Title: Antidepressant-like effect of losartan involves TRKB transactivation from angiotensin receptor type 2 (AGTR2) and recruitment of FYN

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ABSTRACT

Renin-angiotensin system (RAS) is commonly associated to peripheral fluid homeostasis and cardiovascular function, but recent evidence has also drawn its functional role in the brain. RAS has been described to regulate physiological and behavioral parameters related to stress response, including depressive symptoms. Apparently, RAS can modulate levels of brain derived neurotrophic factor (BDNF) and TRKB, which are important to neurobiology of depression and antidepressant action. However, interaction between BDNF/TRKB system and RAS in depression has not been investigated before. Accordingly, in the forced swimming test, we observed an antidepressant-like effect of systemic losartan but not with captopril or enalapril treatment. Moreover, infusion of losartan into ventral hippocampus (vHC) and prelimbic prefrontal cortex (PL) mimicked systemic losartan effect, whereas K252a, a blocker of TRK, infused into these brain areas impaired the systemic effect of losartan. PD123319, an antagonist of AT2 receptor (AGTR2), infused into PL but not into vHC, also prevented systemic losartan effect. Cultured cortical cells of rat embryos indicate angiotensin II (ANG2) binding to AGTR2 activates TRKB, possibly by recruiting FYN, a SRC family kinase. The higher levels of AGTR2 in cortical cells were inverted after insult with glutamate, and under this condition an interaction between losartan and ANG2 was achieved. Occurrence of TRKB/AGTR2 heterodimers was also observed, since GFP-tagged AGTR2 co-immunoprecipitated with TRKB. Therefore, antidepressant-like effect of losartan is proposed to occur through a shift of ANG2 towards AGTR2, followed by coupling of TRK/FYN and putative TRKB transactivation. Thus, AGTR1 show therapeutic potential as novel antidepressant drug therapy.

INTRODUCTION

Renin-angiotensin system (RAS) functional role has been historically implicated in cardiovascular and fluid homeostasis. Firstly, precursor molecule angiotensinogen is cleaved by renin into angiotensin I, which is then converted into Angiotensin II (ANG2) by angiotensin-converting enzyme (ACE) [1]. Main actions of the ANG2 are mediated by angiotensin II receptors type 1 and 2 - AGTR1 and AGTR2, respectively [1].

Other reports, however, has pointed out all components of renin-angiotensin being produced inside central nervous system (CNS) [2]. Thus, AGTR1 and AGTR2 in circumventricular organs and in cerebrovascular endothelial cells may respond to circulating ANG2 of peripheral origin, whereas receptor lying in neurons inside blood brain barrier respond to RAS of brain origin [2]. AGTR1 and AGTR2 have been found expressed inside blood brain barrier structures such as hippocampus and frontal cortex [3,4], both considered crucial limbic structures associated to the neurobiology of depression [5].

In fact, several piece of evidences introduce ANG2 as a hormone regulator of peripheral and central physiological changes regarding stress exposure, including behavioral consequences. For instance, both acute and chronic stress increased ANG2 and AGTR1 expression levels in the hypothalamic-pituitary-adrenal axis (HPA axis) [6–8]. Moreover, candesartan (AGTR1 antagonist) treatment prevented stress effect of increasing pituitary adrenocorticotrophic and adrenal corticosterone hormone levels [9], and treatment with ACE inhibitors (ACEi) or AGTR1 antagonists reversed or prevented animal behavioral responses to stress [10–15]. In the same way, animals lacking angiotensinogen showed antidepressant-like phenotype [16].

The neurotrophin brain-derived neurotrophic factor (BDNF), found mostly in the central nervous system, is important for neural plasticity, including synapse formation, neuronal differentiation and growth [17]. Functional role of BDNF and its receptor (TRKB, *tropomyosin-related kinase B receptor*) has been linked to the pathophysiology of several psychiatric disorders, such as depression, and to the mechanism of action of antidepressant drugs [18]. Apparently, RAS may modulate BDNF and TRKB brain levels. For instance, candesartan treatment prevented both infarct volume and neurological deficit in animals suffering middle cerebral artery occlusion while increased protein and mRNA levels of TRKB in the brain [19]. In addition, telmisartan (AGTR1 antagonist) chronic treatment was able to prevent retinal damage and decrease of BDNF levels, such as observed in diabetic animal model [20]. Valsartan, another AGTR1 antagonist, counteracted the consequences of stress on depressive and anxiogenic-like behavior, as well as on BDNF levels in hippocampus and frontal cortex [12]. Moreover, some case reports describe relief of depressive symptoms in hypertensive patients treated with the ACEi captopril [21–23].

Despite scarce evidence, it is plausible to consider that drugs acting on RAS promote antidepressant-like effects. However, such properties have not been linked to the modulation of BDNF/TRKB system. In this sense, the present work aimed at investigating behavioral effects of AGTR1 antagonist losartan and ACEi in a model predictive of antidepressant-like effect, i.e. forced swimming test, and the requirement of BDNF/TRKB for such effect. Since AGTR1 activation was related to brain injury [24] and activation of AGTR2 has been supposed to employ balancing neuroprotective outcomes, especially when AGTR1 are blocked [25,26], we hypothesized that activation of AGTR2 could underlie the antidepressant-like effects of losartan. In vitro analysis from cultured cortical cells were also performed to provide a mechanistic insight to the behavioral data.

