

Results

Fluoxetine, phenelzine and imipramine disrupt interaction of TRKB, CAPON and NOS1 *in vitro*

Interaction between TRKB receptor and NOS1 has not been previously studied. Thus, using Uniprot database, we analysed NOS1 and TRKB protein domains (represented in figure 1) and we identified no binding domains that could potentially mediate a direct interaction. However, NOS1 has been reported to interact with other proteins through accessory proteins. For example, it has been shown that NOS1AP (NOS1 adaptor protein), also known as CAPON (carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase protein), couples NOS1 protein to NMDA receptor (Courtney et al., 2014). Thus, we investigated by *in silico* analysis if the same principle would be valid for NOS1 and TRKB receptor. The analysis of CAPON protein domains showed potential binding sites for both NOS1 (PDZ-binding site in the C-terminus of CAPON) as expected, and for TRKB (phosphotyrosine interaction domain (PID) domain, in the N-terminus). Thus, we considered a model in which CAPON would couple NOS1 to TRKB receptor (NOS1 would bind to CAPON by PDZ:PDZ binding, while CAPON and TRKB would bind together by CAPON's PID interaction site and TRKB's protein kinase domain).

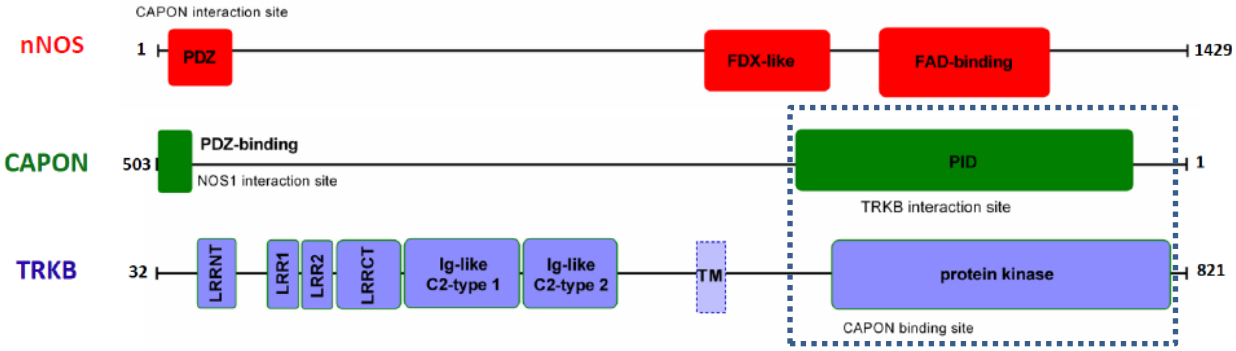

Figure 1 Proposed model of TRKB , NOS1 and CAPON interaction. NOS1 and CAPON interact with PDZ:PDZ binding and TRKB and CAPON bind with PID:protein kinase binding.

We wanted to test if the treatment with AD belonging to different groups, fluoxetine (selective serotonin reuptake inhibitor), phenelzine (monoamine oxidase inhibitor) and imipramine (tricyclic antidepressant), could disrupt interaction of TRKB and CAPON in cortical cell culture. We found that drug treatment had a statistically significant effect on TRKB:CAPON interaction (one-way ANOVA, ($F_{3,\ 26}$=19.86, $p < 0.0001$). Dunnett's multiple comparisons test showed that all treatments, fluoxetine ($p < 0.017$), phenelzine ($p < 0.0001$) and imipramine ($p < 0.0001$) decreased TRKB:CAPON interaction compared to vehicle in the ELISA assay (Fig. 2.)

