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Protein translocation into dendrites: implication of glia-derived angiotensins in noradrenergic brainstem neurons.

Michaël Ogier^{*,†}, Giampiero Bricca[†], Fabrice Navarro^{*}, Jean-Marc Pequignot^{*}, Anne Morales^{*} and Laurent Bezin^{*}.

** Department of Integrative Cellular and Molecular Physiology, CNRS and Université Claude Bernard Lyon 1 (UCBL), UMR 5123, 43 bd du 11 Novembre 1918, 69622 Villeurbanne, France ;[†] UCBL EA 3740, Faculté de médecine Laënnec, 8 rue G. Paradin, 69372 Lyon Cedex 08, France.*

Running Title: Glia-derived angiotensins control TH protein sorting

Corresponding author : Laurent Bezin, PhD
CNRS-UCBL UMR 5123,
Bâtiment R. Dubois, 43 boulevard du 11 Novembre 1918
69622 Villeurbanne Cedex, France
Tel : (33) 4 72 44 58 41 – Fax : (33) 4 72 43 11 72
E-mail : Laurent.Bezin@univ-lyon1.fr

Summary

Noradrenaline (NA) released by dendrites of locus coeruleus (LC) neurons controls LC neuron activity, and hence, NA released from noradrenergic fibers in terminal fields. NA synthesis is dependent on tyrosine hydroxylase (TH), whose dendritic presence is controlled precisely in LC neurons during postnatal development. However, the mechanisms controlling specifically dendritic sorting of TH without affecting its axonal transport remained to be elucidated. Here, by using transgenic rats with astrocyte-directed depletion of angiotensinogen, we show that dendritic but not axonal TH protein sorting is initiated after weaning by cerebral angiotensins in LC neurons, but not in dopaminergic neurons of the substantia nigra (SN). Cerebral angiotensins trigger dendritic translocation of TH protein by stimulating phosphorylation of the myristoylated alanine-rich C kinase substrate (MARCKS), known to facilitate macromolecule export from the soma. Protein trafficking within either axons or dendrites of LC neurons is thus controlled by specialized mechanisms, some having a glial origin.

Keywords : Transgenic rats, angiotensins, ontogeny, tyrosine hydroxylase, gene expression, dendritic trafficking.

Introduction

In nucleus locus coeruleus (LC), which is the major group of central noradrenergic neurons, three proteins in particular play an important role in noradrenergic transmission: tyrosine hydroxylase (TH) is the rate-limiting enzyme in noradrenaline (NA) biosynthesis (Nagatsu et al., 1964), dopamine- β -hydroxylase (DBH) transforms dopamine into NA, and NA transporter (NET) is involved in NA clearance from the extracellular compartment (Nestler et al., 1999). The transport of these proteins from perikarya to dendrites requires to be precisely controlled, because it determines activity and functions of LC neurons. Indeed, noradrenaline (NA) is synthesized in and released not only by axons of LC neurons, but also by dendrites (Singewald and Philippu, 1998; Berridge and Waterhouse, 2003). NA released by dendrites requires to be controlled because it exerts an autocrine inhibition of LC neuronal activity (Cedarbaum and Aghajanian, 1976) mediated by α_2 -adrenergic receptors (Aghajanian and VanderMaelen, 1982) located on the somatodendritic surface of LC neurons (Lee et al., 1998 a,b), and alters NA released in terminal fields (Van Gaalen et al., 1997; Mateo et al., 1998; Mateo and Meana, 1999). A means to control locally the dendritic release of NA relies basically on regulating into this neuronal compartment the presence of TH protein. However, because TH protein is synthesized within the perikarya and can be directed either to dendrites or axons, noradrenergic neurons may have developed molecular strategies to regulate selectively the targeting of TH protein to dendrites without affecting its axonal transport. However, such molecular mechanisms remained poorly understood.

Differential transport of TH protein within axons and dendrites of LC neurons was initially revealed during postnatal development (Specht et al., 1981; Bezin et al., 1994a; Bezin et al., 1994b; Bezin et al., 1994c; Bezin et al., 1997). At adulthood, the precise analysis of TH protein distribution within nucleus LC allows to discriminate two regions within the periventricular grey: one is delimited by the densely packed noradrenergic perikarya of LC neurons, called “perikaryal area of nucleus LC” (PKLC) (Specht et al., 1981; Bezin et al., 1994a; Bezin et al., 1997), the other, called “pericoerulean area” (PCA), is mainly constituted of dendrites of LC neurons that extend

considerable distances far beyond the PKLC (Bezin et al., 1994b; Bezin et al., 1994c; Bezin et al., 1997). Early in development, TH protein is preferentially concentrated in the cell body of LC neurons and is exclusively sorted to axons, although dendrites are already differentiated (Specht et al., 1981), allowing NA to be synthesized in terminals of LC neurons in order to support correct maturation of target cells (Bear and Singer, 1986). It is during the 4th postnatal week only, just after weaning, that sorting of TH protein to dendrites is initiated (Bezin et al., 1994a; Bezin et al., 1994b; Bezin et al., 1997). As a result, TH protein level substantially increases in nucleus LC (PKLC plus PCA), 80% being contained within dendrites (Bezin et al., 1994b). Thus, postnatal development, and the 4th postnatal week in particular, appears to be the appropriate model to elucidate some of the endogenous factors harmonizing transport of TH protein within processes of LC neurons.

Angiotensins appear to be factors potentially involved in the control of protein trafficking within LC neurons. *In vitro* studies revealed that angiotensin II enhances transport of TH, DBH and NET proteins from perikarya to neurites of cultured noradrenergic hypothalamus and brainstem neurons (Figure 1) (Lu et al., 1998). This requires the activation of angiotensin-type 1 (AT1) receptors and the phosphorylation of the myristoylated alanine-rich C kinase substrate (MARCKS) (Lu et al., 1998; Yang et al., 2002a). Activation of AT1 receptors can also stimulate *in vitro* the expression of TH, DBH and NET genes, by stimulating the Ras-Raf-Mitogen-activated protein kinase (Ras-Raf-MAPK) intracellular pathway (Lu et al., 1996; Yu et al., 1996). However, these *in vitro* observations needed to be verified *in vivo*. The hypothesis that cerebral angiotensins can control TH phenotype *in vivo* within LC neurons is justified by the observation that angiotensinogen (Chai et al., 1993; Mungall et al., 1995), angiotensins receptors (Speth et al., 1991; Lenkei et al., 1996; Lenkei et al., 1997; Lenkei et al., 1998; Nuyt et al., 1999) and angiotensins II (Weyhenmeyer and Phillips, 1982; Fuxe et al., 1988; Lenkei et al., 1997), which are major components of the central angiotensinergic system, are present within nucleus LC. In addition, pioneer studies demonstrated that angiotensins can modulate noradrenergic neurotransmission within the rat LC by rising NA turnover (Summers et Phillips, 1983).

