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ORIGINAL ARTICLE

Angiotensin-(1–7) through Mas receptor up-regulates neuronal norepinephrine transporter via Akt and Erk1/2-dependent pathways

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Abstract

As angiotensin (Ang) (1–7) decreases norepinephrine (NE) content in the synaptic cleft, we investigated the effect of Ang-(1–7) on NE neuronal uptake in spontaneously hypertensive rats. [^{3}H]-NE neuronal uptake was measured in isolated hypothalami. NE transporter (NET) expression was evaluated in hypothalamic neuronal cultures by western-blot. Ang-(1–7) lacked an acute effect on neuronal NE uptake. Conversely, Ang-(1–7) caused an increase in NET expression after 3 h incubation (40 ± 7%), which was blocked by the Mas receptor antagonist, a PI3-kinase inhibitor or a MEK1/2 inhibitor suggesting the involvement of Mas receptor and the PI3-kinase/Akt and MEK1/2-ERK1/2 pathways in the Ang-(1–7)-stimulated NET expression. Ang-(1–7) through Mas receptors stimulated Akt and ERK1/2 activities in spontaneously hypertensive rat neurons. Cycloheximide attenuated Ang-(1–7) stimulation of NET expression suggesting that Ang-(1–7) stimulates NET synthesis. In fact, Ang-(1–7) increased NET mRNA levels. Thus, we evaluated the long-term effect of Ang-(1–7) on neuronal NE uptake after 3 h incubation. Under this condition, Ang-(1–7) increased neuronal NE uptake by 60 ± 14% which was blocked by cycloheximide and the Mas receptor antagonist. Neuronal NE uptake and NET expression were decreased after 3 h incubation with an anti-Ang-(1–7) antibody. Ang-(1–7) induces a chronic stimulatory effect on NET expression. In this way, Ang-(1–7) may regulate a presynaptic mechanism in maintaining appropriate synaptic NE levels during hypertensive conditions.

Keywords: angiotensin-(1–7), hypertension, Mas receptor, norepinephrine transporter, norepinephrine uptake.
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The renin-angiotensin system (RAS) is an essential regulator of blood pressure and fluid homeostasis. A complete and functional RAS exists in the brain and comprises all necessary precursors and enzymes required for the synthesis and metabolism of its components (Veerasingham and Raizada 2003; Xu *et al.* 2011). In addition to the known effector angiotensin (Ang) II, the heptapeptide Ang-(1–7) is a biologically active component of the RAS which limits the pressor and proliferative effects of Ang II. Thus, Ang-(1–7) produces vasodilation, natriuresis and diuresis, inhibits angiogenesis and cell growth (Ferrario *et al.* 2010; Ferreira *et al.* 2010; Xu *et al.* 2011).

At the central level, Ang-(1–7) facilitates the baroreflex control of blood pressure and acts at cardioregulatory brain areas, such as hypothalamic and brainstem nuclei,

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Abbreviations used: Ab, antibody; Ang, angiotensin; RAS, renin-angiotensin system; NE, norepinephrine; NET, NE transporter; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto.

contributing to blood pressure regulation (Xu *et al.* 2011). This action may result from its activity on neurotransmitter release; that is, the peptide not only diminishes hypothalamic norepinephrine (NE) release but also blocks the stimulatory effect of Ang II on this mechanism (Gironacci *et al.* 2004a,b). In addition, Ang-(1–7) stimulates the degradation of L-tyrosine hydroxylase as well as inhibits its enzymatic activity in hypothalami and neuronal cultures from spontaneously hypertensive ratas (SHR), reducing in consequence NE biosynthesis (Lopez Verrilli *et al.* 2009). Altogether, these effects lead to a decrease in NE levels at the synaptic cleft, thus contributing to blood pressure regulation.

Synaptic neurotransmission requires the precise control of the duration and the magnitude of neurotransmitter action at specific molecular targets. Uptake of monoamine neurotransmitters into pre-synaptic terminals through the transporters is the main mechanism for monoaminergic neurotransmission ending. NE transporter (NET) regulates noradrenergic signaling in central and peripheral nervous systems by mediating the clearance of NE (Eisenhofer 2001; Torres *et al.* 2003; Bönisch and Brüss 2006; Kvetnansky *et al.* 2009). Thus, changes in the activity of NET should have a significant impact on the concentration and duration of NE present in the synaptic cleft and consequently alter NE signaling. In this way, NET is essential for a fine-tuned control of sympathetic activity. In fact, alterations in NE clearance and NET density are observed in cardiovascular diseases and brain disorders (Bönisch and Brüss 2006; Kvetnansky *et al.* 2009). Based on previous reports that Ang-(1–7) diminishes NE release and synthesis in hypothalami from SHR (Gironacci *et al.* 2004a; Lopez Verrilli *et al.* 2009) we hypothetized that Ang-(1–7) elicits an stimulatory effect on neuronal NE removal. Thus, our aim was to investigate the effect of Ang-(1–7) on neuronal NE uptake and NET expression centrally in SHR rats. In addition, the mechanism of action of Ang-(1–7) on NET expression was also evaluated. We carried out our study in SHR because we previously reported that Ang-(1–7) does not affect neuronal NE uptake in normotensive rats (Gironacci *et al.* 2000). In addition, it has been shown that Ang-(1–7) effects are enhanced in situations of Ang II overactivity as in hypertension (Ferrario *et al.* 1997).

Methods

Materials

Horse serum and Dulbecco's modified Eagle's medium were purchased from Invitrogen (Carlsbad, CA, USA). Deoxiribonuclease I (Dnase I), hydrocortisone, pargyline, desipramine, actimomycin D, cycloheximide and cytosine arabinoside were from Sigma Chemical Co. (St Louis, MO, USA). Losartan was from Dupont (Boston, MA, USA). PD123319 was a gift from Dr Jack Hodges at Parke Davis (Detroit, MI, USA). LY294002 was from Cell Signaling (Beverly, MA, USA) and U0126 was from Calbiochem (Gibbstown, NJ, USA). Ang-(1–7) and [D-Ala^7]-Ang-(1–7) were synthesized in our laboratory by the Merrifield solid-phase procedure, as previously described (Lopez Verrilli *et al.* 2009). Peptide purity (> 97%) was confirmed by matrix assisted laser desorption mass spectrometry. All other chemicals were analytical grade reagents of the highest purity available.

Animals

Male Wistar-Kyoto (WKY) or SHR rats were used in our study. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Norepinephrine neuronal uptake

NE neuronal uptake was evaluated as previously described (Gironacci *et al.* 2000) using the same experimental model where we have previously evaluated Ang-(1–7) effects on NE release (Gironacci *et al.* 2004a). Briefly, minced isolated hypothalami from 3-month-old male rats were incubated at 37°C in Krebs solution (pH 7.4) continuously bubbled with 95% O_2/5% CO_2 in the absence (basal) or presence of 0.1 or 1 μmol/L Ang-(1–7) during 5 min or 3 h, followed by the incubation with 125 pmol/L L-[7-^{3}H]-NE (New England Nuclear, Boston, MA, USA) during 5 min. The reaction media also contain 100 μmol/L pargyline and 100 μmol/L hydrocortisone to avoid L-[7-^{3}H]-NE degradation and non-neuronal uptake, respectively. After four consecutive 15-min washes with cool Krebs solution containing 15 μmol/L desipramine (to block NET activity), tissues were homogenized, centrifuged and tritium was quantified by liquid scintillation counting. NE neuronal uptake was expressed as % of change with respect to basal conditions, considering basal levels as 100%.

In another set of experiments, minced isolated hypothalami from 3-month-old male rats were incubated at 37°C during 3 h in the absence (basal) or presence of a specific anti-Ang-(1–7) antibody in a concentration as previously reported (Höcht *et al.* 2008) (1/50) and NE neuronal uptake was evaluated as described above. Anti-Ang-(1–7) antibody was obtained from rabbit and previously characterized to be specific for Ang-(1–7) (Lopez Verrilli *et al.* 2009).

Cell culture

Neuronal cultures were obtained from hypothalamus-brainstem areas of 1-day-old rats, as previously described (Yu *et al.* 1996; Lopez Verrilli *et al.* 2009). Cells were used after 8–10 days of the initiation of culture. After 7 days, synapses between neurons as well as synaptic vesicles in the pre-synaptic neuron were observed by electron microscopy (data not shown).