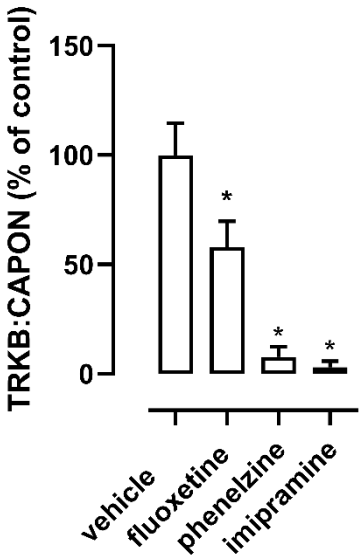

Figure 2 Antidepressant drugs disrupt TRK:CAPON interaction *in vitro*. Fluoxetine, phenelzine and imipramine disrupt interaction of TRKB and CAPON proteins significantly in 10-minute treatment ($p < 0.017$, $p < 0.0001$ and $p < 0.0001$, respectively), n=8. $F_{3, 26}$=19.86. Error bar represent SEM. Dashed line represents background value. Statistical analyses were performed by one-way ANOVA, followed by Dunnett's multiple comparisons test. *$p < 0.05$.

Effects of antidepressant drugs on proposed TRKB:CAPON:NOS1 protein complex was further investigated by analysing effects fluoxetine, imipramine and phenelzine on TRKB and NOS1 interaction. Fluoxetine treatment had a general effect of TRKB:NOS1 interaction (one-way ANOVA, $F_{3, 25}$=25.30) depending on the treatment time. Fluoxetine didn't display statistically significant effect compared to vehicle when treated for 10 minutes but the effect became visible with 15- and 30 minutes treatment ($p < 0.0001$) (Fig 3. A.). Treatment with phenelzine and imipramine disrupted TRKB:NOS1 interaction significantly ($p < 0.0001$ and $p < 0.01$, respectively. One-way ANOVA, $F_{2, 12}$=25.84) (Fig. 3. B.)

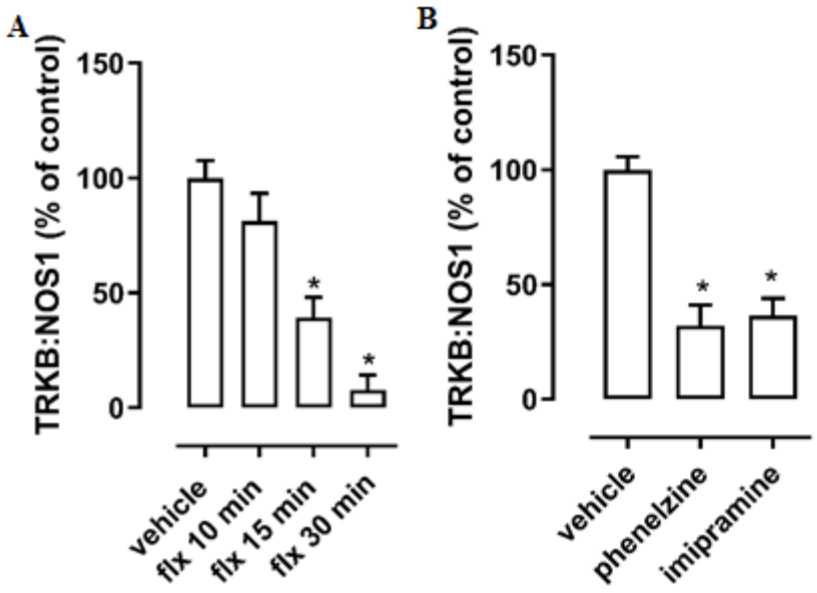

Figure 3 Antidepressant drugs disrupt TRKB:NOS1 interaction *in vitro*. Cortical cell cultures were treated with fluoxetine for different time periods (*A*) Fluoxetine has statistically significant effect when treated for 15 or 30 minutes but not on 10-minute treatment ($F_{3, 25}$=25.30). n=5 in 10-min treatment, n=8 in others. (*B*) Both phenelzine and imipramine have a statistically significant effect in 10-minute treatment compared to vehicle. $F_{2, 12}$=25.84. n=5 in all groups. Error bar represent SEM. Statistical analyses were performed by one-way ANOVA, followed by Dunnett's multiple comparisons test. *$p < 0.05$.