In this study, we used TGR(ASrAOGEN) rats (TGR), with depletion of angiotensins specifically restricted to brain tissue (Schinke et al., 1999), to study *in vivo* the relationship existing between cerebral angiotensins and noradrenergic neurons. Here, we show that depletion of cerebral angiotensins of glial origin is associated with a sharp decrease in TH protein translocation into dendrites of LC neurons and with an important decrease in TH gene expression during the 4th postnatal week. No alteration of TH protein presence was observed in the axonal compartment of LC neurons. Our results highlight that astrocytes contribute to the maturation of TH phenotype by triggering specifically the dendritic transport of TH protein in noradrenergic neurons, through a mechanism involving angiotensin-mediated phosphorylation of MARCKS.

Results

Cerebral AOGEN depletion is associated with a sharp decreased presence of TH protein in dendritic, but not axonal, processes of LC neurons at 4 weeks

Depletion of AOGEN protein occurring in TGR depends on the expression of an anti-sense (AS) RNA directed against AOGEN mRNA (Figure 2A). AS RNA expression, observed during the whole postnatal period (Figure 2B), is under control of the promoter sequence for glial fibrillary acidic protein (GFAP); this was verified in the LC region of TGR by the highly significant linear correlation established between GFAP mRNA (x) and AS RNA (y) levels determined over the entire postnatal period (Figure 2C).

TH protein is detected in control rats by immunohistochemistry within both perikarya of LC neurons, located in the PKLC, and dendrites of these neurons, located in the PCA (Figure 3A), as previously described (Bezin et al., 1994a; Bezin et al., 1994b; Bezin et al., 1994c; Bezin et al., 1997). In TGR, dendrites of LC neurons are almost devoid of TH protein at 4 weeks compared to controls (Figure 3B). By contrast, detection of TH protein in brain areas receiving a dense noradrenergic innervation originating from LC neurons, i.e. the molecular layer of the frontal cortex (MLFC) (Morrison et al., 1981) and the subgranular zone (SGZ) of the dentate gyrus (Loy et al.,

1980), is not modified (Figure 3C, 3D, 3E and 3F). The alteration of the dendritic presence of TH protein observed in the LC of TGR is similar to that observed in the noradrenergic nucleus tractus solitarius (NTS) (Ogier et al., 2003), and may thus be specific to brainstem noradrenergic structure, since no obvious modification is observed in TGR within the dopaminergic substantia nigra (SN) compared to controls (Figure 3G and 3H). After quantification, the density of elements expressing TH protein within the PCA of TGR is decreased by up to 78 %, depending on the anatomical coordinates (mean of 66% when quantified at interaural coordinates -0.80 mm (Paxinos and Watson, 1998), where TH-immunolabelled PCA is the largest; Figure 3I). No change was found in terminal fields of LC neurons and in the pars reticulata of the SN of TGR compared to controls (Figure 3I). Thus, under tonic cerebral angiotensins depletion, presence of TH protein is specifically affected in dendritic processes of brainstem noradrenergic neurons, in particular LC neurons.

Cerebral AOPEN depletion is associated with altered expression of TH gene in the LC at 4 weeks

The decreased presence of TH protein observed in dendritic processes of LC neurons in TGR could result from a reduced expression of the gene encoding the enzyme. Analysis of TH mRNA levels within the whole nucleus LC (PKLC plus PCA along the entire caudorostral axis) reveals a decrease in TGR, reaching almost -30% as compared to controls (Figure 4A). Expression of other main markers of noradrenergic neurons, known to be stimulated *in vitro* by cerebral angiotensins (Lu et al., 1996; Yu et al., 1996), is also reduced in the LC of TGR. Indeed, DBH and NET mRNA are decreased by 37 and 34 %, respectively (Figure 4A).

Phosphorylation of MARCKS stimulated by glia-derived angiotensins may support TH protein translocation into dendritic processes of LC neurons at 4 weeks

If it is considered that the intensity of the immunological labelling can reflect the concentration of the detected protein, it can thus be concluded that TH protein is evenly distributed between cell bodies and dendrites of LC neurons in control rats at 4 weeks (Figure 4B). By contrast, the brown

dark labelling that is restricted to the perikarya in TGR, associated with the absence of labelled dendrites, suggests that, in these rats, TH protein is sequestered in the perikarya of LC neurons (Figure 4C). However, the observation that TH protein targeted toward axonal processes of LC neurons is not affected in TGR argues in favour of the hypothesis that TH protein is not sequestered in the perikarya, but rather that TH protein translocation into dendrites is specifically altered at 4 weeks in TGR.

In cultured noradrenergic neurons, activation of angiotensin receptors stimulates phosphorylation of MARCKS. This mechanism is required for angiotensin II to trigger transport of TH, DBH and NET proteins towards neurites *in vitro* (Lu et al., 1998; Yang et al., 2002a). While MARCKS is abundantly present in LC neurons (Ouimet et al., 1990), this study is the first to evidence the presence of its phosphorylated form (Figure 4E and 4F). We found that phosphorylated-MARCKS protein (P-MARCKS) colocalizes with TH protein in cell bodies and dendrites of LC neurons in control rats at 4 weeks (Figure 4D, 4E and 4F), while P-MARCKS is nearly undetectable in TGR at the same period (Figure 4G, 4H and 4I). Taken together, these results indicate that phosphorylation of MARCKS protein is crucial for TH protein translocation into dendrites of LC neurons at 4 weeks.