MATERIALS AND METHODS

Animals

Male Wistar rats used in behavioral studies (250-350g) were housed in pairs in a temperature controlled room (24±1ºC) under standard laboratory conditions with access to food and water *ad libitum* and a 12h light/12h dark cycle (light on at 6:30a.m.). In vivo experiments were conducted in conformity with local Ethical Committee (protocol 147/2017), which is in accordance with Brazilian Council for the Control of Animals under Experiment (CONCEA), and ARRIVE guidelines [27] for the care and use of laboratory animals. Both comply with international laws and politics. Additional information about the experimental procedures accordingly to the ARRIVE guidelines is given in the supplementary material.

Cell Culture

Mouse fibroblasts stably overexpressing full-length TRKB (MG87.TRKB) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1% l-glutamine and 400 mg/ml G418). Cell lines were maintained at 5% CO2, 37ºC until reaching 70% confluence for experiments. For primary neuronal cultures, cortices from E18 rat embryos were dissected and had tissue dissociated with papain solution in PBS (10min, 37°C). Cells were suspended in DMEM medium containing Ca^{2+}/Mg^{2+} free HBBS, 1mM sodium pyruvate, 10mM HEPES (pH 7.2) and DNAse, and plated onto poly-l-lysine (Sigma–Aldrich) coated 24- or 96-well culture plates at a cell density of 125000 cells/cm^2. Primary neurons were maintained in Neurobasal medium (supplemented with 2% B27, 1% penicillin/streptomycin and 1% l-glutamine) and supplemented with fresh medium every 3rd day.

Drug Treatments

Losartan potassium (losartan; AGTR1 antagonist; Pharmanostra, BRAZIL) was administered intraperitoneally - ip (10, 30 and 45mg/Kg), intracerebrally (0.1, 1 and 10nmoL/site) or in cell cultures (10uM; Tocris #3798). Captopril (ACEi; 3, 10 and 30mg/Kg - Pharmanostra, BRAZIL) and enalapril (ACEi; 1, 3 and 10mg/Kg - Pharmanostra, BRAZIL) were injected ip. K252a was used for intracerebral (TRK inhibitor; 20mM/site – Sigma-Aldrich, #K2015) or in culture (10uM) treatment. PD123319 (PD; AGTR2 antagonist; Tocris, #1361) was used for intracerebral (200uM/site) or culture (200nM) treatment. ANG2 (10uM; Tocris #1158), BDNF (2.5ng/mL), NGF (10ng/mL), TRKB.Fc (200ng/mL; R&D systems, #688-TK-100) were used only for cell culture treatment. K252a and PD123319 intracerebral doses were chosen based on previous works [28,29]. 2,5% 2,2,2 tribromoethanol (ip, Sigma-Aldrich, #T48402) and subcutaneous local anesthetic lidocaine (PROBEM 3%, 0.2 mL) were used for stereotaxic surgery. Chloral hydrate (0.75g/Kg, ip, Sigma-Aldrich, #C8383) was used to euthanize animals for perfusion. Subcutaneous banamine (Schering-Plough, 0.25%, 0.1mL/100g) and intramuscular oxytetracycline (Pfizer, 20%, 0.1mL/100g) were used once to postoperative recovery. Losartan, tribromoethanol, choral and banamine were freshly prepared in in saline solution, whereas all other drugs in 0.1% DMSO in saline.

Surgery, Intracerebral Injections and Histology

Surgery and intracerebral drug injections were performed as described earlier [30]. Briefly, rats were anesthetized with tribromoethanol and fixed in a stereotaxic frame. Further, stainless steel guide cannulas (0.7mm OD) aimed at the dorsal hippocampus (dHC; coordinates: AP= −4.0mm from bregma, L=2.8mm, DV=2.1mm), ventral hippocampus (vHC; coordinates: AP= −5.0mm from bregma, L=5.2mm, DV=4.0mm) or prelimbic ventromedial prefrontal cortex (PL; coordinates: AP= +3.3mm from bregma, L=1.9mm, DV=2.4mm; lateral inclination of 22°) were implanted according to Paxinos and Watson´s atlas [31] and attached to skull bone with stainless steel screws and acrylic cement. A stylet inside guide cannula prevented obstruction. Five to seven days after surgery, intra-cerebral injections were performed with dental needle (0.3mm OD) in a volume of 200nl (mPFC) or 500nl (dHC or vHC) infused for 1min using a micro-syringe (Hamilton) and infusion pump (Kd Scientific).

After behavioral tests, rats were anesthetized with chloral hydrate and 200nL of methylene blue was injected through the guide cannula. The brains were removed and injection sites verified. All histological sites of injection were inserted in diagrams (Figure S2) based on the atlas of Paxinos and Watson [31].

Forced Swimming Test (FST)

Animals were placed individually to swim in a Plexiglas cylinder (24cm diameter by 60cm with 28cm of water at 25±1ºC) for 15min (pretest). Twenty-four hours later, animals were replaced in the cylinder for 5min swim test session and immobility time was measured. Water was changed between each test. After swimming, animals were towel-dried before returning to home cages. Test was videotaped and analyzed by a trained observer blind to treatment.