Fluoxetine treatment reduces nitrated TRKB receptors *in vivo*

To understand AD, TRKB and NO interplay *in vivo*, mice were treated with fluoxetine acutely or over the span of one week to see how fluoxetine affects nitration of TRKB receptor. Two-way ANOVA identified fluoxetine decreasing amount of nitrated TRKB receptors significantly in mouse visual cortex both in acute and continued treatments ($F_{1, 12}$=8.797, p=0.0118). Neither the different time of treatments ($F_{1,12}$=0.096, p=0.76) nor interaction between the variables ($F_{1,12}$=0.096, p=0.76) had significant effect (Fig. 4.).

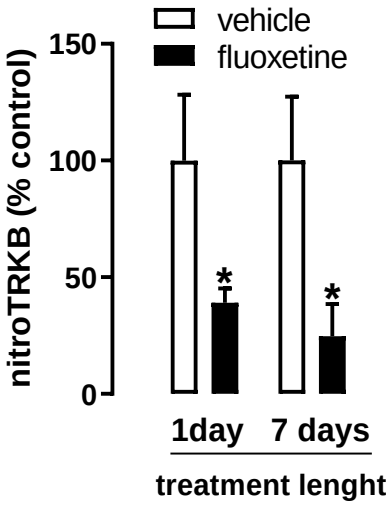

Figure 4 Treatment with fluoxetine reduces amount of nitrated TRKB in mouse visual cortex ($F_{1, 12}$=8.797, p=0.0118). Neither different time of treatment ($F_{1,12}$=0.096, p=0.76) nor treatment and drug interaction ($F_{1,12}$=0.096, p=0.76) had significant effect in two-way ANOVA. n=5 in all groups. *p < 0.05.

Effect of NOS1 inhibition on fear extinction training

The selective NOS inhibitor 7-nitroindazol (7-NI) has been shown to facilitate fear extinction in wildtype mice in contextual fear conditioning experiment (Lisboa et al., 2015). Thus, we wanted to test if another selective NOS1-inhibitor, N^{ω}-propyl-L-arginine (NPA), could induce similar effect in contextual fear conditioning experiment and if such effect is dependent of BDNF. BDNF-deficient ($BDNF^{+/-}$) mice and wild-type littermates were randomised to treatment groups (vehicle or NPA) and their initial freezing tendency was measured for 2 minutes in the fear conditioning apparatus without delivering electric shocks. Basal freezing was almost absent in both genotype groups without conditioning (Fig 5. A.). After receiving 5 foot shocks, both groups began to show freezing prior to drug treatment. Freezing was higher in the $BDNF^{+/-}$ group compared to wildtype mice (Fig 5. B.).