Alterations observed in the LC region of TGR are specific to noradrenergic neurons

Absence of TH protein within dendrites of LC neurons and reduced expression of TH, DBH and NET in TGR cannot result from a global alteration of neuronal maturation caused by the depletion of cerebral angiotensins (Yang et al., 2002b). Indeed, neither the quantity of mRNA encoding microtubule-associated protein 2c (MAP2; Figure 5A), a specific marker for neuronal cell bodies and dendrites (Bernhardt et al., 1985), nor the distribution of this protein (Figure 5B and 5C) are modified in the LC region of TGR, compared to controls. In addition, the quantity of the mRNA encoding GFAP and AOPEN (Figure 5A), two specific markers for glia, and the morphology of GFAP-positive astrocytes (Figure 5D and 5E) in the LC region are similar in both strains of rats,

indicating that the expression of the transgene does not impair neither elementary functions of astrocytes, nor the contacts that they may establish with neighbouring cells, including LC neurons.

TH protein translocation into dendrites of LC neurons is restored in adult TGR

We examined whether the alterations of TH protein distribution that we observed in 4-week old TGR remained constant until adulthood. We found that TH protein is distributed in perikarya (Figure 6A and 6B), dendrites (Figure 6A and 6B) and axons (Figure 6C, 6D, 6E and 6F) of LC neurons in both strains of rats at 12 weeks. The density of immunolabelled dendrites observed in the PCA is similar in controls and TGR (Figure 6G). Moreover, we failed to detect P-MARCKS within noradrenergic neurons of LC, both in controls and TGR (Figure 6H, 6I, 6J, 6K, 6L and 6M). Thus, in the adult rat LC, both dendritic presence of TH in control rats and restoration of TH protein translocation into dendritic processes in TGR are independent of P-MARCKS, suggesting that pathways other than those activated by cerebral angiotensins are involved in the dendritic trafficking of TH protein in LC neurons at the adult stage.

Discussion

This work highlights that cerebral angiotensins trigger during the 4th postnatal week TH protein translocation into dendrites of LC neurons without affecting TH axonal trafficking. This effect of cerebral angiotensins involves both activation of TH gene expression and inhibition of the intracellular pathway restraining the movement of TH from the perikarya to dendrites (Figure 7). This study also underscores that astrocytes, which represent the endogenous source for the precursor of all cerebral angiotensins, are not energetic supports for neurons only; here, we show that they are directly involved in the control of mechanisms that are crucial for central (noradrenergic) neurotransmission.

TGR(ASrAOGEN): a useful tool to investigate the interactions between locally-synthesized angiotensins and catecholaminergic neurons in the CNS

Angiotensinogen (AOGEN), the only known precursor of all cerebral angiotensins, can be synthesized by both glial and neuronal cells *in vivo* (Sernia, 1995). Almost 90 % of the AOGEN detected in brain tissue are derived from glial cells. Within brain catecholaminergic structures, and particularly the LC region, only glial cells have been immunostained for AOGEN throughout postnatal development and at the adult stage (Mungall et al., 1995). Therefore, to determine whether angiotensins derived from glial AOGEN may participate in regulating TH phenotype in LC neurons, we used the transgenic TGR(ASrAOGEN)680 strain of rats (TGR) specifically depleted in cerebral AOGEN (Schinke et al., 1999). In these rats, depletion of AOGEN relies on a sharp decrease in the translation of its mRNA that is strictly restricted to astrocytes. This is due to an anti-sense RNA (AS RNA) directed against AOGEN mRNA, the expression of which is driven by GFAP promoter. This is particularly demonstrated in this study within the LC by the linear correlation established between levels of the transcripts encoding GFAP and AS RNA throughout the entire postnatal development.

It is likely that the alterations observed in TGR originated from glial AOGEN depletion, and not from the overall dysregulation of astrocyte metabolism due to the expression of the transgene (AS RNA) since (i) the morphology and the density of GFAP-immunopositive astrocytes and (ii) the level of GFAP and AOGEN transcripts remained unchanged in TGR as compared to controls. Our work also pointed out that the overall distribution of neuronal elements located within the periventricular grey was maintained in TGR. This observation suggests that AOGEN depletion *in vivo* did not dramatically alter during development the survival, differentiation and migration of neuronal populations, contrasting with conclusions raised for angiotensin II, the active peptide of the angiotensinergic system, from earliest cell culture studies (Laflamme et al., 1996; Yamada et al., 1996; Stroth et al., 1998; Shenoy et al., 1999; Grammatopoulos et al., 2002; Yang et al., 2002b).

Glia-derived angiotensins control specifically dendritic translocation of TH protein in LC neurons through MARCKS-mediated actin cross-linking

Studies specifically aimed at characterizing angiotensin actions upon noradrenergic neurons have established that angiotensin II applied chronically to brainstem neurons *in vitro* stimulates via AT1 the expression of key proteins of NA neurotransmission (Lu et al., 1996; Lu et al., 1998; Yang et al., 2002a). TH and DBH, involved in NA synthesis, and NET, involved in NA clearance from the extracellular compartment, have been shown to have their expression increased following AT1 activation. The enhanced expression of these proteins is mediated by the activation of the Ras-Raf-MAPK signalling pathway (Lu et al., 1996). In the present work, we have observed that the lack of cerebral AOPEN was associated with a significant decrease in the level of the transcripts encoding TH, DBH and NET in LC neurons at 4 weeks, indicating that the Ras-Raf-MAPK pathway may be involved *in vivo* at this developmental stage in the basal expression of the corresponding proteins.

The decreased level of transcript encoding TH in TGR at 4 weeks was associated with the absence of TH protein within dendrites of LC neurons. We first thought that the reduced transcript level supported a reduced synthesis of TH protein, which would be in that case transported in priority toward axons. Indeed, we did not observe any alteration in the density of TH-immunolabelling in selected terminal fields of LC neurons. However, the observation that TH-immunolabelling over perikarya of LC neurons was greater in TGR as compared to controls provided additional insights into the mechanism preventing TH protein trafficking toward dendrites of LC neurons. Indeed, the accumulation of TH protein within LC perikarya, together with the observation that TH protein transport into axons was maintained, demonstrates that TH protein was not sequestered within the perikarya of LC neurons at 4 postnatal weeks in TGR, but rather that TH protein could not translocate from perikarya into dendrites selectively (Figure 7).