Western blot

Protein expression was measured by western blot as previously described (Lopez Verrilli *et al.* 2009). The following antibodies were used: anti-NET (1/3000) (Santa Cruz Biotechnology Laboratories, Santa Cruz, CA, USA) or anti-Mas R (1/500) (Alomone, Jerusalem, Israel) or anti-phosphospecific antibodies against Akt (1/1000) (Cell Signaling) or ERK1/2 (1/2000) (Cell Signaling). Total Akt and ERK 1/2 protein content (non-phosphorylated) was evaluated by reblotting membranes with anti-Akt (1/1000) or anti-ERK 1/2 (1/2000) antibodies (Cell Signaling). Akt or ERK 1/2

phosphorylation were normalized to protein content in the same sample and expressed as % of change with respect to basal conditions, considering basal levels as 100%. Protein loading was evaluated by stripping and reblotting membranes with β-actin antibody (1/1000) (Sigma Chemical Co.).

mRNA levels determination

Quantification of NET mRNA expression was performed by real-time PCR and normalized by β-actin housekeeping gene expression. Briefly, for cDNA synthesis, 2 μg of total RNA and 1 μg of random hexamer primers (Promega Corp., Madison, WI, USA) were heated at 70°C for 5 min, followed by incubation at 4°C. After adding 5 μL of M-MLV RT 5× buffer (250 mmol/LTris–HCl, pH 8.3, 375 mmol/L KCl, 15 mmol/L $MgCl_2$, 50 mmol/L dithiothreitol), 25 U RNasin (Promega Corp.), 1.25 μL of 10 mmol/L dNTPs (Invitrogen), and 200 U of MMLV reverse transcriptase (Promega Corp.), the reaction was carried out at 37°C for 1 h, followed by inactivation of the enzyme at 95°C for 10 min. Real-time PCR was performed using a Bio-Rad iQ iCycler Detection System (Bio-Rad Laboratories Ltd, CA, USA) with SYBER green fluorophore. Reactions (in duplicates) were carried out in a total volume of 20 μL. A three-step protocol (95°C for 20 s, 60°C, annealing for 30 s, 72°C for 40 s) was repeated for 40 cycles. Rat primers (Invitrogen) were designed as previously described (Habecker *et al.* 2006):

(+)GCTGACCAGCACCATCAAC, (-)GGCGACATCCTCAATCTTGAC. A melt curve analysis was performed following every run to ensure a single amplified product for every reaction. The authenticity of the amplicon generated was confirmed by their size in 2% agarose gel. In accordance with the literature, we did not find any difference in the β-actin used as housekeeping gene, between the experimental groups.

Statistical analysis

The data are presented as mean ± SEM. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Bonferroni post-tests (GraphPad Prism 4; GraphPad Software, Inc.). p values < 0.05 were considered significant.

Results

Ang-(1–7) does not evoke acute effects on NE neuronal uptake

As shown in Fig. 1, basal neuronal NE uptake was evaluated in hypothalami from WKY and SHR rats. No difference was observed between both strains. Incubation with 0.1 μmol/L or 1 μmol/L Ang-(1–7) during 5 min, did not elicit any change in neuronal NE uptake in hypothalami from SHR (Fig. 1).

Ang-(1–7) increases NET expression through Mas receptor, via Akt and ERK1/2-dependent pathways

Basal endogenous NET levels were similar in hypothalamic-brainstem neuronal cultures from SHR and WKY (Fig. 2a). A concentration of 0.1 μmol/L Ang-(1–7), chosen from previous experiments (Gironacci *et al.* 2004a; Lopez Verrilli *et al.* 2009), was used to test time-dependent effects on

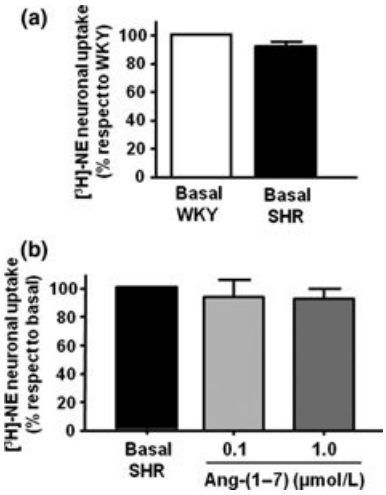

Fig. 1 (a) Basal [^{3}H]-NE neuronal uptake in hypothalami from WKY and SHR rats as described in Methods. Results are expressed as percentage of the response detected in hypothalami from WKY, taking them as 100%. Data are means ± SEM of six experiments. (b) Effect of Ang-(1–7) on [^{3}H]-NE neuronal uptake in hypothalami from SHR. Hypothalami were incubated with [^{3}H]-NE in the absence (basal) or in the presence of 0.1 μmol/L or 1 μmol/L Ang-(1–7) during 5 min and NE neuronal uptake was measured as described in Methods. Results are expressed as percentage of the response detected in basal conditions, taking them as 100%. Data are mean ± SEM of 6–8 experiments.

neuronal cultures. Treatment of SHR neuronal cultures with 0.1 μmol/L Ang-(1–7) induced an increase in NET expression after 1–5 h of incubation (Fig. 2b).

Concentration–response assays were performed by incubation of neuronal cultures with Ang-(1–7) during 3 h (Fig. 2c). No effect on NET expression was observed with 0.01 μmol/L Ang-(1–7). Conversely, 0.1 and 1 μmol/L Ang-(1–7) increased NET expression 40 ± 8% and 70 ± 24% above basals, respectively (Fig. 2c).

Then, we investigated the Ang-receptor subtype that was coupled to the stimulatory activity of Ang-(1–7) on NET expression. Figure 3a shows that 1 μmol/L [D-Ala^7]-Ang-(1–7), a Mas receptor antagonist, blocked the stimulatory effect of Ang-(1–7) on NET expression, suggesting the involvement of Mas receptor. In fact, Mas R are expressed in neuronal cultures from SHR and in a greater amount than that in WKY (2.7-fold increase) (Fig. 3b). However, the effect of Ang-(1–7) on NET expression was modified by neither 1 μmol/L PD123319, an AT_2 receptor antagonist, nor 1 μmol/L losartan, an AT_1 receptor antagonist (Fig. 3a), demonstrating that AT_1 or AT_2 receptors are not involved in the Ang-(1–7) stimulatory effect.

As it has been demonstrated that PI3-kinase/Akt and MEK 1/2-ERK 1/2 signal transduction pathways are coupled to Mas receptor activation (Giani *et al.* 2007; Sampaio *et al.* 2007; Dias-Peixoto *et al.* 2008; Zimpelmann and Burns

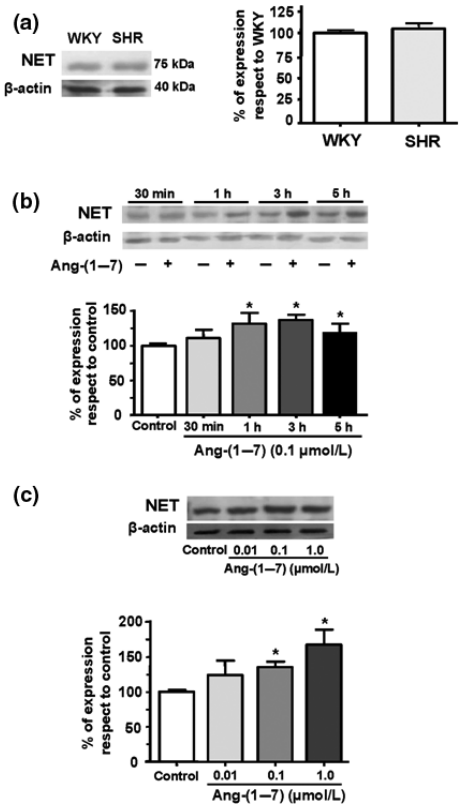

Fig. 2 (a) Basal expression of NET in both WKY and SHR hypothalami-brainstem neuronal cultures. NET expression was measured by western blot as described in Methods and is expressed as a percentage of the response detected in WKY cells, taking them as 100%. Each bar represents the mean ± SEM of three determinations from six separate cell culture preparations. (b) Time-dependent effect of Ang-(1–7) on NET expression in hypothalami-brainstem neuronal cultures from SHR. Neuronal cultures were incubated in the absence (control) or presence of 0.1 μmol/L Ang-(1–7) for the indicated time periods. NET expression was measured by western blot as described in Methods and is expressed as the percentage of the response detected in control cells, taking them as 100%. Each incubation period with Ang-(1–7) had its respective control. The control band is shown only once to simplify the graph. Each bar represents the mean ± SEM of three determinations from five separate cell culture preparations. * $p < 0.05$ as compared with the corresponding control. (c) Concentration-response effect of Ang-(1–7) on NET expression in hypothalami-brainstem neuronal cultures from SHR. Neuronal cultures were incubated for 3 h with increasing concentrations of Ang-(1–7) (0.01–1 μmol/L). NET expression was measured by western blot as described in Methods and is expressed as a percentage of the response detected in control cells, taking them as 100%. Data are mean ± SEM of three determinations from four separate cell culture preparations. *$p < 0.05$ as compared with control. A representative western blot is presented over each bar graph.