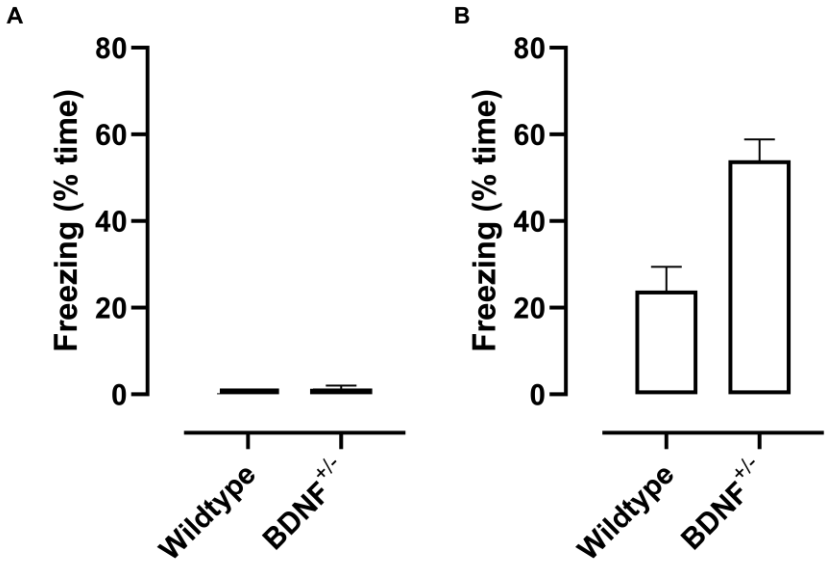

Figure 5 Basal freezing levels measured before injecting vehicle or NPA. (A) Non-conditioned basal freezing levels shows no difference between genotype groups, but (B). after conditioning, $BDNF^{+/-}$ mice show more freezing behaviour than their wildtype counterparts. Wildtype n = 9, $BDNF^{+/-}$ n = 10.

Treatment with NPA reduces freezing in wildtype but not in BDNF heterozygous mice

Due to genotype-dependent difference in fear response at basal freezing (Fig. 5.B.), the effect of NOS1-inhibitor NPA was further analysed in both genotypes independently. $BDNF^{+/-}$ mice showed statistically significant effect of time ($F_{1, 24}$=8.872, p =0.0013) but not drug treatment ($F_{1, 24}$=0.08625, p=0.7715) or interaction of drug and time ($F_{2,24}$ = 0.5285, p = 0.5962) in the freezing reaction to the context (Fig. 6. B.). Wildtype mice showed significant effect of both drug treatment ($F_{1, 21}$ = 5.682, p =0.0257) and time ($F_{2, 21}$=7.602, p=0.0033) but not interaction ($F_{2,21}$ = 0.6023, p = 0.5567) (Fig. 6. A.).

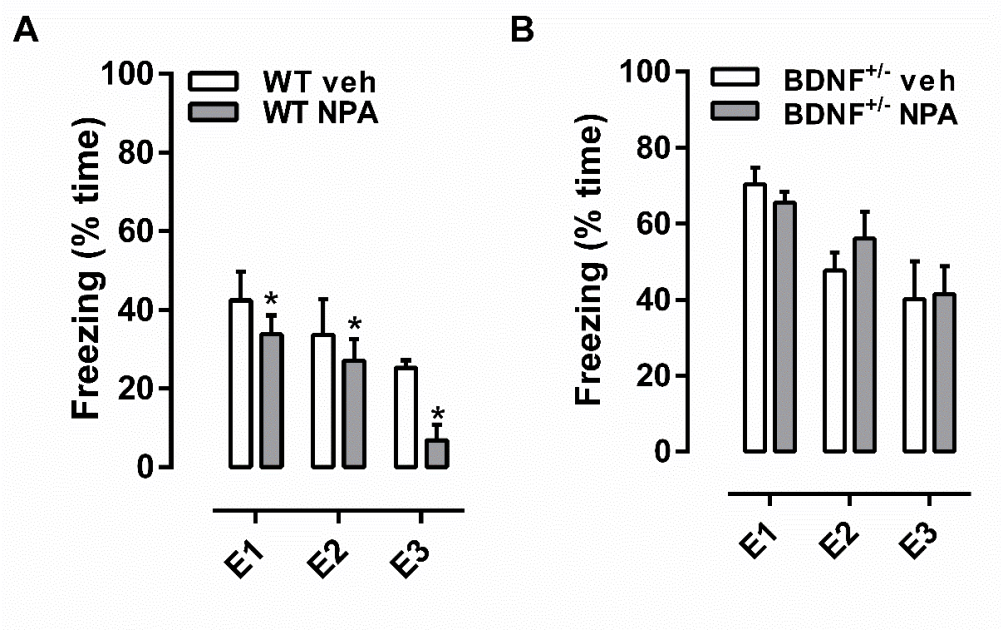

Figure 6. A) NPA facilitates the decrease in freezing response by extinction training in wildtype but B) not in $BDNF^{+/-}$ group. Fear extinction training's efficacy depends on time in both wildtype and $BDNF^{+/-}$ groups (2-way ANOVA). $BDNF^{+/}$ mice do not show response to drug treatment at all in Sidak's multiple comparisons test (Fig. 6. B.). n = 5 in all groups except wildtype vehicle treated n = 4. Statistically significant effect of drug treatment marked with *. *p < 0.05. Error bars represent SEM.