F-actin has been shown to mediate protein trafficking in dendritic spines (Halpain, 2000; Matus, 2000) and recent studies have suggested that F-actin depolymerization may provide access of proteins towards dendritic spines in hippocampal neurons (Ouyang et al., 2005). Interestingly,

TH, DBH and NET have been shown *in vitro* to have their transport toward neurites increased following AT1 activation, which induces phosphorylation of MARCKS by the PKC β (Lu et al., 1998; Yang et al., 2002a). MARCKS phosphorylation prevents MARCKS-mediated actin cross-linking and thus facilitates transport of macromolecules from soma to neuronal processes (Hartwig et al., 1992). However, the existence of such a mechanism remained to be demonstrated *in vivo* within LC neurons. We found that during the 4th postnatal week, transport of TH protein toward LC dendrites occurred in presence of P-MARCKS in LC neurons. By contrast, the sharp reduction of TH protein presence in dendrites of LC neurons in TGR at 4 weeks was associated with disappearance of P-MARCKS immunoreactivity, suggesting that phosphorylation of MARCKS is required for TH protein transport within dendritic processes of LC neurons. Altogether, the present data demonstrate that glia-derived angiotensins stimulate not only the expression of key proteins involved in noradrenergic transmission but also the phosphorylation of MARCKS by PKC β , required to trigger, at this developmental stage, TH protein translocation into dendrites, but not axons, of LC neurons.

Glial angiotensins initiate TH protein translocation into dendrites of (nor)adrenergic neurons specifically at critical stages of postnatal development

Two major questions that could not be addressed by *in vitro* studies remained to be answered: (i), are glia-derived angiotensins required to maintain TH protein translocation into dendritic processes of noradrenergic neurons during the whole postnatal period, and (ii), is this mechanism shared by all central catecholaminergic structures?

Dendritic trafficking of TH protein was restored in LC neurons of TGR at adulthood (12 weeks), despite the fact that P-MARCKS was still lacking. This unexpected result was reinforced by the absence of P-MARCKS immunolabeling in noradrenergic neurons of LC in adult control rats, which have previously been shown to express the unphosphorylated form of the protein (Ouimet et al., 1990). Altogether, these results demonstrate that TH protein translocation into

dendrites of LC neurons is not controlled by angiotensin-dependent phosphorylation of MARCKS at the adult stage. However, one cannot exclude the possibility that MARCKS phosphorylation may be required in adult control rats when enhanced dendritic trafficking of TH protein is needed to support greater somatodendritic release of noradrenaline.

In a previous study (Ogier et al., 2003), we also found that depletion of glial AOPEN was associated at 4 weeks with an altered translocation of TH protein into dendrites in the nucleus tractus solitarius and the rostral-ventral lateral medulla, both located in the medulla oblongata. Hence, we first hypothesized that cerebral angiotensins controlled TH gene expression and initiated after weaning the dendritic transport of TH protein in all central catecholaminergic structures. However, we did not find here in TGR any notable alteration of TH presence in dendrites of the dopaminergic substantia nigra neurons, which express high concentrations of AT receptors (Lenkei et al., 1997; Nuyt et al., 2001). Prior studies already mentioned a differential response of dopaminergic and noradrenergic neurons to angiotensin II injected intracerebroventricularly, with enhancement of the turnover of NA, but not of dopamine, within various brain regions including nucleus LC (Sumners and Phillips, 1983). Thus, central catecholaminergic systems may have developed specialized mechanisms to control catecholamine metabolism, and particularly the translocation of TH protein toward dendritic processes. Our results demonstrate that angiotensins may be one of the key mediators controlling such a mechanism in (nor)adrenergic neurons specifically, while those operating in dopaminergic neurons remain to be elucidated.

Regulating TH protein translocation into LC dendrites: functional relevance

NA released by dendrites requires to be precisely regulated, because it reduces LC neuronal activity (Cedarbaum and Aghajanian, 1976), and hence NA released in terminal fields (Van Gaalen et al., 1997; Mateo et al., 1998, 1999). Thus, controlling TH protein translocation into dendrites may contribute to the local regulation of dendritic synthesis of NA. Here, by comparing control rats to TGR, we show that glia-derived angiotensins play a crucial role in regulating the translocation of

TH protein into dendrites of LC neurons just after weaning. Angiotensin receptors (Nuyt et al., 1999, 2001; Speth et al. 1991) are expressed in the LC early in development, and AOPEN/angiotensins (Mungall et al., 1995) are present during postnatal development in the microenvironment of LC neurons. The activity of the cerebral angiotensinergic system is thus not sufficient before weaning to allow TH protein sorting to dendrites. However, cerebral angiotensins are required after weaning to set the precise timing of TH protein translocation into LC dendrites. The accurate control of dendritic sorting of TH protein in LC neurons before weaning may support hyper-functioning of LC neurons and elevated release of NA in their terminal fields to promote learning, as clearly evidenced in models of infant attachment (Moriceau and Sullivan, 2005).

This study also highlights the importance of studying molecular mechanisms controlling protein transport to dendrites and axons *in vivo*, by taking into account the complexity of interactions that neurons maintain with their neighbouring cells, and astrocytes in particular. Cell culture studies uncovered appealing mechanisms by which angiotensin II modulates the transport of TH protein in neurites of noradrenergic neurons through phosphorylation of MARCKS. Using a rat model with extensive downregulation of cerebral synthesis of angiotensins, we found that these neurohormones participate in the control of TH protein sorting to dendrites only, and not to axons, of LC neurons (Figure 7).

Finally, our results should provide additional insights into a better understanding of psychiatric disorders that have been associated in the past with alterations of either central angiotensinergic (Meira-Lima et al., 2000; Gard, 2002) or noradrenergic system (Iversen, 2000; Berridge and Waterhouse, 2003). These disorders could actually depend on an alteration of the relationship existing between these two systems, and may involve a deficient phosphorylation of MARCKS (Lenox and Watson, 1994; Lenox et al., 1998), and a reduced dendritic translocation of proteins involved in noradrenergic neurotransmission.

Experimental procedures

Animals and tissue preparation

All studies were performed according to the *guiding principles in the care and use of animals* corresponding to the American physiological society guidelines. All TGR rats were obtained from our breeding colony, and control Sprague Dawley rats (mother strain) were purchased from Charles River (France).

For biochemical analysis, brains of rats were removed and fresh-frozen. Then, LC was micro-punched (bilaterally) from 500 μm -thick fresh-frozen brain sections using a 2-mm-diameter needle. For immunohistochemical assays, rats were perfused transcardially with a 4% paraformaldehyde solution prepared in PB 0.1M (pH 7.4). Brains were then removed from the skulls, post-fixed, cryoprotected, frozen in isopentane and stored at -80°C .