Overexpression of GFP-AGTR2

MG87 cells were transfected to express GFP-tagged AGTR2 using lipofectamine [28]. Briefly, at a confluence of 70%, the cells were incubated with a mixture of 2.5% lipofectamine 2000 (Thermo Scientific, #11668019) and 5ug/mL of the plasmid in OptiMEM medium. Following 48h after transfection, cells were treated, lysed and submitted for immunoprecipitation as described below.

Sample collection

For immunoassays, cells were washed with ice-cold PBS, lysed [137mM NaCl; 20mM Tris-HCl; 1%NP40; 0.5mM NaF; 10% glycerol; pH=7.4; supplemented with protease/phosphatase inhibitor cocktail (Sigma-Aldrich, #P2714; #P0044) and sodium orthovanadate (0.05%, Sigma-Aldrich, #S6508] and the samples were centrifuged at 10000g for 15min at 4ºC. Supernatant was collected and stored at -80ºC until use. For polymerase chain reaction, the cells were washed with PBS and treated with Qiazol Lysis Reagent™ (Qiagen). Lysate was collected in a clean tube and incubated with chloroform for 3min at RT. After centrifuged at 15200g for 10min at 4ºC, the aqueous phase was mixed with isopropanol for 10min at RT and centrifuged at 15200g for 10min at 4ºC. The pellet was washed with 75% EtOH two times, than with 100% EtOH once, air-dried and dissolved in 20µL MQ-water.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Quantitative Polymerase Chain Reaction (qPCR)

Concentration and purity of each RNA sample were determined using NanoDrop (Thermo Scientific). Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (#K1672, Thermo Scientific) was used to synthesize cDNA from the samples. Primers were designed via https://eu.idtdna.com/pages/scitools and purchased from Sigma-Aldrich (Germany). The following primers were used for qPCR: AGTR1a-(NM_030985.4) forward: CATCAGTCTCCCTTTGCTATGT, reverse: AGTGACCTTGATTTCCATCTCTT. AGTR2-(NM_012494.3) forward: CCTTCCATGTTCTGACCTTCTT, reverse: GCCAGGTCAATGACTGCTATAA. Actin-(NM_031144) forward: TGTCACCAACTGGGACGATA, reverse: GGGGTGTTGAAGGTCTCAAA.

The PCR method used SYBR Green as probe. Briefly, maxima®SYBR Green qPCR Master Mix (Thermo Scientific, #K0253) was used according to manufacturer's instructions in Hard-Shell™ 96-well PCR plate (BioRad). The reaction was conducted in duplicates, using thermal cycler (BioRad CFX96 Real-Time System), with initial denaturation at 95ºC for 10min. Denaturation and amplification were carried out by 45 cycles of 95ºC for 15s, 63ºC for 30s and 72ºC for 30s. 'No template control' (NTC) was included to the reaction and melting curve analysis was done. Results were analyzed using Ct values. Levels of beta-actin mRNA was used for normalization of the results and $2^{-\Delta\Delta Ct}$ values were calculated for each gene.

Immunoprecipitation

Lysate from transfected MG87.TRKB/GFP-AGTR2 cells were incubated with antibody against TRK (Santa Cruz, #sc7268) overnight (1ug of Ab: 500ug of total proteins) at 4ºC. Following incubation with Protein-G Sepharose (Life Technologies, #101242) for 2h at 4ºC, samples were centrifuged (10000g/2min) and the precipitate was stored at -80°C until use. This cycle was repeated twice, using lysis buffer to wash samples. Supernatant was discarded.

Western-blotting and ELISA

For western-blotting, protein precipitated by anti-TRK and supernatant were separated in SDS-PAGE and transferred to PVDF membranes. Following blocking with 3% BSA in TBST, membranes were incubated with antibody against GFP (1:1000, Santa Cruz, #sc-8334) or total TRK (1:1000, Santa Cruz, #sc11-Rb). Membranes were incubated with secondary antibody conjugated to HRP (1:10000, Bio-Rad, #170-5046) and chemiluminescence emitted after addition of ECL was detected by CCD camera. Immunoblot bands were measured using NIH ImageJ 1.32. For ELISA, samples (120ug total proteins) were incubated (overnight at 4ºC) in 96-well plates, previously coated with anti-TRK (1:500, Santa Cruz, #sc7268, overnight at 4ºC) and blocked with 3%BSA (2h at RT) in PBST. Following wash with PBST, anti-pTRK.Y816 (1:2000, Cell Signaling, #4168), biotin-conjugated anti-pY (1:2000, AbD Serotec, UK, #MCA2472B) or anti-FYN (1:2000, Santa Cruz, #sc16) was incubated overnight at 4ºC. After wash with PBST, plate was incubated with HRP-conjugated tertiary antibody (1:5000, Bio-Rad, #170-5046) or HRP-conjugated streptavidin (1:10000, Thermo Fisher, #21126). Chemiluminescence emitted after addition of ECL was detected by a plate reader (Varioskan Flash, Thermo-Fisher). Signal from each sample, discounted blank, was normalized and expressed as percentage of control-group.

Surface expression of TRK

Cells from rat E18 cortex were cultivated in 96-well plates as described above (DIV8). Detection of surface TRKB was performed by ELISA (Zheng et al, 2008). Cells were fixed with

4%PFA for 20min at RT. After washing with PBS wells were blocked with 5% non-fat dry milk and 5% normal goat serum in PBS for 1h at RT. Then, primary antibody against extracellular portion of TRK (1:500, Santa Cruz, #sc8316) was incubated overnight at 4ºC. Following wash with PBS, cells were incubated with HRP-conjugated antibody (1:5000; Bio-Rad, #170-5046) for 2h at RT. Signal detected after addition of ECL, discounted blank, was normalized by the average of vehicle-treated samples and expressed as percentage of control-group.