In the last extinction training (E3), analysis of freezing response minute-by-minute is different between genotype groups. BDNF$^{+/-}$ mice showed no difference between time points, drug treatment groups or their interaction. Wildtype mice display a significant interaction of time ($F_{4, 24} = 5.324$, $p = 0.0033$), drug ($F_{1, 6} = 10.94$, $p = 0.0163$) and interaction ($F_{4, 24} = 3.3$, $p = 0.0273$). In the wildtype group, post-hoc analysis (Sidak's multiple comparisons test) shows significant effect of the drug only during the first minute of the third extinction session (Fig. 7.).

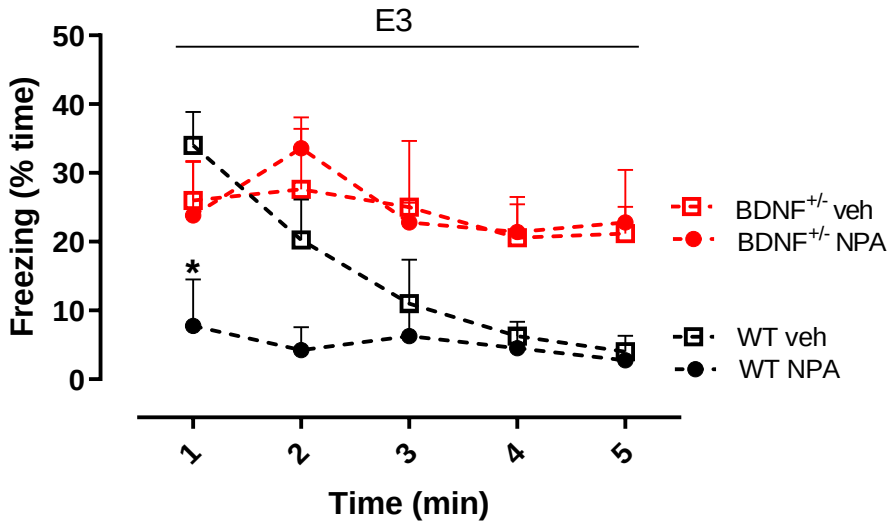

Figure 7 The last extinction training (E3), minute by minute. BDNF$^{+/-}$ mice show no reduction in freezing time within the 5 min period, wildtype mice show lower levels of freezing. During the first timepoint (1 min), wildtype mice treated with NPA show significantly less freezing than their vehicle treated counterparts. This drug induced difference disappears on the consequent timepoints. Error bars represents SEM. *$p < 0.05$.

Discussion

Antidepressant drugs are among the most widely used drugs worldwide (Mars et al., 2017; OECD, 2017; Pratt et al., 2017). The first antidepressant drug, the monoamine oxidase inhibitor iproniazid, was discovered by serendipity over 60 years ago (López-Muñoz et al., 2007). Since then, many other drugs were developed but, despite that, the mechanism of action of those drugs was never completely elucidated. Over the years, many theories were proposed, the most well-known being the Monoaminergic theory (Schildkraut, 1965) and the Neurotrophic Theory (Duman et al., 1997). The first one proposes that ADs act by facilitating noradrenergic, serotonergic and/or dopaminergic neurotransmission. The second one proposes that increased production of neurotrophic factor BDNF is the main mechanism behind the effect of AD. However, it has been later understood that, although modulation of monoaminergic neurotransmission and increase in total levels of BDNF might be important for the effect of those drugs, it is quite unlikely that those are the primary molecular effects (Liu et al., 2017). To date, the search for a better understanding of how these drugs work continues. Interestingly, activation of TRKB receptors, even in the absence of increased BDNF levels, has been consistently found as a crucial piece in that puzzle. We show for the first time that ADs share the ability of preventing TRKB nitration by modulating the interaction with NOS1 and CAPON.

After binding of an endogenous ligand, TRK receptors are endocytosed in a clathrin-dependent manner by endocytic adaptor protein (AP-2) and they elicit signals before driven towards ubiquitination and degradation (Grimes et al., 1997; Beattie et al., 2000; Doherty and McMahon, 2009; Kononenko et al., 2017). Previously, our group found a putative interaction sites between TRKB receptor and cargo-docking subunit of AP-2 complex (AP-2M) and intriguingly one of the potential interaction sites is tyrosine residue 816 in TRKB (Fred et al., 2019), the same residue phosphorylated upon AD treatment, leading to increased PLCγ1-signalling (Rantamäki et al., 2007). Several ADs, including fluoxetine, phenelzine and imipramine, were shown to reduce interaction between TRKB and AP-2, leading to increased surface availability of TRKB receptors (Fred et al., 2019). Additionally, our group has shown that NO may directly nitrate Y816 in TRKB (Biojone et al., 2015) and NO-donor sodium nitroprusside SNP impairs BDNF-TRKB signalling by reducing TRKB:PLCγ1 interaction and increasing TRKB interaction with AP-2 and ubiquitin (Biojone et al., *in preparation*). We were interested how treatment with AD fluoxetine would affect nitrated TRKB receptors *in vivo* and found that both acute and 7-day treatment reduced nitration of TRKB receptors. Fluoxetine induces conformational change in TRKB, promoting BDNF signalling (Casarotto et al., 2019, *preprint in BioRxiv*) and possibly dislocating NOS1 from TRKB-protein complex (see below).

NOS1 and CAPON interactions have been studied for decades in their association with NMDA receptors. Keeping in mind that TRKB activation induces NO synthesis *per se* (Nott et al., 2008), we wanted to know if NO-synthesising protein, NOS1, could interact with TRKB receptor. Based on an *in silico* -analysis of protein

interaction domains, we propose a model where accessory protein of NOS1 (CAPON), would bind to TRKB and NOS1 would be bound to CAPON. We wanted to know if antidepressant drugs could modulate interactions of this proposed protein complex. We show that treatment with different classes of antidepressant drugs fluoxetine (a SSRI), phenelzine (a MAOi) and imipramine (a TCA) disrupt interactions of TRKB:CAPON:NOS1 complex *in vitro*. This evokes an interesting idea that fluoxetine – or other antidepressant drugs – could induce its effect by uncoupling NOS1 from TRKB and counteract nitration of Y816.

Interestingly, it has been shown that NOS inhibitors are able to facilitate TRKB signalling (Joca et al., 2019) and we found that treatment with NOS inhibitor NPA increases phosphorylation of Y816 in TRKB *in vitro* (Biojone et al., *in preparation*). Furthermore, we have shown that NPA facilitates extinction learning in the fear condition model, similarly to what has been previously shown with ADs (Karpova et al., 2011). In fact, many different types of nitric oxide synthase inhibitors induce behavioural effects that are very similar to those induced by ADs in many animal models (Harkin et al., 1999; Yildiz et al., 2000a, 2000b; Spiacci et al., 2008), which is consistent with the idea that both NOS inhibition and ADs share a common molecular mechanism. Moreover, similar to ADs, NOS inhibition also modulates many plastic events in the central nervous system, such as long-term potentiation (Bon and Garthwaite, 2003; Leßmann et al., 2011), synaptic remodeling (Sunico et al., 2005), organization of neuronal projections (Wu et al., 1994; Ernst et al., 1999) and neurogenesis (Moreno-López et al., 2004).

In conclusion, we propose that ADs prevent TRKB nitration by uncoupling it from NOS1-CAPON, which results in facilitation of TRKB phosphorylation, signalling and plasticity. Our data gives a new perspective on the molecular mechanism underlying AD effects, but also opens a new avenue of investigation on disruption of NOS1-TRKB as a new target to enable plasticity in central nervous system.

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