Immunohistochemistry

Paraformaldehyde-fixed 25 μm -thick coronal sections were incubated in a mouse monoclonal anti-TH antibody solution diluted at 1:1,000 (Roche Diagnostics), or in a mouse monoclonal anti-MAP2 antibody diluted 1:6,000 (Sigma), or in a rabbit polyclonal anti-GFAP antibody diluted at 1:2,000 (Chemicon). Sections were then incubated in a biotinylated horse anti-mouse IgG antibody solution diluted at 1:1,000 (Vector) for TH and MAP2 detection and in a biotinylated donkey anti-rabbit IgG antibody diluted at 1:1,000 (Jackson Immunoresearch) for GFAP detection. TH, MAP2 and GFAP signals were revealed using a peroxidase-conjugated avidin-biotin complex (Vector) and diaminobenzidine (Sigma). Sections were then dehydrated, defatted and coverslipped.

Dual immunohistofluorescent labelling was used to colocalize TH and the phosphorylated form of MARCKS (P-MARCKS). Anti-TH antibody used was the one cited above. P-MARCKS was detected using a polyclonal antibody raised in goat (Santa Cruz Biotechnology; 1:250), which recognizes Ser159-Ser162 phosphorylated isoform of MARCKS. Both antibodies were added in blocking buffer (2% donkey serum/0.3% Triton X-100 in PBS 0.1M) and incubated with sections at

4°C for 48 h. The secondary antibodies, AlexaFluor 488-conjugated donkey anti-mouse IgG (Molecular Probes, 1:1,000) and Cy3-conjugated rabbit anti-goat IgG (Sigma, 1:300), were added in the same blocking buffer and incubated with sections at 4°C for 24 h. Fluorescence signals were detected with a Leica TCS SP2 confocal system set at wavelengths of 488 nm (excitation) and 497/527 nm (emission) for AlexaFluor 488 (green), and at wavelengths of 532 nm (excitation) and 550/663 nm (emission) for Cy3 (red). Adobe Photoshop 7.0 software (Adobe Systems Incorporated) was used to colocalize from selected pictures both markers on the same section.

Image analysis

Sections immunolabelled for TH protein were digitalized, and the density of TH-immunolabelled elements was quantified using the Leica QWin softwareTM (Leica). For this purpose, sections referred to the same anatomical plane were superimposed using different anatomical landmarks. Once the best overlap was attained, a system of reference, previously described in details (Bezin et al., 1994c), common to these stacked sections, allowed one to memorize the orientation of each section with the others, and to position precisely “measurement boxes”. For each section, a color code was used to highlight the TH-specific immunohistochemical labelling. Then, the software provided the percentage of the surface area of TH-specific labelling within each measurement boxes, as an index of the density of TH-immunopositive elements.

Reverse transcription and PCR amplification

Total RNA extraction was performed using Trizol (Invitrogen). Expand RTTM DNA polymerase (Roche, France) was used to reverse-transcribe total mRNA from LC homogenates using a mix of random and oligo dT (12-18) primers (Invitrogen), and to reverse-transcribe the angiotensinogen antisens transgene RNA (AS-RNA) using a mix containing a reverse specific primer used for PCR amplification (see below; Schinke et al., 1999). PCR amplification was performed on the LightCyclerTM (Roche) with the Quantitect SYBR Green PCR kit (Qiagen). Results for all genes

have been normalized against an external heterologous non-competitive standard (Patent # WO2004.092414). Sequences of the forward and reverse primers used for PCR were respectively: 5' CGC AGG TGC TCT TGC TGT AG 3' and 5' ATA GCT GTG CTT GTC TGG GC 3' for AS RNA; 5' CAC GAC TTC CTG ACT TGG AT 3' and 5' GAG TTC AAG GAG GAT GCT GT 3' for AOPEN mRNA (L00090); 5' CGA AAT CTG GAA TCC GCA TCT 3' and 5' TCA TTG CCG AAC CGG TTT ACT 3' for DBH mRNA (L12407); 5' GGT GCT GAG TAT GTC GTG GA 3' and 5' GCC ATG CCA GTG AGC TTC CC 3' for GAPDH mRNA (X02231.1); 5' TAG CTA CAT CGA GAA GGT CC 3' and 5' AAG AAC TGG ATC TCC TCC TC 3' for GFAP mRNA (RNU03700); 5' CCC AAG AAC CAA CAA GAT GAA 3' and 5' AAT CAA GGC AAG ACA TAG CGA 3' for MAP2 mRNA (NM_013066.1); 5' TTG CTA TGC CTG ATG GTC GTT 3' and 5' AAA TCC AGC TCC CAG GGA AAA 3' for NET mRNA (AB0221970); 5' ACT GTC CGC CCG TGA TTT TC 3' and 5' TCC CCA TTC TGT TTA CAT AGC CC 3' for TH mRNA (M10244). References in parenthesis correspond to the sequence accession number in GenBank.

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Figure legends

Figure 1. Neuromodulatory effects of angiotensins in cultured noradrenergic neurons.

In cultured hypothalamus and brainstem noradrenergic neurons, angiotensin II binds to angiotensin-type 1 receptors (AT1) located at the neuronal plasma membrane, and then stimulates the Ras-Raf-MAP Kinase pathway. This triggers a rise in the expression of the gene encoding TH, following translocation of the complex formed by AT1-Ras-Raf-MAPK to the nucleus of noradrenergic neurons. AT1 stimulation also leads to the activation of the beta subunit of PKC (PKC β) and to the subsequent phosphorylation of MARCKS. Increased presence of phosphorylated form of MARCKS (P-MARCKS) reduces MARCKS-actin crosslinking, which facilitates the translocation of TH protein from perikarya into neuritic extensions of cultured noradrenergic neurons.

Figure 2. Expression of the anti-sense RNA (AS RNA) to AOGEN-mRNA correlates with GFAP-mRNA during the whole postnatal period in TGR(ASrAOGEN) rats.

(A) The transgene (Tg) construct used to specifically downregulate glial AOGEN synthesis is constituted of the promoter sequence of the human GFAP gene (hGFAP prom.) and of the 200 base pairs of the 5' end of the rat AOGEN cDNA sequence, all cloned in reverse sense in a bacterial plasmid.

(B) The anti-sense RNA to AOGEN mRNA is detected in the LC region of transgenic rats during the whole postnatal period, as seen using RT-PCR.

(C) AS RNA expression correlates with the expression of GFAP-mRNA in transgenic rats during postnatal development: $[AS\ RNA] = 0.818 [GFAP\text{-}mRNA] + 0.216$, $r = 0.703$, $P < 0.05$.