12 2009) we evaluated their involvement in the Ang-(1–7)-stimulated NET expression. SHR neuronal cultures were incubated with 0.1 μmol/L Ang-(1–7) during 3 h in the presence of 25 μmol/L LY294002, a PI3-kinase inhibitor, or 10 μmol/L U0126, a MEK1/2 inhibitor. As shown in Fig. 3c, Ang-(1–7)-stimulated NET expression was abolished when PI3-kinase and MEK1/2 pathways were inhibited, suggesting that Akt and ERK1/2 are both involved in Ang-(1–7) effect. The inhibitors did not modify NET expression by themselves.

To verify that Ang-(1–7) induces activation of Akt and ERK1/2 and that these effects are mediated via Mas receptor, we assessed the effect of Ang-(1–7) on Akt and ERK1/2 phosphorylation in SHR neuronal cultures in the absence or presence of [D-Ala^7]-Ang-(1–7), a Mas receptor antagonist. Following PI3-kinase stimulation, Akt becomes phosphorylated at Thr^{308} and Ser^{473}, which results in its activation (Alessi *et al.* 1996). As shown in Fig. 4a, 0.1 μmol/L Ang-(1–7) increased Akt phosphorylation at Thr^{308}, which was maximal after 5 min of incubation. This effect was blocked by 1 μmol/L [D-Ala^7]-Ang-(1–7), a Mas receptor antagonist, or by 25 μmol/L LY294002, a PI3-kinase inhibitor. These results suggest that Ang-(1–7) activates Mas receptors, thus leading to PI3-kinase activation with the subsequent Akt phosphorylation at Thr^{308}.

MEK1/2 are the kinases directly upstream of ERK1/2 responsible for the dual phosphorylation of ERK1/2 on tyrosine and threonine residues and hence, its activation (Pearson *et al.* 2001). Figure 4b shows that Ang-(1–7) 15 stimulated ERK1/2 phosphorylation after 10 min of incubation and this effect was abolished by 1 μmol/L [D-Ala^7]-Ang-(1–7), the Mas receptor antagonist, or 10 μmol/L U0126, the MEK1/2 inhibitor. These results demonstrate that Ang-(1–7), through Mas receptor activation, induced MEK1/2 activation with the subsequent ERK1/2 phosphorylation.

Neither total Akt nor ERK1/2 contents were modified by the different treatments (Fig. 4b).

Ang-(1–7) increases NET expression by stimulating NET synthesis

As the increase in NET expression caused by Ang-(1–7) may be due to an increase in NET synthesis, we examined the effect of Ang-(1–7) on NET expression in the presence of inhibitors of gene transcription and translation. The increase in NET expression induced by 0.1 μmol/L Ang-(1–7) was blocked by either actinomycin-D (0.5 and 1.0 μmol/L), a gene transcription inhibitor, or cycloheximide (0.5 and 1.0 μmol/L), a protein synthesis inhibitor, suggesting that Ang-(1–7) stimulates NET gene transcription and NET mRNA translation (Fig. 5a and b). Dimethylsulfoxide (actynomycin solvent) did not modify NET expression by itself (Fig. 5a).

To corroborate whether Ang-(1–7) induced a stimulatory effect on NET *de novo* synthesis, we evaluated the effect of the peptide on NET mRNA levels. As shown in Fig. 5c, 0.1 μmol/L Ang-(1–7) increased NET mRNA levels in neuronal cultures after 3 h incubation.

LOW RESOLUTION FIG

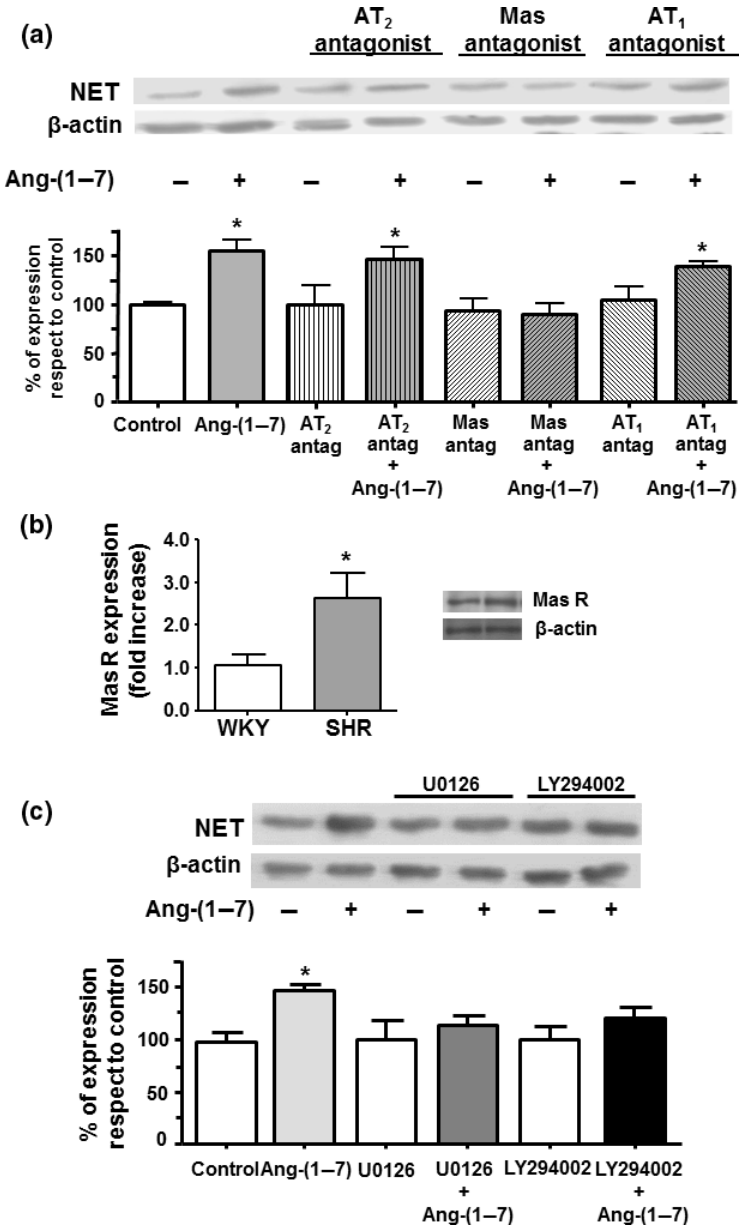

Fig. 3 (a) Effect of AT_2-, AT_1- and Mas receptor antagonists on the Ang-(1–7)-stimulated NET expression in hypothalami-brainstem neuronal cultures from SHR. Cells were incubated in the absence (control) or presence of 0.1 μmol/L Ang-(1–7) during 3 h. When indicated the AT_2 receptor antagonist PD 123319 (AT_2 antag) (1 μmol/L), or the Mas receptor antagonist [D-Ala^7]-Ang-(1–7) (Mas antag) (1 μmol/L), or the AT_1 receptor antagonist losartan (AT_1 antag) (1 μmol/L) was added alone or simultaneously with Ang-(1–7). NET expression was measured by western-blot as described in Methods and is expressed as a percentage of the response detected in control cells, taking them as 100%. Each bar represents the mean ± SEM of three determinations from five separate cell culture preparations. *$p < 0.05$ as compared with control. A representative western blot is presented. (b) Mas R expression in hypothalami-brainstem neuronal cultures from WKY and SHR. Each bar represents the mean ± SEM of relative increase over basal of three determinations from four separate cell culture preparations. A representative western blot is presented. (c) Effect of PI3-kinase and MEK1/2 inhibitors on the Ang-(1–7)-stimulated NET expression in hypothalami-brainstem neuronal cultures from SHR. Cells were incubated in the absence (control) or presence of 0.1 μmol/L Ang-(1–7) during 3 h. When indicated, the MEK1/2 inhibitor U0126 (10 μmol/L), or the PI3-kinase inhibitor LY294002 (25 μmol/L) was added alone or simultaneously with Ang-(1–7). NET expression was measured by western blot as described in Methods and is expressed as a percentage of the response detected in control cells, taking them as 100%. Each bar represents the mean ± SEM of three determinations from six separate cell culture preparations. *$p < 0.05$ as compared with control. A representative western blot is presented.