Data Analysis

Statistical analyses were carried out using two-tailed Student's t-test, one-way analysis of variance (ANOVA) followed by Fisher´s LSD *post-hoc* test, or two-way ANOVA test. Criteria for statistical significance was $p<0.05$.

RESULTS

Antidepressant-like Effect of Losartan

Different classes of drugs, with discrepant mechanisms concerning RAS modulation, were used to evaluate a possible drug-induced antidepressant effect. As depicted in figure 1, one-way ANOVA indicates a significant effect of systemically injected losartan ($F_{3,25}=3.74$, $p<0.05$; Figure 1c); but not captopril ($F_{3,29}=1.83$, non-significant NS; Figure 1a) or enalapril ($F_{3,16}=0.46$, NS; Figure 1b), decreasing the immobility time in the FST, interpreted as an antidepressant-like effect. Losartan was found effective at 10 and 45mg/kg dose (Fisher's LSD, $p<0.05$ for both). In fact, immobility time of rats exposed to swimming session is increased after uncontrollable stress and the treatment with antidepressant drugs decrease this parameter [32]. Moreover, provided that known antidepressants decrease immobility time in FST, a good predictive validity is attributed to it, thus supporting FST as a screening test for putative new antidepressant drugs and their mechanisms. Since only systemic losartan exhibited antidepressant-like effect, this drug was infused into dHC, vHC or PL to address which of these structures may underlie such effect. The data depicted in figure 1d-f indicates that losartan infused into vHC $F_{3,11}=10.66$, $p<0.05$; Figure 1e) and PL ($F_{3,19}=2.72$, $p<0.05$; Figure 1f), but not into dHC ($F_{3,24}=0.50$, NS; Figure 1d), was able to decrease immobility time in the FST.

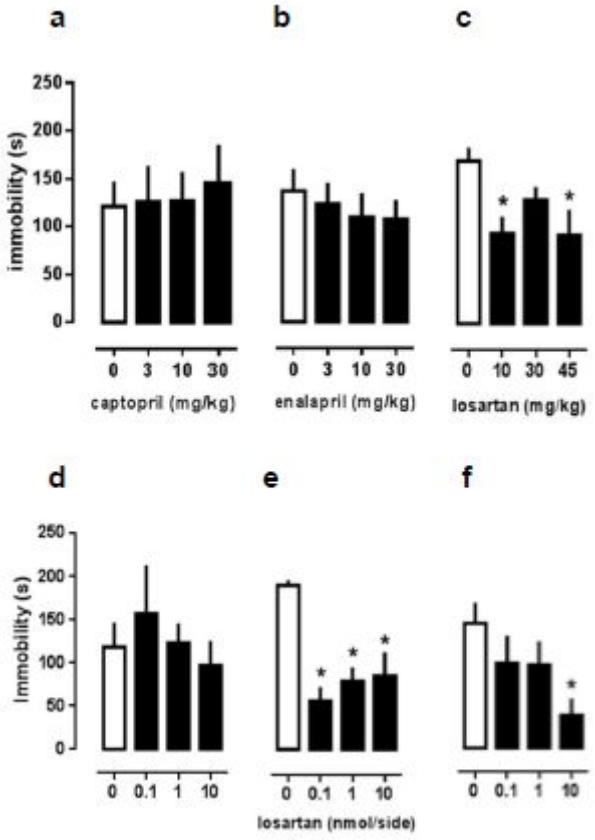

Figure 1. Antidepressant-like Effect of Losartan. (a) Animals were treated i.p 24h, 5h and 1 hour before FST with captopril at 0, 3, 10 or 30mg/kg (n=7-9/group) doses or with enalapril at 0, 1, 3 or 10mg/kg (n=5/group) doses. (c) Losartan was administered i.p 1h before FST at 0, 10, 30 or 45mg/kg (n=7-8/group). (d-f) Losartan was bilaterally infused at 0, 0.1, 1 and 10nmol/side into (d) dorsal hippocampus (n=4-8/group), (e) ventral hippocampus (n=3-4/group) or (f) prelimbic prefrontal cortex (n=5-7/group). Data are expressed as mean ± SEM of immobility time (s); *p<0.05 compared to control group.

Interaction between losartan and TRK or AGTR2: *in vivo* data

In this experimental set, requirement of TRK and/or AGTR2 activation for antidepressant effect of losartan was examined. As shown in figure 2a-b, K252a, an antagonist of TRK receptors, infused into vHC or PL was able to modulate the effect of systemically injected losartan. Two-way ANOVA revealed interaction between factors (systemic and intracerebral injection) for both structures (vHC: $F_{1,15}$=8.62; PL: $F_{1,13}$=6.58, p<0.05 for both). Regarding vHC experiments, pairwise comparisons unveiled immobility time of animals treated with losartan/ctrl is decreased compared to control group (Fisher's LSD, p<0.05), whereas losartan/K252a group was considered significantly different of losartan/ctrl group (Fisher's LSD, p<0.05), suggesting that K252a pretreatment prevents antidepressant-like effect of losartan. Regarding PL experiments, pairwise comparisons unveiled immobility time of animals

treated with losartan/ctrl is lower than ctrl/ctrl group (Fisher's LSD, $p<0.05$), whereas losartan/K252a group was found different from losartan/ctrl group (Fisher's LSD, $p<0.05$), suggesting that K252a also prevents losartan effect in this brain region.