Figure 3. TH protein presence into dendrites of LC neurons is altered at 4 weeks in TGR deprived of glia-derived angiotensins.

(A,B) TH immunohistochemical detection reveals the extended presence of TH protein into dendrites of LC neurons laying within the PCA in control rats (CTRL; A). In transgenic rats (TGR, B) reduced dendritic presence of TH is observed within the PCA.

(C-F) No difference in TH protein presence was observed between control rats (CTRL; C,E) and transgenic rats (TGR; D,F) in terminal fields of LC neurons, i.e. the MLFC (double arrow in C,D) and the SGZ (observed between arrowheads and the dashed line in E,F).

(G,H) Distribution of TH protein is similar between control rats (G) and TGR (H) in both the perikaryal area of the substantia nigra (SNc) and in the dendritic field of SN neurons (SNr).

(I) Quantification of the density of TH containing elements in the PCA, MLFC, SGZ and SNr confirms qualitative observations (**P<0.001; n = 3-5; Student *t* test).

Results are the mean \pm SEM. Scale bars : 500 μ m (A,B) ; 100 μ m (C-F and G,H).

Abbreviations: MLFC: molecular layer of the frontal cortex; PCA: pericoerulean area of nucleus LC; PKLC: perikaryal area of nucleus LC; SGZ: sub-granular zone of the dentate gyrus; SN (substantia nigra) pars compacta (SNc) and pars reticulata (SNr).

Figure 4. Decreased presence of TH protein within dendrites of LC neurons is associated with reduced TH gene expression and dendritic absence of phosphorylated MARCKS in TGR.

(A) Messenger RNA encoding TH and other noradrenergic cell markers, such as DBH and NET, are all decreased in the LC of TGR as compared to controls (*P<0.05; n=4-5; Student *t* test).

(B,C) In the perikaryal area of nucleus LC, TH protein is present within both perikarya and processes of noradrenergic neurons in control rats (CTRL; B), while it is quasi-exclusively confined to the perikarya in TGR (C).

(D-I) Dual immunofluorescent labelling of TH (D,G) and the phosphorylated form of MARCKS (P-MARCKS; E,H) reveals that both markers are colocalized (F,I) in perikarya (arrow) and

dendrites (arrowheads) of LC neurons in control rats (CTRL; **D-F**). By contrast, labelling of LC dendrites in transgenic rats was characterized by both the absence of P-MARCKS and the reduced presence of TH (TGR; **G-I**).

Results are the mean \pm SEM. Scale Bars: 80 μ m (**B,C**); 20 μ m (**D-I**).

Figure 5. Phenotypic alterations observed in 4 week-old transgenic rats are specific to noradrenergic neuronal system.

(**A**) At 4 weeks, expression of mRNAs encoding either neuron-specific MAP2 or glia-specific GFAP and AOPEN are similar between transgenic rats (TGR) and controls.

(**B,C**) Immunohistochemical detection of MAP2 in the LC of control rats (CTRL; **B**) and TGR (**C**) shows no difference between both strains of rats in the perikaryal area of the structure (PKLC) and the pericoerulean area (PCA).

(**D,E**) Immunohistochemical detection of GFAP in the LC shows no difference in the morphology of astroglial cells between control rats (**D**) and TGR (**E**).

Results are the mean \pm SEM. Scale bars : 500 μ m (**B,C**) ; 40 μ m (**D,E**).

Figure 6. TH presence is restored in dendrites of LC neurons in adult transgenic rats in absence of the phosphorylated form of MARCKS.

(**A,B**) TH immunohistochemical detection reveals presence of TH protein within dendrites of LC neurons laying in the PCA both in control rats (CTRL; **A**) and transgenic rats (TGR; **B**) at 12 weeks.

(**C-F**) TH protein is present both in controls (**C,E**) and TGR (**D,F**) within axons in terminal fields of LC neurons, i.e. the MLFC (double arrow in **C,D**) and the SGZ (between arrowheads and the dashed line in **E,F**).

(**G**) After quantification with an image analysis system, the density of elements immunolabelled for TH contained within the PCA appears to be similar in both strains of rats at 12 weeks.

(H-M) In the LC of adult rats, the phosphorylated form of P-MARCKS is barely detectable in control rats (I) and transgenic rats (L) within both perikarya (arrow) and dendrites (arrowhead) of noradrenergic neurons.

Results are the mean \pm SEM. Scale Bars: 500 μ m (A,B); 100 μ m (C-F); 20 μ m (H-M).

Figure 7. Glia-derived angiotensins control the expression and trigger translocation of TH into dendrites of brainstem (nor)adrenergic neurons at critical stages of development.

Angiotensinogen is synthesized and released by astrocytes, and angiotensins are formed within the extracellular compartment through successive degradations of angiotensinogen. After weaning, glia-derived angiotensins control the presence of TH protein within dendritic but not axonal processes of brainstem (nor)adrenergic neurons by regulating both the expression of TH mRNA and the intracellular trafficking of the protein. Both mechanisms require previous activation of angiotensin receptors (AT), expressed at the plasma membrane of noradrenergic neurons. Glia-derived angiotensins control the expression of TH mRNA, likely by recruiting the Ras-Raf-MAPK intracellular pathway, and control TH protein translocation into dendrites by stimulating the phosphorylation of MARCKS through the beta subtype of PKC (PKC β). TH protein translocation into axons of brainstem (nor)adrenergic neurons is regulated by other factors that remain to be determined. At the adult stage, other factors than glia-derived angiotensins are involved in the regulation of TH phenotype in noradrenergic neurons, since the expression of TH mRNA and TH protein translocation into dendrites are restored in angiotensinogen-depleted rats.