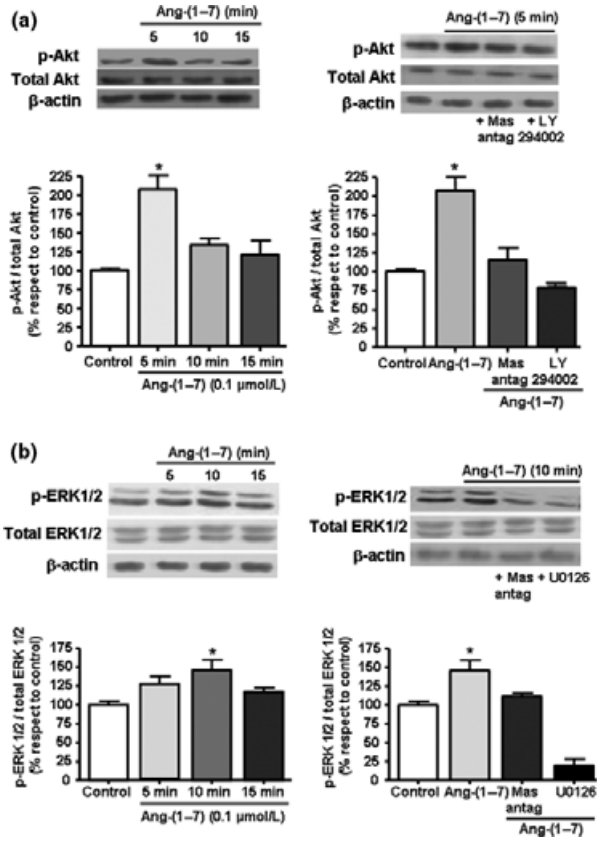

Fig. 4 Effect of Ang-(1–7) on Akt (a) and ERK 1/2 phosphorylation (b) in SHR hypothalami-brainstem neuronal cultures. Left pannels. SHR neuronal cultures were treated with 0.1 μmol/L Ang-(1–7) for 5–15 min. Akt-phosphoThr^{308} (p-Akt) (**a**) or ERK 1/2-phosphoThr^{202}/Tyr^{204} (p-ERK 1/2) (**b**) were measured by western blot as described in Methods and normalized to Akt and ERK 1/2 content in the same sample. Results are expressed as the percentage of the response detected in control cells, taking them as 100%. Each bar represents the mean ± SEM of three determinations from five separate cell culture preparations. *$p < 0.05$ as compared with control. A representative western blot is presented. Right pannels. (a) SHR neuronal cultures were incubated with Krebs buffer alone (control) or 0.1 μmol/L Ang-(1–7) for 5 min. When indicated, the Mas receptor antagonist 1 μmol/L [D-Ala^7]-Ang-(1–7) (Mas antag) or the PI3-kinase inhibitor LY294002 (25 μmol/L) was simultaneously added with Ang-(1–7). (b) SHR neuronal cultures were incubated with Krebs buffer alone (control) or 0.1 μmol/L Ang-(1–7) for 10 min. When indicated, the Mas receptor antagonist 1 μmol/L [D-Ala^7]-Ang-(1–7) (Mas antag) or the MEK 1/2 inhibitor U0126 (10 μmol/L) was simultaneously added with Ang-(1–7). Akt-phosphoThr^{308} (a) or ERK 1/2-phosphoThr^{202}/Tyr^{204} (b) were measured by western blot as described in Methods and normalized to Akt and ERK 1/2 content in the same sample. Total Akt and ERK 1/2 abundance was measured by reblotting the same membranes with anti-Akt or anti-ERK 1/2, respectively, and no changes were observed. Results are expressed as the percentage of the response detected in control cells, taking them as 100%. Each bar represents the mean ± SEM of 3 determinations from four separate cell culture preparations. *$p < 0.05$ as compared with control. A representative western blot is presented.

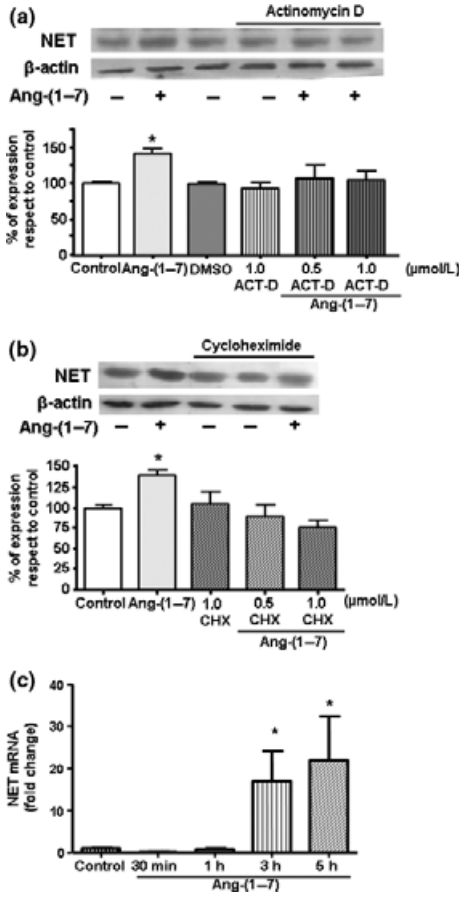

Fig. 5 (a) Effect of Ang-(1–7) on NET expression in hypothalami-brainstem neuronal cultures from SHR. Cells were incubated in the absence (control) or presence of 0.1 μmol/L Ang-(1–7) during 3 h. When indicated, the transcription inhibitor actinomycin-D (ACT-D 0.5 and 1.0 μmol/L) was added alone or simultaneously with Ang-(1–7). NET expression was measured by western blot as described in Methods and is expressed as a percentage of the response detected in control cells, taking them as 100%. Each bar represents the mean ± SEM of three determinations from five separate cell culture preparations. *$p < 0.05$ as compared with control. A representative western blot is presented. (b) Effect of Ang-(1–7) on NET expression in hypothalami-brainstem neuronal cultures from SHR. Cells were incubated in the absence (control) or presence of 0.1 μmol/L Ang-(1–7) during 3 h. When indicated, the protein synthesis inhibitor cycloheximide (CHX 0.5 and 1.0 μmol/L) was added alone or simultaneously with Ang-(1–7). NET expression was measured by western blot as described in Methods and is expressed as a percentage of the response detected in control cells, taking them as 100%. Each bar represents the mean ± SEM of three determinations from four separate cell culture preparations. *$p < 0.05$ as compared with control. A representative western blot is presented. (c) Effect of Ang-(1–7) on NET mRNA levels in hypothalami-brainstem neuronal cultures from SHR. Cells were incubated in the absence (control) or presence of 0.1 μmol/L Ang-(1–7) during different times. NET mRNA was measured as described in Methods. Values are mean ± SEM and expressed as fold change with respect to control. *$p < 0.05$ as compared with control.

Ang-(1–7) elicites a stimulatory long-term effect on neuronal NE uptake

Considering that Ang-(1–7) increased NET expression through Mas receptor stimulation (as shown in Figs 2 and 3a), we hypothesized that this effect would lead to a long-term increase in neuronal NE uptake. To test this hypothesis, we measured the effect of Ang-(1–7) on neuronal NE uptake after 3 h of incubation, the period time after which Ang-(1–7) induced an increase in NET expression and NET mRNA levels. As shown in Fig. 6a, Ang-(1–7) (0.1 μmol/L) significantly increased NE neuronal uptake in hypothalami from SHR, and this effect was abolished in the presence of either 1.0 μmol/L cycloheximide or the Mas receptor antagonist (1.0 μmol/L).

Endogenous Ang-(1–7) contributes to NET up-regulation

To test whether endogenous Ang-(1–7) contributes to NE neuronal uptake, hypothalami were incubated with a specific anti-Ang-(1–7) antibody (Ab) and neuronal uptake was evaluated 3 h later. As shown in Fig. 6b, when endogenous Ang-(1–7) was blocked with the Ab, a decrease in NE neuronal uptake was observed. In accord, when neuronal cultures were incubated with the Ab during 3 h, a decrease in NET expression was observed (Fig. 6c).

Altogether, these results confirm the long-term sympatoinhibitory effect of Ang-(1–7) via regulation of NET expression.

Discussion

This study demonstrates that Ang-(1–7) does not evoke an acute effect on neuronal NE uptake in hypothalami from SHR. Conversely, Ang-(1–7) causes a long-term stimulatory effect on NE neuronal uptake by increasing NET. Moreover, Ang-(1–7)-stimulated NET expression is coupled to Mas receptor activation acting through a PI3-kinase/Akt and MEK 1/2-ERK1/2-dependent pathway.