Next, we analyzed if antidepressant effect of losartan relies on AGTR2 activity. As shown in figure 2c-d, the AGTR2 antagonist PD123319 infused into PL, but not into vHC, was able to mitigate the effect of systemically injected losartan. Accordingly, Two-way ANOVA revealed interactions between the compounds in PL ($F_{1,28}=5.11$, $p<0.05$) but not in vHC ($F_{1,26}=0.18$, NS) structure. In addition, pairwise comparisons concerning PL experiments unveiled immobility time of animals treated with losartan/ctrl is reduced compared to ctrl/ctrl group (Fisher's LSD, $p<0.05$), however losartan/PD123319 group was not found different from losartan/ctrl (Fisher's LSD: $t_{28}=1.95$, $p=0.06$). Therefore, activation of AGTR2 in PL, but not in vHC, is necessary to mediate the antidepressant-like effect of losartan.

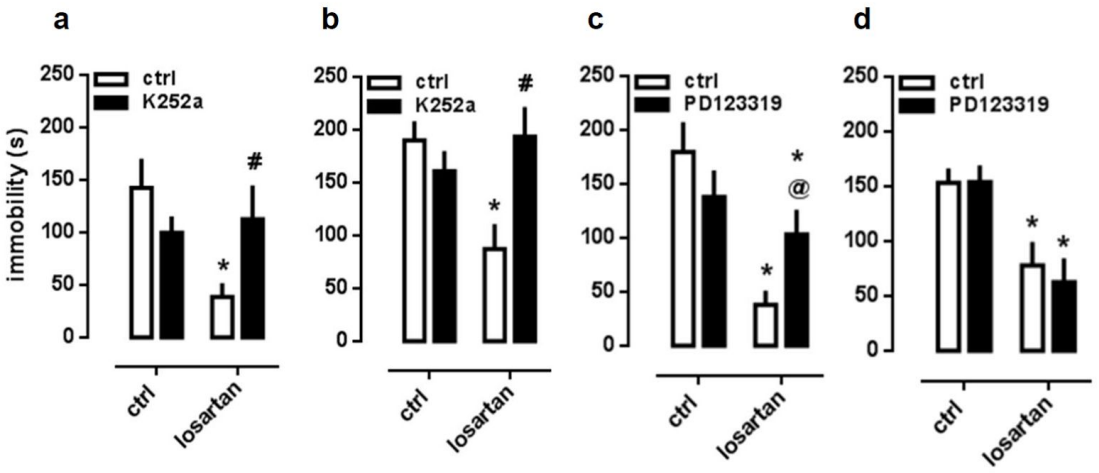

Figure 2. Interaction between losartan and TRK or AGTR2: *in vivo* data. Antidepressant-like effect of losartan was attenuated by K252a infused into (a) PL (n=4-5/group) and (b) vHC (n=4-6/group). The previous infusion of AGTR2 antagonist PD123319 into (c) PL (n=7-9/group) attenuated the effect of systemically injected losartan, but no change was observed in vHV (n=4-6/group). Losartan was administered 1h before FST and either K252a or PD was bilaterally infused 20min before swimming test. Data are expressed as mean ± SEM of immobility time (s). *$p<0.05$ compared to ctrl/ctrl group, unless otherwise stated; #$p<0.05$ compared to ctrl/losartan group. @$p=0.06$ compared to ctrl/losartan group.

Interaction between TRK and AGTR2: *in vitro* data

Since losartan antidepressant-like effect may depend mutually on TRKB and AGTR2 signaling in PL, we hypothesized losartan treatment would allow a shift of ANG2 from AGTR1 towards AGTR2 to forward TRKB activation. In order to test this possibility, losartan, PD123319 or K252a (1st factors) was added to the primary cell cultures previously to ANG2 (2nd factor) and levels of pTRK were analyzed. In fact, two way ANOVA indicated an interaction between both

factors ($F_{3,28}$=6.77, p<0.05) and pairwise comparisons assert that both PD123319 and K252a, prevented ANG2 effect of increasing pTRK levels (Fisher's LSD, NS for both; Figure 3a) and no additive effect was observed in the combination of ANG2 and losartan. Next step was to check if ANG2 effect was dependent on BDNF release. With this intent, a soluble BDNF scavenger - TRKB.Fc (1st factor) - was added to the medium of the cell culture before ANG2 (2nd factor). Two way ANOVA indicated no interaction between both factors ($F_{1,20}$=0.82, NS), suggesting an effect of ANG2 on pTRK levels is independent of BDNF release (Figure 3b).

To further investigate that, PD123319 (1st factor) was added to culture medium before BDNF (2nd factor) and levels of pTRK were analyzed. Two-way ANOVA showed a significant interaction between factors ($F_{1,20}$=36.18, p<0.05). Pairwise comparisons indicate PD123319 abrogated BDNF effect of increasing pTRK levels (Fisher's LSD, p<0.05 p<0.05; Figure 3c), indicating AGTR2 participates in BDNF-induced TRK activation. In addition, previous administration of PD123319 (1st factor) was not able to prevent NGF (2nd factor) effect of increasing pTRK levels (Figure 3d), since no interaction between both factors was observed ($F_{1,13}$=0.60, NS).

