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Antihypertensive drug Valsartan promotes dendritic spine density by altering AMPA receptor trafficking

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Abstract

Recent studies demonstrated that the antihypertensive drug Valsartan improved spatial and episodic memory in mouse models of Alzheimer's Disease (AD) and human subjects with hypertension. However, the molecular mechanism by which Valsartan can regulate cognitive function is still unknown. Here, we investigated the effect of Valsartan on dendritic spine formation in primary hippocampal neurons, which is correlated with learning and memory. Interestingly, we found that Valsartan promotes spinogenesis in developing and mature neurons. In addition, we found that Valsartan increases the puncta number of PSD-95 and trends toward an increase in the puncta number of synaptophysin. Moreover, Valsartan increased the cell surface levels of AMPA receptors and selectively altered the levels of spinogenesis-related proteins, including CaMKII and phospho-CDK5. These data suggest that Valsartan may promote spinogenesis by enhancing AMPA receptor trafficking and synaptic plasticity signaling.

Keywords

Valsartan; CaMKII ; AMPA receptor; spinogenesis

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Authors' contributions

HSH designed and performed the experiments, and wrote the manuscript. YS, NL, and AC analyzed the data, generated the figures, and wrote the manuscript. DTSP, JS, RST supplied reagents and wrote the manuscript.

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1. Introduction

Hypertension is the abnormal state of high blood pressure, and it is one of the most common risk factors for cardiovascular disease [1]. Although the pathogenesis of this disorder is not yet fully understood, it is known that the renin-angiotensin system (RAS) has a key role in regulating blood pressure, and that irregular activation of RAS leads to hypertension and cardiovascular morbidity [2]. Interaction between angiotensin II (AII) and AII type 1 receptors (AT1) in the kidney can be a cause of hypertension [2]. It has also been reported that mice with an overexpression of angiotensinogen and a high salt diet developed hypertension [3]. Thus, an inhibitor of the angiotensin receptor may be a useful drug for the prevention or treatment of hypertension. To support this idea, treatments that act as inhibitors of AT1 receptors for hypertension have been developed and synthesized. For example, Valsartan, an angiotensin receptor blocker (ARB), has been found to reduce hypertension [4]. Candesartan is another type of ARB that has been shown to effectively reduce the adverse effects of hypertension when used in combination with dihydropyridine calcium channel blockers [5]. Telmesartan has been shown to decrease blood levels in mice with hypertension induced by high-fat diets [6]. As previous studies have shown, preventing the action of AII by blocking its AT1 receptor effectively lowers blood pressure and serves as a treatment for hypertension [4].

Several recent reports have found that hypertension is a significant risk factor for dementia [4]. A 15-year longitudinal study revealed that there is an increased risk for Alzheimer's Disease (AD) for 70-year-old subjects with high blood pressure, possibly due to the formation of white matter lesions in brain by high blood pressure [7]. Furthermore, a strong relation was determined between high blood pressure at middle age and the development of AD at late age [8], whereas receiving antihypertensive treatment consisting of enalapril and/or hydrochlorothiazide is correlated with reduced risk of developing dementia [9]. Interestingly, Valsartan also decreases A β levels and AD pathology and increases spatial memory in a mouse model of AD (the Tg2576 line) [10]. Therefore, these data suggest that ARBs, such as Valsartan, may have therapeutic benefit for individuals with or at risk for AD.

An important unanswered question is the mechanism by which Valsartan improves spatial learning in Tg2576 mice. We hypothesized that this effect occurs by regulating the number of dendritic spines, the primary sites of excitatory synaptic transmission in the CNS. Here, we found that Valsartan-treated primary hippocampal neurons exhibited significantly increased dendritic spine density at developing and mature stages. Additionally, Valsartan boosted the number of excitatory synapses and elevated the cell surface levels of AMPA receptors. Moreover, Valsartan selectively increased the levels of the kinases CaMKII and phosphorylated CDK5 (p-CDK5). Overall, our data suggest that Valsartan may promote dendritic spine formation by enhancement of surface AMPA receptors as well as spinogenesis-related signaling pathways.

2. Materials and methods

2.1. Primary hippocampal neuronal culture and transfection

Primary hippocampal neurons from Sprague–Dawley rats at embryonic day 19 (E19) were cultured at 150 cells/mm². Primary hippocampal neurons were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) with GFP for 24 hours and treated with Valsartan (10 μ M) or vehicle (1% DMSO) for another 24 hours.