NE release from the hypothalamic nuclei contributes to blood pressure regulation by enhancing sympathetic nervous system activity (De Wardener 2001). In this context, NE uptake is an essential process that limits the action of NE and prevents sympathetic overactivity. Thus, disturbances in NET function are involved in the genesis and development of hypertension (Bönisch and Brüss 2006). For instance, it has been reported that the impaired uptake of NE by the NET was closely related with the origin of essential hypertension in a substantial number of patients (Esler *et al.* 1981; Bönisch and Brüss 2006). In addition, a decrease in NE uptake has been reported in cases of hypertension, orthostatic intolerance and tachycardia (Esler *et al.* 1981; Hahn *et al.* 2003; Lambert *et al.* 2008). In all these situations, an impaired NET function leads to disturbances of the homeostasis of the sympathetic nervous system. Furthermore, a genetic study has shown the association between the presence of a variation in human NET promoter and essential hypertension (Ono *et al.* 2003). Taking into account that sympathetic overactivity results from an increase in synaptically released neurotransmitter, we may speculate that NET

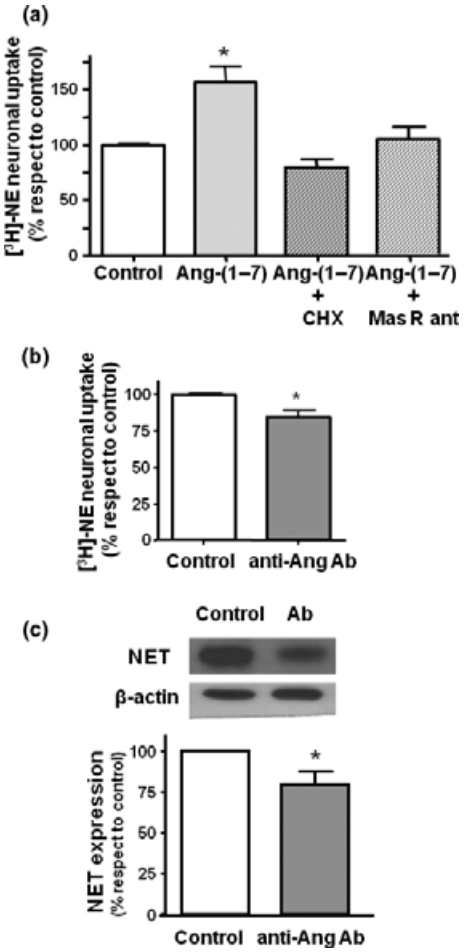

Fig. 6 (a) Long-term effect of Ang-(1–7) on [^{3}H]-NE neuronal uptake in hypothalami from SHR. Hypothalami were incubated in the absence (control) or in the presence of 0.1 μmol/L Ang-(1–7) during 3 h and NE neuronal uptake determined as described in Methods. When indicated, the protein synthesis inhibitor cycloheximide (CHX) (1.0 μmol/L) or the Mas receptor antagonist (Mas ant.) (1.0 μmol/L) were added simultaneously with 0.1 μmol/L Ang-(1–7). Results are expressed as percentage of the response detected in control conditions, taking them as 100%. Data are means ± SEM of 6–8 experiments. *$p < 0.05$ as compared with control. (b) [^{3}H]-NE neuronal uptake in hypothalami from SHR. Hypothalami were incubated for 3 h in the absence (control) or in the presence of a specific anti-Ang-(1–7) antibody (Anti-Ang Ab) and NE neuronal uptake determined as described in Methods. (c) NET expression in SHR hypothalami-brainstem neuronal cultures in the absence (control) and presence of a specific anti-Ang-(1–7) antibody (Anti-Ang Ab). NET expression was measured by western blot as described in Methods and is expressed as a percentage of the response detected in control cells, taking them as 100%. Each bar represents the mean ± SEM of three determinations from five separate cell culture preparations.

activity should be augmented in a hypertensive state as a compensatory feed back mechanism of control. However, Lu *et al.* (1996) demonstrated that both basal NET activity and NET mRNA levels are similar in brainstem, hypothalamus and neuronal cultures from normotensive WKY and SHR. In accordance, our present results show that the centrally basal neuronal NE uptake and NET expression in central areas of the sympathetic nervous system did not differ between SHR and normotensive WKY rats. As an increase in NE release and synthesis leads to sympathetic overactivity in SHR (De Wardener 2001; Veerasingham and Raizada 2003), the fact that NET activity and expression were not altered in this hypertensive model (present results and Lu *et al.* 1996) suggest that NET function may be disregulated, leading to neurotransmitter accumulation. Another possible explanation may be that an enhanced NE release and synthesis predominates over NE uptake, and despite that Ang-(1–7) exerts a counterbalancing effect (Gironacci *et al.* 2004a; Lopez Verrilli *et al.* 2009; and present results), others pressor agents act to favor the enhanced NE release and synthesis, such as Ang II or endothelin-1 and -3 (Veerasingham and Raizada 2003; Di Nunzio *et al.* 2004).

We have previously reported that Ang-(1–7) decreases NE synthesis and release in hypothalami from SHR (Gironacci *et al.* 2004a; Lopez Verrilli *et al.* 2009). This Ang-(1–7)-induced NE metabolism alterations result in a reduction of NE availability into the synaptic cleft. Moreover, changes in the transporter activity or expression should impact on the length time and concentration of NE in the synaptic cleft. These changes, in turn, may influence on pre- and post-synaptic receptors, enhancing or diminishing the final response to the released neurotransmitter. Ang-(1–7) did not evoke an acute effect on neuronal NE uptake in hypothalami from SHR (present results), neither in hypothalami from normotensive rats (Gironacci *et al.* 2000). Conversely, Ang-(1–7) evoked a long-term effect on NE neuronal uptake by increasing NET expression (present results). The fact that this effect was cycloheximide-sensitive and that Ang-(1–7) induced an increase in NET mRNA levels demonstrates that Ang-(1–7) long-term stimulation of neuronal NE uptake involves an increase in mRNA translation and/or stability. Cycloheximide has been reported not only to inhibit protein translation but also to increase mRNA stabilization (Yamazaki and Takeshige 2008). In agreement, Apparsundaram *et al.* (2001) described a similar action for insulin: no acute effect on NE neuronal uptake but a long-term action on that mechanism involving an increase in NET biosynthesis. Our results about the inhibitory role of Ang-(1–7) on the NET system were reinforced by the fact that when endogenous Ang-(1–7) was blocked by its specific antibody, a decrease in NE neuronal uptake as well as in NET expression were observed.

Ang-(1–7) long-term stimulation of neuronal NE uptake involves an increase in NET mRNA translation and/or stability after 3 h, whereas at a shorter time (1 h), Ang-(1–7) may induce a decrease in NET protein degradation. The fact that the time course of NET protein and mRNA expression is not the same may be due to a decrease in NET protein degradation induced by Ang-(1–7). For instance, Ang-(1–7) affects tyrosine hydroxylase expression by regulating its turnover (Lopez Verrilli *et al.* 2009). Tyrosine hydroxylase is the rate-limiting enzyme involved in norepinephrine synthesis.