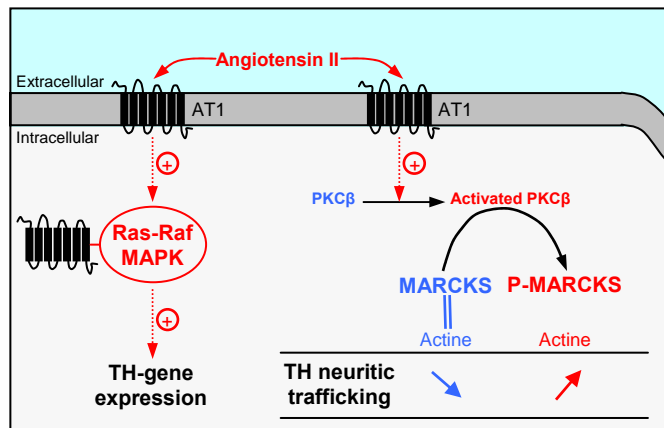


Figure 1

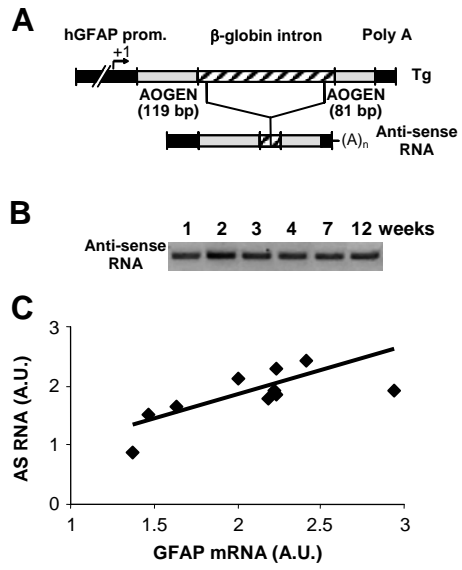


Figure 2

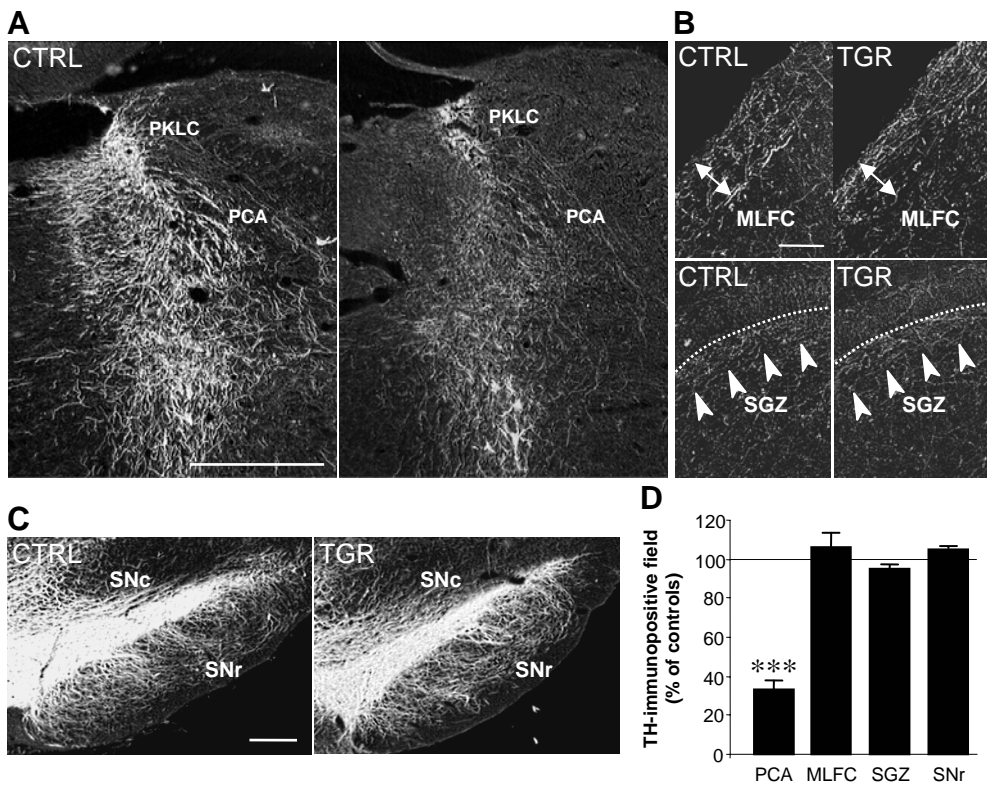


Figure 3

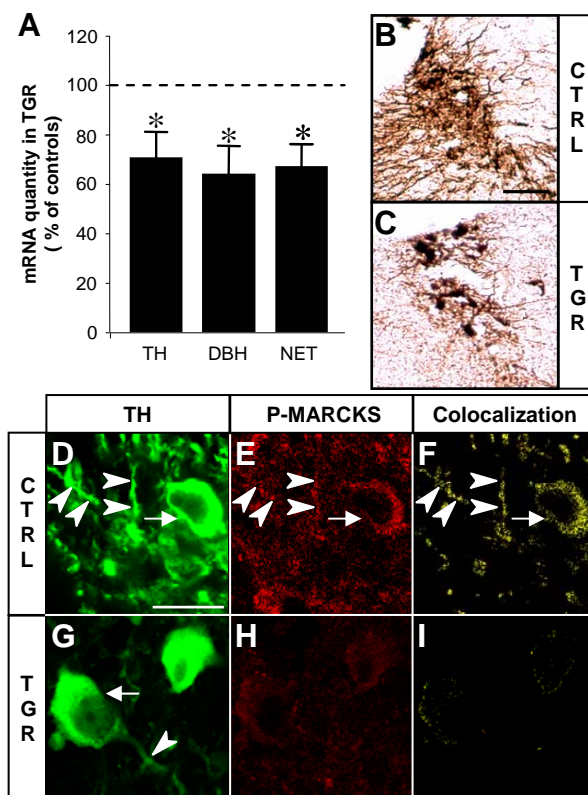