2.2. Live cell surface immunostaining

To measure the cell surface levels of AMPA receptors, we conducted live cell surface staining. Briefly, live primary hippocampal neurons were incubated with N-terminal directed GluA1 or GluA2 antibodies (10 µg/mL in conditioned medium) for 10 minutes at 37°C, and then briefly fixed in 4% paraformaldehyde (non-permeabilizing conditions) for 5 minutes. Surface-labeled GluA1 or GluA2 were detected with Alexa fluor-555 secondary antibodies. After surface staining, the cells were permeabilized in methanol (−20°C, 90 s), followed by incubation with anti-GFP antibody to identify transfected neurons.

2.3. Immunostaining

To measure synaptic protein expression profiles, which are associated with dendritic spine formation or retardation, we conducted immunostaining in primary hippocampal neurons by treating them with Valsartan or vehicle for 24 hours. Afterwards, the following antibodies were used: mouse anti-GFP (Novus Biologicals, 9F9.F9), rabbit anti-GFP (Invitrogen, A11122), rabbit anti-GluA1 (Calbiochem, PC246), mouse anti-GluA2 (BD Pharmagen, 556341), mouse anti-GluN1, rabbit anti-GluN2A, mouse GluN2B, mouse anti-postsynaptic density (PSD)-95 (NeuroMabs, Davis, CA, USA), mouse anti-synaptophysin (Sigma Aldrich, s5768), anti-RasGRF1 (Santa Cruz, C-20; BD Biosciences, 610149), rabbit anti-SynGAP, mouse anti-CaMKII, rabbit anti-CaMKII, anti-p-ERK1/2 (Invitrogen, 36880), anti-p-CREB (Millipore, 06-519), rabbit anti-p-JNK (Cell signaling, #9251S), rabbit anti-p-AKT (cell signaling, #9271S), rabbit anti-p-CDK5 (Abcam, ab63550), and rabbit anti-PKC (Abcam, ab19031). Images were acquired by LSM 510 laser scanning confocal microscope (Zeiss) and entire dendrite segments were analyzed using Image-J software (Universal Imaging Corporation, Downingtown, PA, USA). To measure the puncta number of synaptophysin and PSD-95, we used MetaMorph software, and we used Scion Image software to measure dendritic spine density.

2.4. Statistical analyses

All data were analyzed with Graphpad Prism 4 software using either a 2-tailed t-test or ANOVA with Tukey's post-hoc test for multiple comparisons, with significance determined at $p < 0.05$. Cumulative distribution plots were analyzed using the Kolmogorov-Smirnov test. Descriptive statistics were calculated with StatView 4.1 and expressed as mean $\pm$ S.E.M.

3. Results

3.1. Valsartan increases dendritic spine density in primary hippocampal neurons

A recent study demonstrated that Valsartan improves spatial memory in a mouse model of AD. To test whether Valsartan affects dendritic spine density, which is correlated with learning and memory, primary hippocampal neurons were transfected with GFP (to visualize dendrite segments and spines) and treated with different doses of Valsartan (1 µM, 5 µM, or 10 µM) or vehicle (1% DMSO) for 24 hours at day in vitro (DIV) 21, representing a relatively mature stage of neuronal development. Interestingly, we found that Valsartan (5 and 10 µM) caused significantly increased dendritic spine number (CTRL $n=25$, Val 1 µM $n=22$, 5 µM $n=22$, 10 µM $n=22$, $*p < 0.05$, $***p < 0.001$, Fig. 1A and B). We also examined whether Valsartan can alter dendritic spine density in younger neurons during the stage of peak synaptogenesis. To test this idea, primary hippocampal neurons (DIV14) were transfected with GFP and treated with Valsartan (10 µM) or vehicle (1% DMSO) for 24 hours. Again, Valsartan significantly increased dendritic spine density (CTRL $n=25$, Val $n=27$, $**p < 0.01$, Fig. 1C and D). These data suggest that Valsartan can enhance dendritic spine density of primary hippocampal neurons in mature stages as well as during periods of active spinogenesis.