Taking into account that Ang-(1–7) opposes many of Ang II actions, including NE release and synthesis (Veerasingham and Raizada 2003; Gironacci *et al.* 2004a; Lopez Verrilli *et al.* 2009; Xu *et al.* 2011) it was surprising to find that Ang-(1–7) effect on NET expression was similar to that displayed by Ang II (Lu *et al.* 1996). Lu *et al.* (1996) showed that Ang II stimulates the NET system both acutely and chronically in neuronal cultures from SHR, the former involving activation of pre-existing transporters and the latter involving NET gene transcription and translation. As Ang II is degraded to Ang-(1–7) by ACE2 activity (Ferrario *et al.* 2010; Ferreira *et al.* 2010; Xu *et al.* 2011), we could not disregard that the effect of Ang II on NET system may be mediated by Ang-(1–7) generation. For instance, it has been shown that some of the central or renal effects of Ang II are caused by Ang III generation (Wright *et al.* 2003; Padia *et al.* 2008). Further experiments should be carried out to test this hypothesis. 14

Post-translational modifications such as glycosylation and phosphorylation regulate monoamine transporter function and expression levels (Mandela and Ordway 2006; Ramamoorthy *et al.* 2011). PI3-kinase pathways are known to regulate long-term expression of neurotransmitter transporters, including monoamine transporters (Ramamoorthy *et al.* 2011). NET has also been shown to be regulated by MAPK (Mandela and Ordway 2006; Ramamoorthy *et al.* 2011). Conversely, protein phosphatase 1/2A down-regulates NET, dopamine and serotonin transporters activity (Mandela and Ordway 2006; Ramamoorthy *et al.* 2011). Thus, direct phosphorylation and/or dephosphorylation of transporters by cellular protein kinases and phosphatases are involved in the dynamic regulation and expression of amine transporters. Our present work shows that the long-term stimulatory effect of Ang-(1–7) on NET expression was coupled to Mas receptor activation acting through a PI3-kinase/Akt and MEK1/2-ERK1/2 mediated pathways. The involvement of PI3-kinase/Akt and MEK1/2-ERK1/2 signaling cascades have been described for Ang II on NE neuromodulation in neuronal cultures from SHR (Gelband *et al.* 1997; Yang and Raizada 1999). In this way, Ang-(1–7) appears to share a similar response and pathway as Ang II. It must be pointed out that Ang-(1–7)-induced Akt or ERK1/2 phosphorylation mediated by Mas receptors has been previously reported at the perypheral level (Giani *et al.* 2007; Sampaio *et al.* 2007; Dias-Peixoto *et al.* 2008; Zimpelmann and Burns 2009) but not centrally, not even in neurons, where Mas receptors are expressed (present results). Furthermore, an increased Mas R

expression of 2.7-fold was observed in hypothalami-brain stem neurons from SHR compared with WKY (present results). This increase may be due to compensate the increased Ang II activity observed in SHR (Gelband *et al.* 1997; Veerasingham and Raizada 2003). In addition, AT1 receptors have been reported to be increased in neuronal cultures from SHR, being 4-fold higher than the corresponding WKY cultures (Raizada *et al.* 1993).

Despite the fact that it has been shown that the PI3K/Akt pathway is involved in the stimulatory action of Ang-(1–7) on NO generation (Sampaio *et al.* 2007; Dias-Peixoto *et al.* 2008) and that the inhibitory effect of Ang-(1–7) on NE release is mediated by NO (Gironacci *et al.* 2004a), we disregard that the Ang-(1–7)-stimulated NET expression may be coupled to NO production, because evidences show that NO reduces NE uptake (Kaye *et al.* 2000; Hope *et al.* 2010), in contrast to what is observed with Ang-(1–7) (present results). Up-to-date effects of NO on NET have not been fully characterized yet.

Amine transporters are essential players in regulating normal and abnormal amine signaling in the CNS and periphery, fine-tuning neurotransmitter levels at the synaptic cleft. Altered pattern or a disturbance in the pre- and post-synaptic regulatory mechanisms could lead to abnormal neurotransmission and hence abnormal behavior, brain disorders or hypertension. Present results demonstrate that Ang-(1–7) does not evoke an acute but a long-term effect on NE neuronal uptake in hypothalami from SHR increasing NET expression, being these effects coupled to Mas receptor activation acting through both MEK1/2-ERK1/2 and PI3-kinase/Akt dependent pathways. In this way, Ang-(1–7) may regulate a pre-synaptic mechanism in maintaining appropriate synaptic NE levels during hypertensive conditions. This study, together with the fact that Ang-(1–7) inhibits NE synthesis and release from SHR hypothalami supports a negative neuromodulatory role for Ang-(1–7) on central sympathetic nervous system activity, thus contributing to the modulation of NE homeostasis. Enhanced sympathetic activity and hypertension are often associated with an activation of the brain RAS. A better understanding of the interaction between these central networks, peptides and monoamines that regulate sympathetic effluents might offer new possibilities for pharmacological intervention in hypertension.

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Conflict of interest

None.

References

Alessi D. R., Andjelkovic M., Caudwell B., Cron P., Morrice N., Cohen P. and Hemmings B. A. (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* **15**, 6541–6551.

Apparsundaram S., Sung U., Price R. D. and Blakely R. D. (2001) Trafficking-dependent and -independent pathways of neurotransmitter transporter regulation differentially involving p38 mitogen-activated protein kinase revealed in studies of insulin modulation of norepinephrine transport in SK-N-SH cells. *J. Pharmacol. Exp. Ther.* **299**, 666–77.

Bönisch H. and Brüss M. (2006) The norepinephrine transporter in physiology and disease. *Handb. Exp. Pharmacol.* **175**, 485–524.

De Wardener H. E. (2001) The hypothalamus and hypertension. *Physiol. Rev.* **81**, 1599–1658.

Di Nunzio A. S., Legaz G., Rodano V., Bianciotti L. G. and Vatta M. S. (2004) Modulatory effect of endothelin-1 and -3 on neuronal norepinephrine release in the rat posterior hypothalamus. *Regul. Pept.* **118**, 51–59.

Dias-Peixoto M. F., Santos R. A. S., Gomes E. R. M. *et al.* (2008) Molecular mechanisms involved in the angiotensin-(1-7)/Mas signaling pathway in cardiomyocytes. *Hypertension* **52**, 542–548.

Eisenhofer G. (2001) The role of neuronal and extraneuronal plasma membrane transporters in the inactivation of peripheral catecholamines. *Pharmacol. Ther.* **91**, 35–62.

Esler M., Jackman G., Bobik A., Leonard P., Kelleher D., Skews H., Jennings G. and Korner P. (1981) Norepinephrine kinetics in essential hypertension. Defective neuronal uptake of norepinephrine in some patients. *Hypertension* **3**, 149–156.

Ferrario C. M., Chappell M. C., Tallant E. A., Brosnihan K. B. and Diz D. I. (1997) Counterregulatory actions of angiotensin-(1–7). *Hypertension* **30** [2], 535–541.

Ferrario C. M., Ahmad S., Joyner J. and Varagic J. (2010) Advances in the renin angiotensin system focus on angiotensin-converting enzyme 2 and angiotensin-(1-7). *Adv. Pharmacol.* **59**, 197–233.

Ferreira A. J., Santos R. A. S., Bradford C. N., Mecca C. N., Sumners C., Katovich M. J. and Raizada M. K. (2010) Therapeutic implications of the vasoprotective axis of the renin-angiotensin system in cardiovascular diseases. *Hypertension* **55**, 207–213.

Gelband C. H., Sumners C., Lu D. and Raizada M. K. (1997) Angiotensin receptors and norepinephrine neuromodulation: implications of functional coupling. *Regul. Pept.* **72**, 139–144.

Giani J. F., Gironacci M. M., Muñoz M. C., Peña C., Turyn D. and Dominici F. P. (2007) Angiotensin-(1-7) stimulates the phosphorylation of JAK2, IRS-1 and Akt in rat heart in vivo: role of the AT1 and Mas receptors. *Am. J. Physiol. Heart Circ. Physiol.* **293**, H1154–H1163.

Gironacci M. M., Rodríguez-Fermepín M., Vatta M., Fernandez B. E., Rubio M. and Peña C. (2000) Angiotensin-(1-7) does not affect norepinephrine neuronal uptake or catabolism in rat hypothalamus and atria. *Cell. Mol. Neurobiol.* **20**, 773–779.

Gironacci M. M., Valera M. S., Yujnovsky I. and Peña C. (2004a) Angiotensin-(1-7) inhibitory mechanism of norepinephrine release in hypertensive rats. *Hypertension* **44**, 783–787.

Gironacci M. M., Yujnovsky I., Gorzalczany S., Taira C. and Peña C. (2004b) Angiotensin-(1-7) inhibits the angiotensin II-enhanced norepinephrine release in coarcted hypertensive rats. *Regul. Pept.* **118**, 45–49.

Habecker B. A., Willison B. D., Shi X. and Woodward W. R. (2006) Chronic depolarization stimulates norepinephrine transporter expression via catecholamines. *J. Neurochem.* **97**, 1044–1051.

Hahn M. K., Robertson D. and Blakely R. D. (2003) A mutation in the human norepinephrine transporter gene (SLC6A2) associated with

orthostatic intolerance disrupts surface expression of mutant and wild-type transporters. *J. Neurosci.* **23**, 4470–4478.

Höcht C., Gironacci M. M., Mayer M. A., Schuman M., Bertera F. M. and Taira C. A. (2008) Involvement of angiotensin-(1-7) in the hypothalamic hypotensive effect of captopril in sinoaortic denervated rats. *Regul. Pept.* **146**, 58–66.