Figure 4

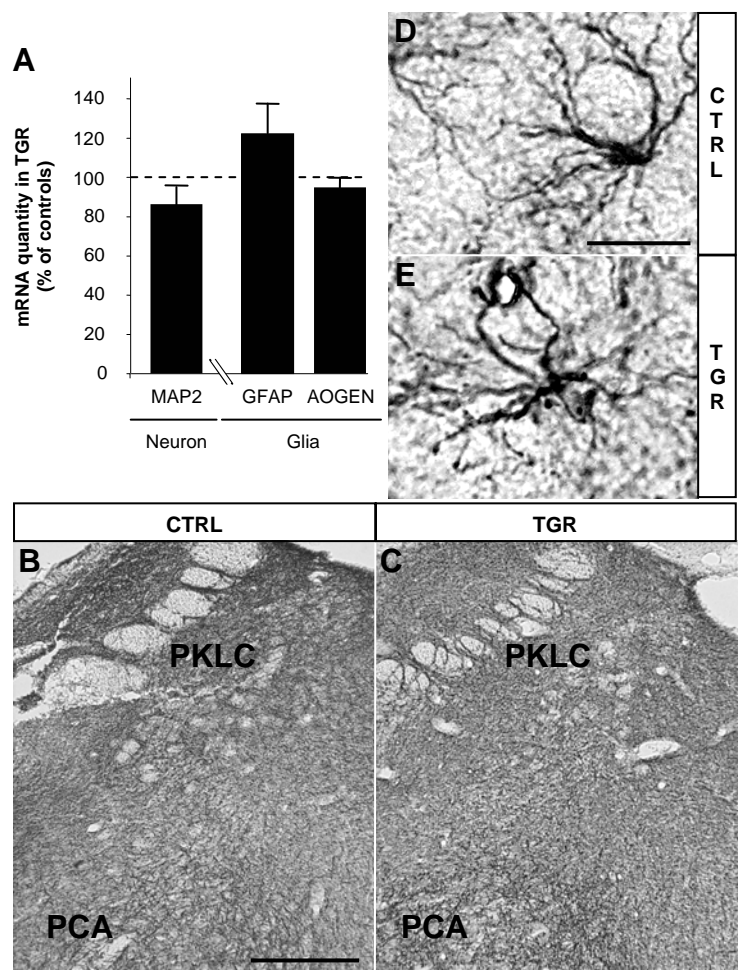


Figure 5

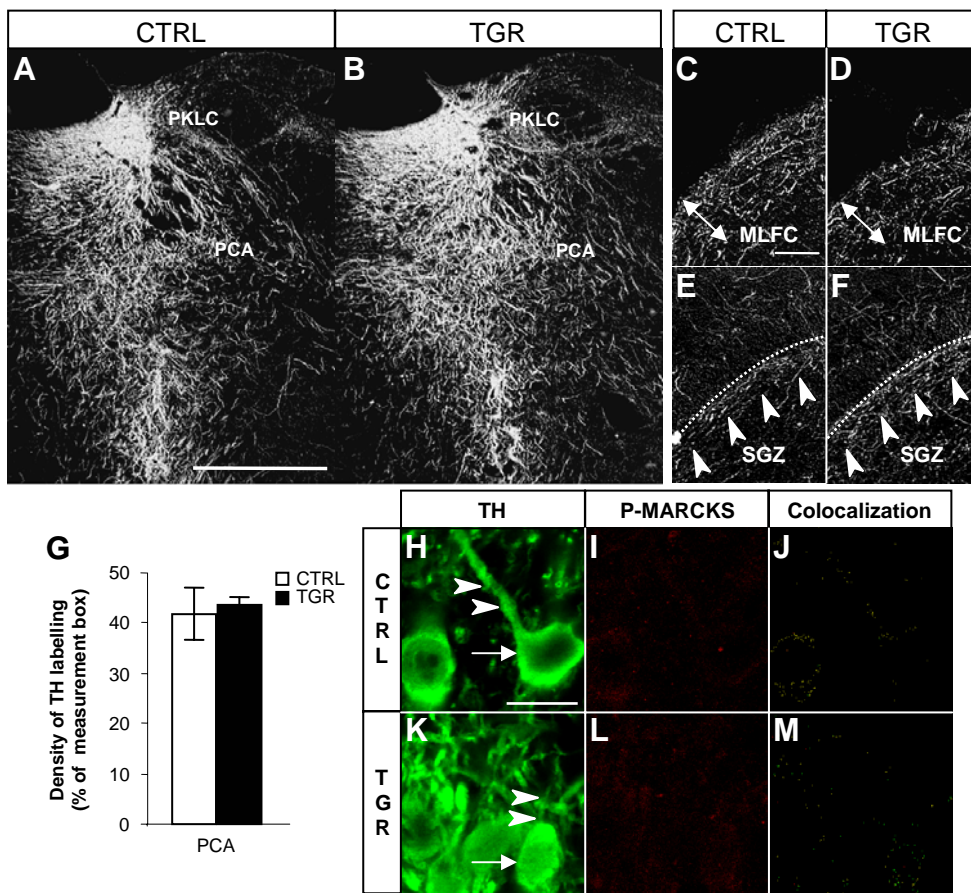


Figure 6

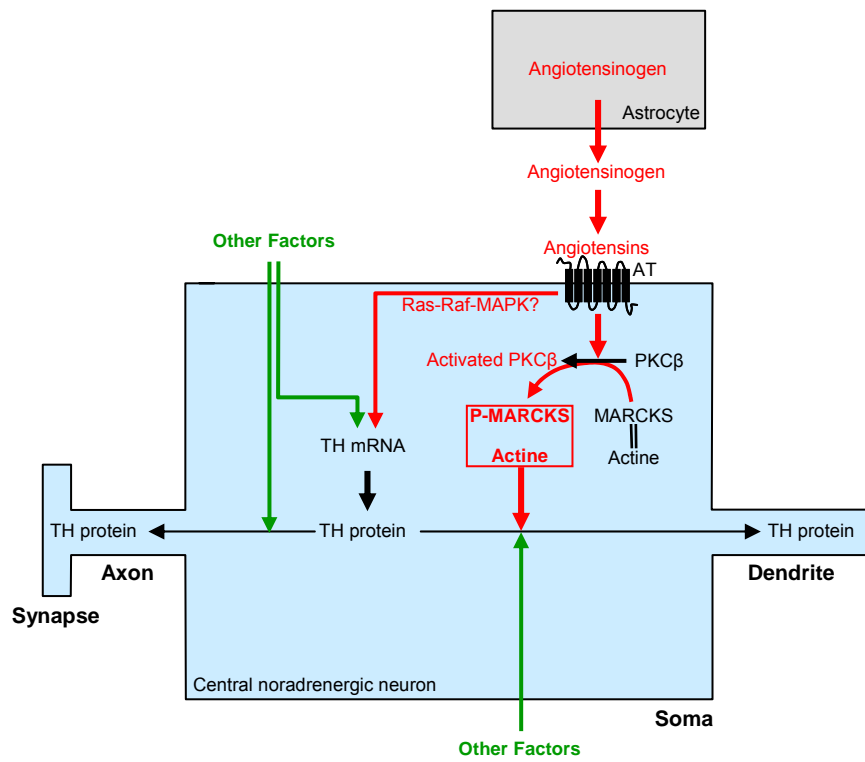


Figure 7