3.2. Valsartan increases the puncta number of PSD-95

To test whether Valsartan regulates the number of excitatory synapses, primary hippocampal neurons were transfected with GFP and treated with Valsartan (10 μ M) or vehicle (1% DMSO) for 24 hours. We used the 10 μ M dosage of Valsartan because it showed the most significant change in dendritic spine formation (Fig. 1). After 24 hours, neurons were immunostained for the presynaptic and postsynaptic markers synaptophysin or PSD-95, respectively (Fig. 2). We found that Valsartan did not alter expression levels of synaptophysin (CTRL n=13, Val n=13) but trended towards an increase in puncta number of synaptophysin (CTRL n=12, Val n=18, Fig. 2A, B, and C). Additionally, Valsartan increased the levels of PSD-95 (CTRL n=14, Val n=14) and significantly increased PSD-95 puncta number (CTRL n=18, Val n=27, * p <0.05, Fig. 2D, E, and F). These data suggest that Valsartan increases excitatory synapse number in primary hippocampal neurons.

3.3. Valsartan selectively regulates expression of NMDA and AMPA receptors

It is well known that NMDA and AMPA receptors are involved in synaptic strength and plasticity. Therefore, we first examined whether Valsartan can regulate NMDA receptor subunit expression levels. To address this question, we transfected primary hippocampal neurons with GFP and treated with Valsartan (10 μ M) or vehicle (1% DMSO) for 24 hours. We then immunostained neurons against GluN1, GluN2A, or GluN2B. Valsartan selectively increased protein expression levels of GluN2A (CTRL n= 6, Val n=14, *** p <0.001) and GluN2B (CTRL n=8, Val n=17, *** p <0.001) without altering the levels of the GluN1 subunit (CTRL n=8, Val n=15, Fig 3A–F). These data suggest that Valsartan can regulate the expression levels of specific NMDA receptor subunits.

We next examined whether Valsartan can alter the cell surface and total levels of AMPA receptor subunits, specifically GluA1 and GluA2. For this experiment, primary hippocampal neurons were transfected with GFP, treated with Valsartan (10 μ M) or vehicle (1% DMSO) for 24 hours, then subjected to live cell surface staining under non-permeabilized conditions with N-terminal directed anti-GluA1 and anti-GluA2 antibodies. We found that Valsartan significantly increased the cell surface levels of both GluA1 (CTRL n=16, Val n=20, * p <0.05) and GluA2 (CTRL n=11, Val n=19, * p <0.05, Fig. 3G, H, K, and L). By performing total immunostaining of neurons with anti-GluA1 and anti-GluA2 under permeabilized conditions, we found that the total level of GluA2 was increased (CTRL n=10, Val n=19, *** p <0.001), but the total level of GluA1 was not altered (CTRL n=9, Val n=25, Fig 3I, J, M, and N). These data suggest that Valsartan can also selectively affect trafficking and expression levels of specific AMPA receptor subunits.

3.4. Valsartan increases the levels of CaMKII α and p-CDK5

In the previous figures, we demonstrated that Valsartan increases spine density, which is accompanied by alterations in NMDA and AMPA receptor subunit expression levels and surface availability. We then further examined whether Valsartan can regulate other synaptic proteins which are involved in dendritic spine formation or maintenance. We initially examined the expression profiles of Ras signaling proteins because Ras signaling is well known to be involved in spinogenesis [11] as well as neurodegeneration. However, we observed that Valsartan altered neither upstream nor downstream Ras signaling protein levels, including RasGRF1 (CTRL n=7, Val n=12), p-ERK (CTRL n=8, Val n=9), and p-CREB (CTRL n=13, Val n=13, Fig. 4A–F). In contrast, we found that Valsartan significantly increased the levels of CaMKII (CTRL n=15, Val n=15, *** p <0.001), which has been reported to be an important protein for spinogenesis [12], but CaMKII levels were not altered (CTRL n=12, Val n=12, Fig. 4G–J). Additionally, we examined the effects of Valsartan on Rap signaling proteins, which are involved in long term depression [13]. We found that Valsartan-treated neurons were significantly decreased in p-JNK (CTRL n=7, Val

n=10, * $p<0.05$), but the levels of p-AKT were not changed, compared to vehicle treatment (CTRL n=13, Val n=18, Fig. 4K–N). Moreover, Valsartan had significantly increased p-CDK5 (CTRL n=10, Val n=11, ** $p<0.01$) and PKC levels (CTRL n=8, Val n=12, *** $p<0.001$), without altering the total levels of CDK5 (CTRL n=7, Val n=8, Fig. 4O–T). These data suggest that Valsartan may promote dendritic spine formation by selectively altering the levels of certain synaptic proteins.

4. Discussion

We demonstrated for the first time that the antihypertensive drug, Valsartan, promotes dendritic spine density in primary hippocampal neurons. We also found that Valsartan selectively regulates the levels of NMDA receptor subunits and increases the cell surface levels of AMPA receptor subunit GluA1 and GluA2. Moreover, Valsartan specifically altered the levels of CaMKII and p-CDK5, which are spinogenesis-related proteins, but not those of Ras signaling proteins examined. This data suggest that Valsartan may regulate dendritic spine formation by altering AMPA receptor trafficking and/or specific spinogenesis-related proteins.

Recent studies have suggested that hypertension may be associated with dementia and with AD. For instance, hypertension may be regulated by the Receptor for Advanced Glycation End products (RAGE) pathway [14, 15]. RAGE mediates the transportation and accumulation of A β [16], which is well known to be a characteristic of AD [17], in the brain. In support of these findings, RAGE knockout (KO) mice exhibit decreased hypertension-induced A β deposition and were rescued from learning and memory impairment [14]. Other studies have found that AD patients with hypertension are more likely to exhibit a faster rate of cognitive decline [18, 19, 20]. These data suggest that there may be a shared pathology and molecular pathway of both diseases, implicating a potential cause-and-effect relationship between hypertension and AD.

A previous study has shown that Valsartan decreases A β levels, resulting in improved spatial memory in a mouse model of AD, possibly by the promotion of A β “sinking” from the brain to the periphery [10]. However, it is unknown how Valsartan can improve cognitive function or memory. To investigate this issue, we examined whether Valsartan could affect dendritic spine formation, which is deterministic of cognitive function, and found that Valsartan promotes spinogenesis *in vitro*. Based on our findings, we hypothesized that Valsartan increased dendritic spine density by increasing the number of excitatory synapses. We measured the puncta number of synaptophysin and PSD-95 because it is well known that PSD-95 is involved in dendritic spine formation at the postsynaptic junction [21] and that synaptophysin is a presynaptic marker related to cognitive function [22]. Interestingly, we observed that Valsartan increased the puncta number of PSD-95, and trended toward an increase in synaptophysin puncta. These data suggest that increased spinogenesis may lead to a greater number of functional synapses.

Here, we found that Valsartan selectively affects the total levels of NMDA receptor subunits GluN2A/B. It is possible that Valsartan may differentially affect the cell surface levels of NMDA receptors as well. However, in the present study, we were unable to examine the effects of Valsartan on NMDA receptor trafficking due to the unavailability of specific antibodies that can detect their cell surface expression levels. Further studies are required to address this question. Interestingly, we also found that Valsartan increases cell surface levels of AMPA receptor subunits GluA1 and GluA2, which are involved in spinogenesis [23] and synaptic plasticity [24]. Taken together, our data suggest that Valsartan may affect spine development through selectively regulating NMDA and AMPA receptor subunits.

How does Valsartan affect spinogenesis? One possible mechanism may be that Valsartan inhibits the AT1 receptor to mediate spine formation. Specifically, it has been found that blockade of AT1 receptor enhances memory by preventing the activation of AII [25], which has been found to cause memory impairment in rats [26]. Because enhanced memory may be indicative of greater synaptic density and dendritic spine growth in the hippocampus, it is possible that Valsartan, which is an ARB, promotes dendritic spine formation by blocking the AT1 receptor.

Another possible mechanism is that Valsartan may regulate spinogenesis-related proteins and/or their distribution from the dendritic shaft to dendritic spines. Interestingly, in our study, we found that Valsartan significantly increased CaMKII expression. Because CaMKII is known to induce LTP by driving surface delivery of GluA1 [27], Valsartan upregulation of CaMKII would affect AMPA receptor trafficking and alter spinogenesis, consistent with our results. Similarly, PKC is thought to be involved in LTP as well as direct phosphorylation of NMDA and AMPA receptor subunits [28], and increased PKC expression observed here could additively contribute to the effects on dendritic spines as well as changes in NMDA and AMPA receptor levels. We also observed decreased p-JNK, suggesting that downregulation of Rap-related signaling pathways implicated in synaptic depression [29] could also lead to enhanced spine density and AMPA receptor surface expression by Valsartan.

Lastly, Valsartan may promote dendritic spine density through phosphorylation and activation of CDK5, which is known to enhance synaptogenesis by phosphorylating and regulating the distribution of PSD-95 [30] and other synaptic proteins such as CASK [31]. In our experiments, Valsartan significantly increased phosphorylated but not total levels of CDK5, which suggests that Valsartan plays a role in CDK5 activation and thereby promotes dendritic spine formation. Further studies are required to fully dissect the multiple molecular mechanisms involved in Valsartan-mediated spinogenesis.