Hope S. I., Nabhen S. L., Soria C., Bianciotti L. G. and Vatta M. S. (2010) Endothelin-1 and -3 modulate the neuronal norepinephrine transporter through multiple signalling pathways in the rat posterior hypothalamus. *Neurochem. Int.* **57**, 306–313.

Kaye D. M., Gruskin S., Smith A. I. and Esler M. D. (2000) Nitric oxide mediated modulation of norepinephrine transport: identification of a potential target for S-nitrosylation. *Br. J. Pharmacol.* **130**, 1060–1064.

Kvetnansky R., Sabban E. L. and Palkovits M. (2009) Catecholaminergic systems in stress: structural and molecular genetic approaches. *Physiol. Rev.* **89**, 535–606.

Lambert E., Eikelis N., Ester M. *et al.* (2008) Altered sympathetic nervous reactivity and norepinephrine transporter expression in patients with postural tachycardia syndrome. *Circ. Arrhythmia Electrophysiol.* **1**, 103–109.

Lopez Verrilli M. A., Pirola C. J., Pascual M. M., Dominici F. P., Turyn D. and Gironacci M. M. (2009) Angiotensin-(1-7) through AT2 receptors mediates tyrosine hydroxylase degradation via the ubiquitin-proteasome pathway. *J. Neurochem.* **109**, 326–35.

Lu D., Yu K., Paddy M. R., Rowland N. E. and Raizada M. K. (1996) Regulation of norepinephrine transport system by angiotensin II in neuronal cultures of normotensive and spontaneously hypertensive rat brains. *Endocrinology* **137**, 763–772.

Mandela P. and Ordway G. A. (2006) The norepinephrine transporter and its regulation. *J. Neurochem.* **97**, 310–333.

Ono K., Iwanaga Y., Mannami T., Kokubo Y., Tomoike H., Komamura K., Shioji K., Yasui N., Tago N. and Iwai N. (2003) Epidemiological evidence of an association between SLC6A2 genepolymorphism and hypertension. *Hypertens. Res.* **26**, 685–689.

Padia S. H., Kemp B. A., Howell N. L., Fournie-Zaluski M. C., Roques B. P. and Carey R. M. (2008) Conversion of renal angiotensin II to angiotensin III is critical for AT2 receptor-mediated natriuresis in rats. *Hypertension* **51**, 460–465.

Pearson G., Robinson F., Beers Gibson T., Xu B., Karandikar M., Berman K. and Cobb M. H. (2001) Mitogen-activated protein kinase pathways: regulation and physiological functions. *Endocr. Rev.* **22**, 153–183.

Raizada M. K., Lu D., Tang W., Kurian P. and Sumners C. (1993) Increased angiotensin II type-1 receptor gene expression in neuronal cultures from spontaneously hypertensive rats. *Endocrinology* **132**, 1715–1722.

Ramamoorthy S., Shippenberg T. S. and Jayanthi L. D. (2011) Regulation of monoamine transporters: role of transporter phosphorylation. *Pharm. Ther.* **129**, 220–238.

Sampaio W. O., Souza dos Santos R. A., Faria-Silva R., da Mata Machad L. T., Schiffrin E. L. and Touyz R. M. (2007) Angiotensin-(1-7) through receptor Mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. *Hypertension* **49**, 185–192.

Torres G. E., Gainetdinov R. R. and Caron M. G. (2003) Plasma membrane monoamine transporters: structure, regulation and function. *Nat. Rev. Neurosci.* **4**, 13–25.

Veerasingham S. J. and Raizada M. K. (2003) Brain renin-angiotensin system dysfunction in hypertension: recent advances and perspectives. *Br. J. Pharmacol.* **139**, 191–202.

Wright J. W., Tamura-Myers E., Wilson W. L., Roques B. P., Llorens-Cortes C., Speth R. C. and Harding J. W. (2003) Conversion of brain angiotensin II to angiotensin III is critical for pressor response in rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **284**, R725–733.

Xu P., Sriramula S. and Lazartigues E. (2011) ACE2/Ang-(1-7)/Mas pathway in the brain: the Axis of Good. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **300**, R804–R817.

Yamazaki S. and Takeshige K. (2008) Protein synthesis inhibitors enhance the expression of mRNAs for early inducible inflammatory genes via mRNA stabilization. *Biochem. Biophys. Acta* **1779**, 108–114.

Yang H. and Raizada M. K. (1999) Role of phosphatidylinositol 3-kinase in angiotensin II regulation of norepinephrine neuromodulation in brain neurons of the spontaneously hypertensive rat. *J. Neurosci.* **19**, 2413–2423.

Yu K., Lu D., Rowland N. E. and Raizada M. K. (1996) Angiotensin II regulation of tyrosine hydroxylase gene expression in the neuronal cultures of normotensive and spontaneously hypertensive rats. *Endocrinology* **137**, 3566–3576.

Zimpelmann J. and Burns K. D. (2009) Angiotensin-(1-7) activates growth-stimulatory pathways in human mesangial cells. *Am. J.Physiol. Renal Physiol.* **296**, F337–F346.

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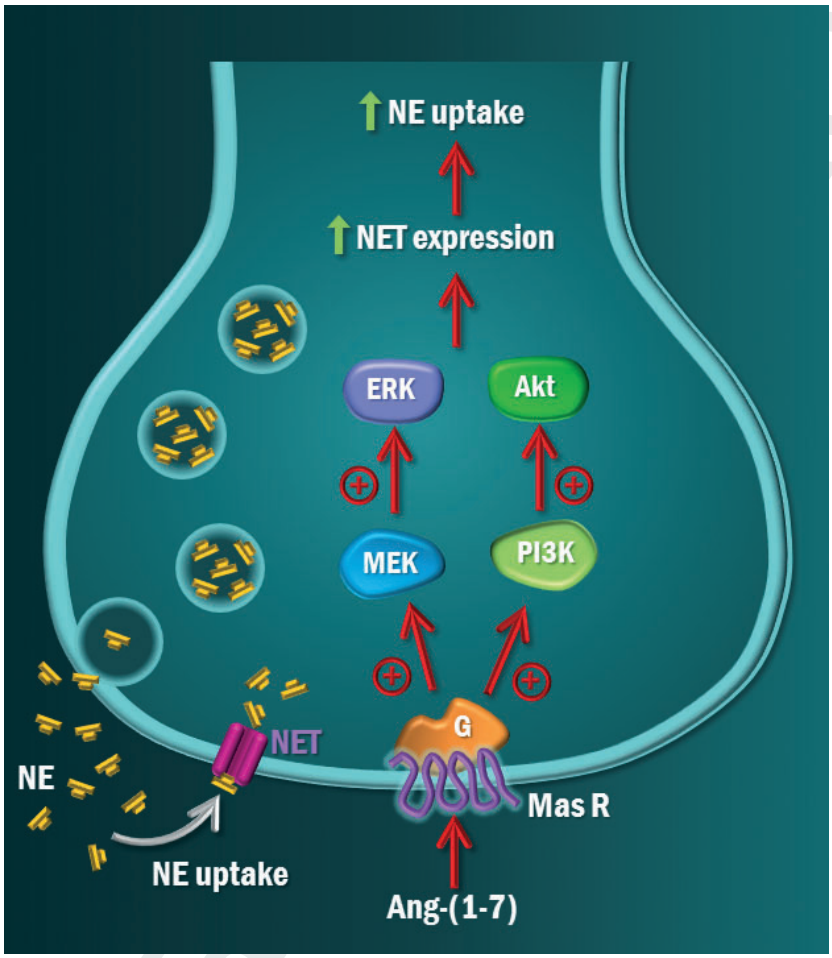

Central NET upregulation by angiotensin-(1-7) in hypertension

As angiotensin (Ang) (1–7) decreases norepinephrine (NE) content in the synaptic cleft, we investigated the effect of Ang-(1–7) on centrally NE neuronal uptake, the main mechanism for NE removal from the synaptic cleft, in hypothalamic neurons from hypertensive rats. We showed that Ang-(1–7) causes a long-term stimulatory effect on NE neuronal uptake by increasing NE transporter expression. This effect is coupled to Mas receptor activation acting through both MEK1/2-ERK1/2 and PI3-kinase/Akt-dependent pathways. In this way, Ang-(1–7) may regulate a pre-synaptic mechanism in maintaining appropriate synaptic NE levels during hypertensive conditions, thus contributing to the modulation of NE homeostasis and blood pressure regulation.