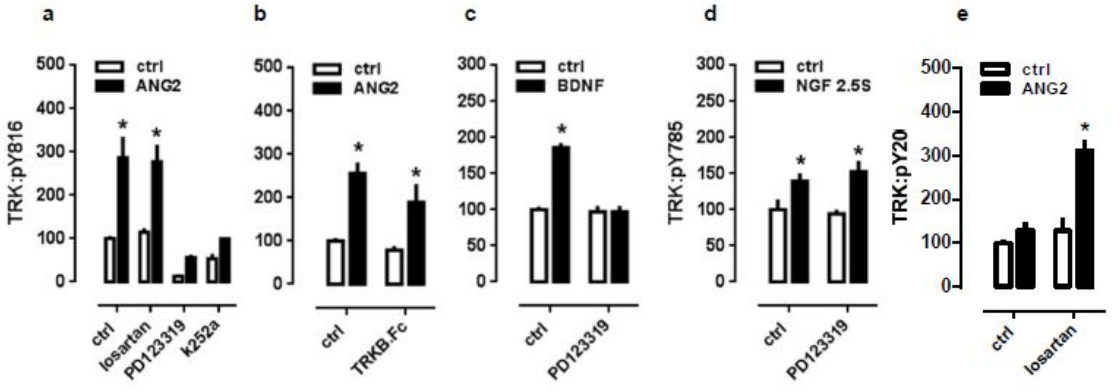

Figure 3. Interaction between TRK and AGTR2: *in vitro* data. (a) Previous administration of PD or K252a, but not losartan, blocked the ANG2-induced increase in TRKB activation in cortical cells of rat embryo - E18; DIV8-10 (n=4-6/group). (b) Previous incubation with TRKB.Fc did not change ANG2-induced increase in TRKB activation in cortical cells (n=6/group). Previous incubation with PD123319 blocked (c) BDNF-, but not (d) NGF-induced activation of TRKB (n=4-6/group). (e) Cells challenged with glutamate (100uM/2h) and tested for ANG2-induced activation of TRKB 24h later were responsive only in the presence of losartan (n=6/group). *p<0.05 compared to ctrl/ctrl group, unless otherwise stated; cells were lysed 10min after last drug administration.

AGTR2-dependent interaction with FYN, surface positioning of TRKB and co-immunoprecipitation of AGTR2 and TRKB

Inasmuch as ANG2 increased pTRK levels independent of BDNF release, we decided to verify if ANG2 and BDNF are able to influence TRK/FYN coupling, since FYN is described as a SRC member responsible for transactivation of TRK [33]. For this purpose, PD123319 (1st factor) was added in the culture medium before ANG2 or BDNF (2nd factors) and TRK/FYN coupling was analyzed. Interestingly, two-way ANOVA indicated a significant interaction between ANG2 and PD123319 ($F_{1,19}$=5.08, $p<0.05$) and, surprisingly, between BDNF and PD123319 ($F_{1,20}$=6.74, $p<0.05$). Respectively, pairwise comparisons showed PD123319 abolished ANG2 and BDNF effect of increasing TRK/FYN coupling (Fisher's LSD, NS for both; Figure 4a). Next, we measured if PD123319 or ANG2 was able to modulate surface levels of TRKB. In fact, cultured cortical cells exposed to PD123319 presented decreased, while ANG2 increased, surface levels of TRKB ($F_{2,30}$=24.33; Figure 4c).

Altogether, these results point to the existence of a heterodimer TRKB/AGTR2, since previous studies have described cross-antagonism (ability of both antagonists of each receptor units in the heterodimer to block signaling of each other agonist) as a fingerprint of heterodimerization [34,35]. In agreement with this idea, as observed in Figure 4d, a labeled GFP-tagged AGTR2 was co-precipitated with TRKB, however no apparent effect of BDNF or ANG2 was found in the levels of such complex.

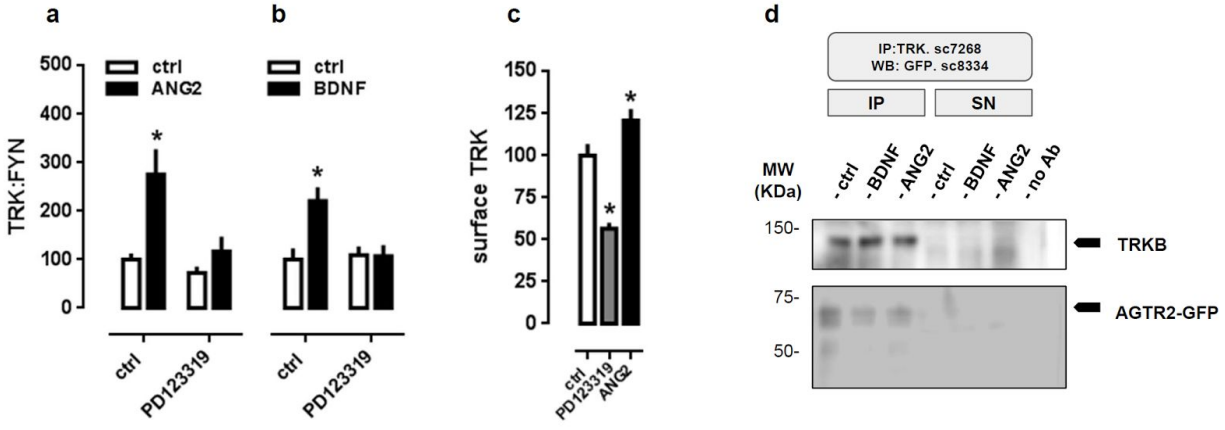

Figure 4. AGTR2-dependent interaction with FYN, surface positioning of TRKB and co-immunoprecipitation of AGTR2 and TRKB. Previous treatment with PD123319 impaired TRK:FYN coupling induced by (a) ANG2 (n=5-6/group) or by (b) BDNF (n=6/group). (c) PD123319 decreased while ANG2 increased the levels of TRKB in the surface of cultured cortical cells (n=9-12/group). (d) Sample from MG87.TRKB fibroblast cell line, overexpressing AGTR2 labelling on blotting membrane from immunoprecipitation of TRKB protein (IP). *p<0.05 compared to ctrl/ctrl group, unless otherwise stated; cells were lysed 10min after last drug administration.

Challenge with glutamate inverts the ratio between AGTRs in cortical cells

Preliminary comparison between the presented *in vitro* and *in vivo* analysis indicates an incompatibility regarding the effects of losartan. This compound, although effective when injected systemic or into mPFC, did not exerted any effect *per se* in cultured cortical cells. Therefore, we considered the putative role of pretest stress in animals. In this scenario, as previously described [36], a single exposure to inescapable stress, in addition to a peak in corticosterone production (lasting for 2h), increases glutamate levels for up to 24h. Therefore, firstly we determined the levels of AGTR1 and AGTR2 mRNA in our cultured cells. The results indicated a 5-times higher expression of AGTR2 compared to AGTR1 (Mean ΔCt value/SEM(n); AGTR1: 17.08/0.09(3); AGTR2: 14.69/0.37(3); t_4= 2.96, p<0.05). Further, we incubated cortical cells with glutamate (10 or 100uM/2h) and determined the levels of AGTRs mRNA. Separate analysis of AGTRs expression following glutamate insult suggests a decrease in AGTR2 mRNA levels (Mean of fold change/SEM from ctrl: 1.00/0.09; glutamate 10uM: 0.88/0.06 and glutamate 100uM: 0.47/0.10; n=5,5,4 respectively) but no change in AGTR1 (fold change from ctrl: 1.00/0.10; glutamate 10uM: 1.39/0.14 and glutamate 100uM: 1.12/0.11; n=5,5,4 respectively). In this condition, there was an inversion in the ratio between AGTR1 and AGTR2 mRNA levels [glutamate 10uM: 1.5-times more AGTR1; glutamate 100uM: 2.54-times more AGTR1]. Then, cortical cells pre-exposed to glutamate (100uM/2h) were tested 24h after the insult for the interaction between losartan and ANG2. Interestingly, 24h after the insult with glutamate, putatively inverting the AGTR1/AGTR2 ratio, losartan was necessary for the effect of ANG2 on pTRK levels (interaction: $F_{1,20}$= 14.46 p<0.05, Figure 3e).

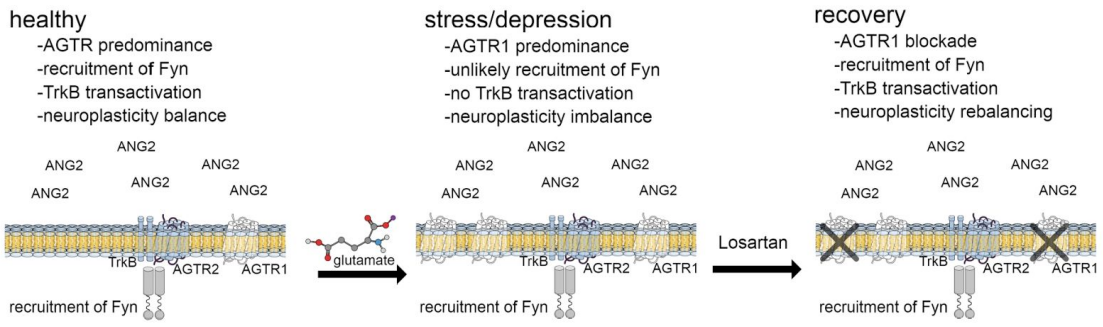

Figure 5. Graphical abstract for the TRKB-dependent antidepressant-like effect of losartan. In basal conditions the low levels of AGTR1 compared to AGTR2 in cortical cells would be responsible to keep TRKB at the cell surface, passive to be activated by BDNF or transactivated by AGTR2. Upon excessive glutamatergic firing under stressful situations the decrease in AGTR2 levels compromises TRKB activation and treatment with losartan, putatively blocking AGTR1, favors the activation of AGTR2, reinstating TRKB activity.

DISCUSSION

Our data indicates systemic treatment with angiotensin receptor blocker (ARB) losartan, but not with angiotensin converting enzyme inhibitors (ACEi) captopril or enalapril, promoted antidepressant-like effect in the FST. Losartan infusion into vHC, or into PL, but not into dHC, was enough to mimic antidepressant-like effect of systemic injection. Both hippocampus and vmPFC are core structures to modulate motivational and emotional behavioral consequences of stress exposure, including depressive disorder [37,38]. In agreement with our data, vHC is suggested to be mainly related to behavioral and physiological consequences of stress exposure, while dHC engages cognitive and learning process concerning spatial navigation [39]. The antidepressant-like effect of losartan probably rely on TRK signaling acting in hippocampus and vmPFC, whereas AGTR2 activation is required only in PL, since TRK inhibitor into vHC and PL, and AGTR2 antagonist into PL decreased that effect. Neither FST data could be a misleading from locomotor activity, since no change was observed in this parameter with treatments in vHC, and a decrease in locomotion was observed after losartan infusion into PL (Figure S1).

In order to corroborate behavioral experiments and further explore the mechanisms involved in losartan effects, we used primary cultures of embryonic cortex to evaluate interaction between RAS and BDNF/TRKB signaling. First, we observed that K252a and PD123319, but not losartan, prevented ANG2 effect of increasing pTRK levels, thus suggesting that ANG2 increases pTRK levels by acting on AGTR2. Since TRKB.Fc did not prevent ANG2 effect on pTRK, it is plausible to consider no involvement of BDNF release in that TRK activation. In this sense, transactivation of TRKB is a reasonable scenario. In fact, previous studies have described that both GPCR ligands adenosine and pituitary adenylate cyclase-activating polypeptide can transactivate TRK [40]. In addition, TRK transactivation by adenosine 2A receptor agonist was blocked by PP1, suggesting an involvement of SRC family tyrosine kinase [41]. Later, FYN was described as the SRC member responsible for TRK transactivation by adenosine [33]. Accordingly, lipid raft localization of TRKB is regulated by FYN [42]. In agreement with this evidence, we observed ANG2 was also able to increase levels of TRK/FYN coupling in cortical cultures. Therefore, we propose that FYN acts as an intermediary molecule capable of inducing TRKB transactivation when ANG2 acts on AGTR2. Moreover, it was observed that showed BDNF itself could promote a greater TRKB/FYN coupling [43]. Corroborating that prospect, our data also showed BDNF increasing TRKB/FYN coupling. Therefore, both ANG2 or BDNF, which are able to increase pTRK levels, can also induce TRK/FYN coupling.

In addition, as expected PD123319 prevented TRKB/FYN coupling from ANG2 action, but unexpectedly PD123319 also prevented such coupling from BDNF action. Also unexpected was

PD123319 preventing BDNF itself effect of increasing pTRKB levels. Besides, generalized interaction of AGTR2 with other TRK members is unlikely, insofar as PD123319 did not prevent NFG action of increasing pTRKA levels. These unforeseen interactions can be explained by observation that PD123319 is able to reduce surface expression of TRKB, whereas ANG2 leads to an increase, thereby suggesting a putative displacement of TRK to surface upon AGTR2 signaling and the decrease of BDNF effectiveness with previous PD123319. Indeed, modulation of TRK surface trafficking is important considering that transactivation of TRKB might happen on cell membrane intracellular domain [40]. In addition, MG87.TRKB cell line, which overexpresses TRKB, allowed us to observe co-immunoprecipitation of GFP-tagged AGTR2 and TRKB, also suggesting an AGTR2/TRKB dimerization. This approach was chosen for two main reasons: as analyzed by the group of Juan Saavedra, commercially available antibodies against AGTRs are far from ideal [44]; second, the cell line used expresses exclusively TRKB, thus being an ideal tool to our purpose.

Preliminary analysis showed that AGTR2 mRNA levels is 5 times higher than AGTR1 in our primary cultures, and this ratio is inverted to 2.5 times more AGTR1 after an insult with glutamate, and this later feature seems to allow a cooperative effect of losartan and ANG2. Using a model of retinal ischemia, it was observed increased expression of AGTR1 receptor mRNA peaked 12h after reperfusion, while the treatment with candesartan was able to prevent ischemia-induced glutamate release [45]. Taken together, these data indicate a possible positive feedback between AGTR1 signaling and glutamatergic transmission. Moreover, in line with our *in vitro* observations, the levels of AGTR1 was increased while AGTR2 was decreased in medulla of stress-induced hypertensive rats [46]. However, an opposed effect of glutamate on AGTR2 mRNA have also been described [47]. In this study, the insult with glutamate led to an increase in AGTR2 mRNA. The precise mechanism where stressful events or excessive glutamate release reduces the levels of AGTR2 receptors are still not comprehended and these apparent discrepancies could rest on methodological differences. For example, the culture method of Makino et al. rely on cortical cells cultivated for 14 days, supplemented with calf serum and mitosis inhibitors; while our cultures were serum-free (substituted by B27), cultivated for 8 days without any drugs to prevent cell proliferation.

In conclusion, as depicted in figure 5, according to our findings we speculate that losartan-induced antidepressant-like effect is possibly mediated by AGTR2 and TRKB transactivation in the mPFC. We disclose a previously unknown TRKB transactivation by AGTR2, involving recruitment of SRC family kinase FYN. Therefore, TRKB and AGTR2 could form a heterodimer that probably docks FYN kinase to promote a crosstalk, putatively inducing pTRKB/PLCγ1 signaling. Considering the high comorbidity between depression and

cardiovascular disorders, drugs such as losartan, could be an interesting therapeutic tool or even available as a novel class of antidepressant drugs.

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