Taken together, our data shed light on a potential therapeutic strategy for neurodegenerative diseases, including AD, by augmenting synaptic potentiation and inducing dendritic spine formation via antihypertensive medication.

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Highlights

- Valsartan increases dendritic spine density in primary hippocampal neurons
- Valsartan increases the puncta number of postsynaptic scaffold PSD-95
- Valsartan selectively regulates expression of NMDA and AMPA receptor subunits
- Valsartan upregulates synaptic signaling molecules CaMKII and phospho-CDK5

A DIV21

B

C DIV14

D

Fig. 1.

Valsartan increases dendritic spine density in primary hippocampal neurons. (A) Neurons (DIV21) were transfected with GFP and were treated with different doses of Valsartan (1 μM, 5 μM, or 10 μM) or vehicle (1% DMSO) for 24 hours. (B) Dendritic spine densities from (A) are presented. (C) Primary hippocampal neurons (DIV14) were transfected with GFP, and were treated with Valsartan (10 μM) or vehicle (1% DMSO) for 24 hours. (D) Dendritic spine densities from (C) are shown. Error bars represent S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 2.

Valsartan increases the puncta number of synaptophysin and PSD-95. (A–C) Primary hippocampal neurons (DIV21) were transfected with GFP and treated with Valsartan (10 μM) or vehicle (1% DMSO) for 24 hours. After 24 hours, immunostaining was conducted with anti-synaptophysin, and protein levels of synaptophysin and puncta number were measured. (B) Protein levels of synaptophysin from (A). (C) Puncta number of synaptophysin from (A). (D–F) Primary hippocampal neurons (DIV21) were transfected with GFP and treated with Valsartan (10 μM) or vehicle (1% DMSO) for 24 hours. After 24 hours, cells were immunostained with anti-PSD-95, and protein levels of PSD-95 and puncta

number were measured. (E) Protein expression level for PSD-95 from (D) is shown. (F) Puncta number of PSD-95 from (D). Error bars represent S.E.M. * $p < 0.05$.

Fig. 3.

Valsartan alters the cell surface levels of AMPA receptors. (A–F) Primary hippocampal neurons (DIV21) were transfected with GFP and treated with Valsartan (10 μ M) or vehicle (1% DMSO) for 24 hours. Neurons were then immunostained with GluN1, GluN2A, or GluN2B antibodies respectively, and total protein levels were measured. (B, D, F) Quantification of protein levels of GluN1, GluN2A, and GluN2B. (G, H, K, L) Primary hippocampal neurons (DIV21) were transfected with GFP, and were treated with Valsartan (10 μ M) or vehicle (1% DMSO) for 24 hours. Live cell surface staining was conducted with N-terminal anti-GluA1 or anti-GluA2 antibodies, and the cell surface levels were measured. (H, L) Quantification of cell surface levels of GluA1 and GluA2. (I, J, M, N) Primary

hippocampal neurons (DIV21) were transfected with GFP and treated with Valsartan (10 μ M) or vehicle (1% DMSO) for 24 hours. Immunostaining with GluA1 or GluA2 antibodies were conducted and their total protein levels were measured. (J, N) Total levels of GluA1 and GluA2 are shown. Error bars represent S.E.M. * $p < 0.05$, *** $p < 0.001$.

Fig. 4.

Valsartan regulates the levels of CaMKII and p-CDK5. Primary hippocampal neurons (DIV21) were transfected with GFP and treated with Valsartan (10 μ M) or vehicle (1% DMSO) for 24 hours. (A–F) Neurons were immunostained using RasGRF1, p-ERK, or p-CREB antibodies as shown. (B, D, F) Protein expression levels of RasGRF1, p-ERK, and p-CREB. (G–J) Neurons (DIV21) were transfected with GFP and treated with Valsartan (10 μ M) or vehicle (1% DMSO) for 24 hours. Neurons were immunostained with CaMKII or CaMKII antibodies. (H, J) Protein expression levels of CaMKII and CaMKII. (K–T) Neurons were immunostained with p-JNK, p-AKT, p-CDK5, CDK5, or PKC antibodies. (L,

N, P, R, T) Levels of p-JNK, p-AKT, p-CDK5, CDK5, and PKC are shown. Error bars represent S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.