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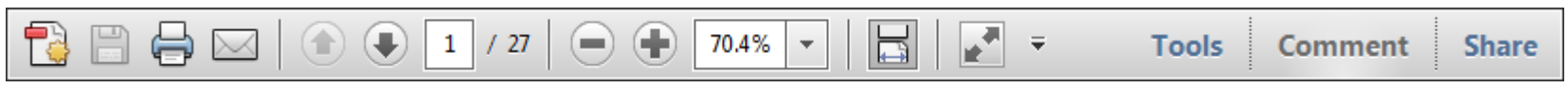

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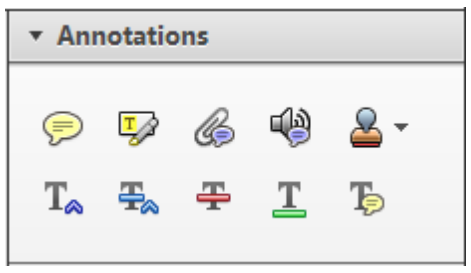

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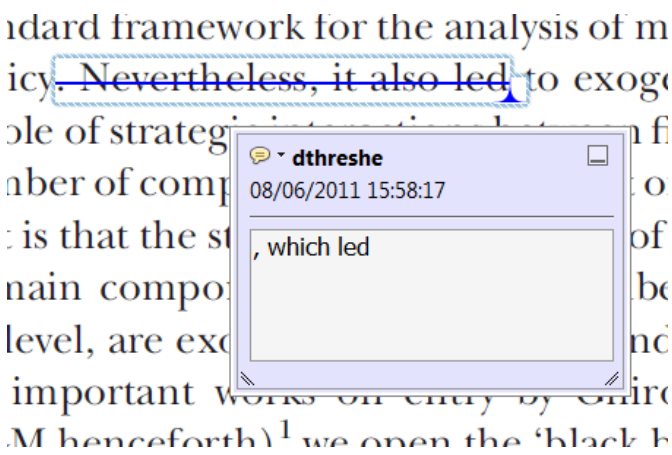

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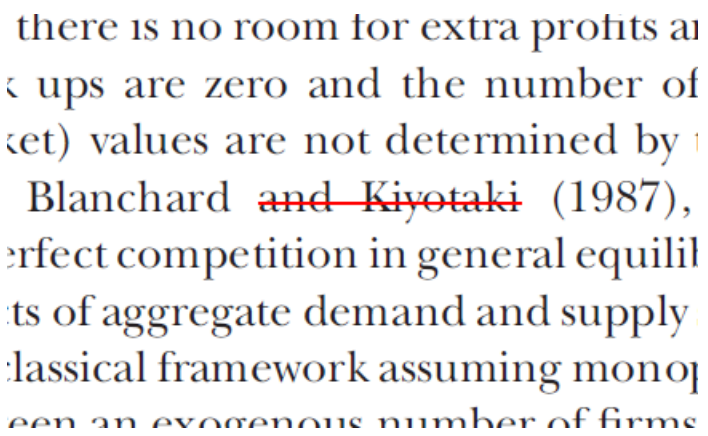

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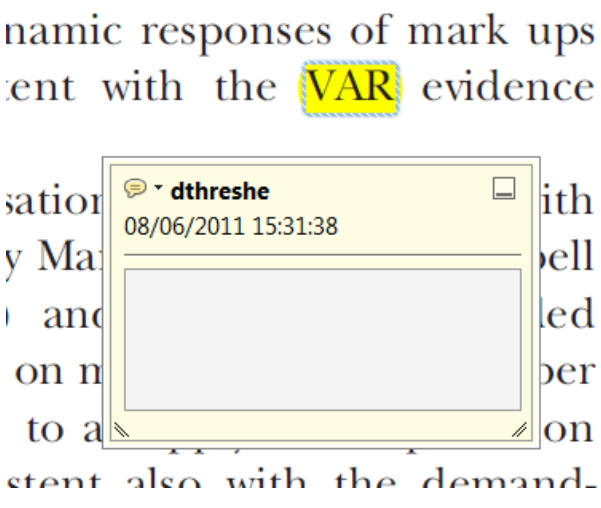

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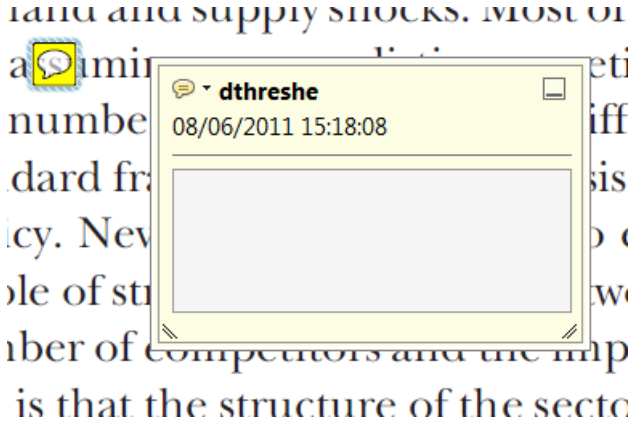

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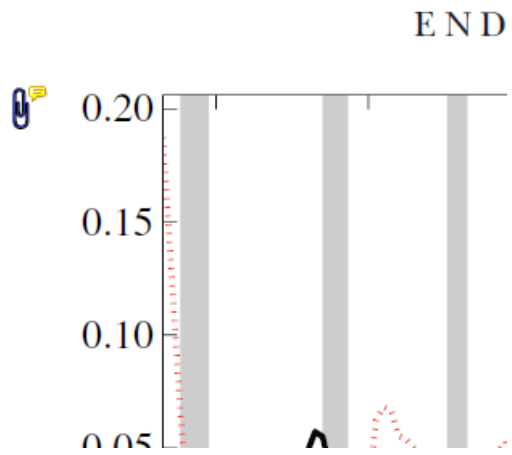
- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

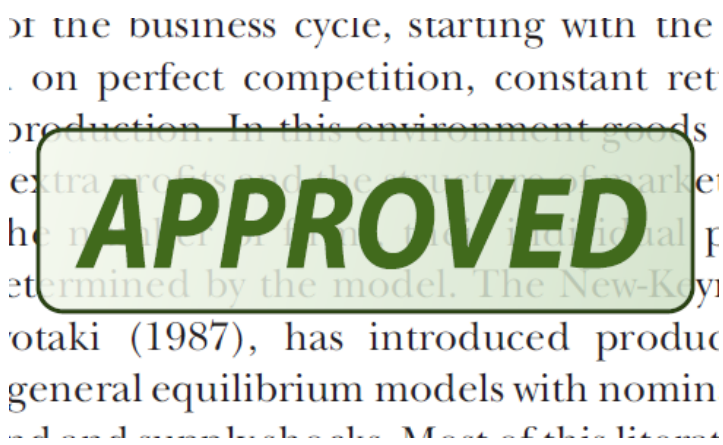


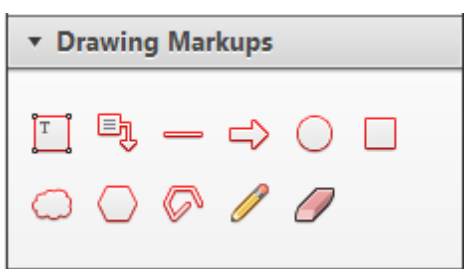
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

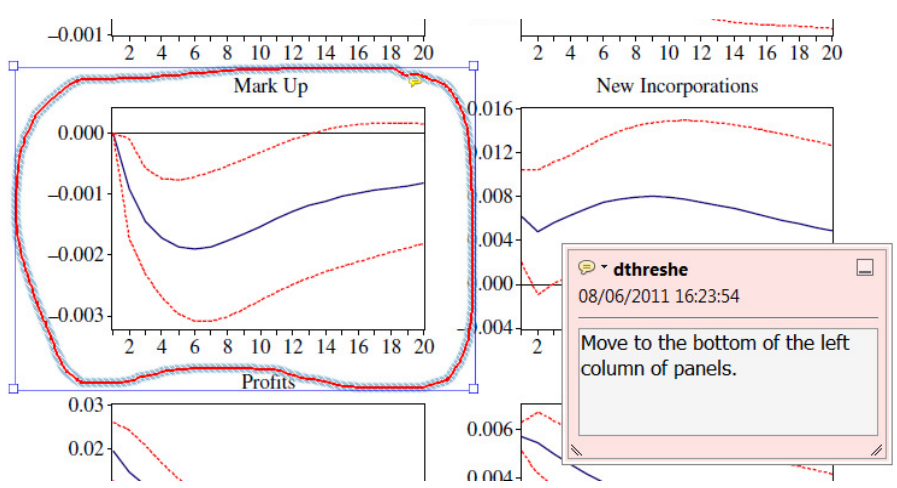
- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).





7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..



How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: