DISSERTATION

ANGIOTENSIN-II SIGNALING IN THE PARS RETICULATA GABA-ERGIC NEURONS IN THE SUBSTANTIA NIGRA AND ITS IMPLICATIONS IN NIGRAL NEUROTRANSMISSION

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ABSTRACT

ANGIOTENSIN-II SIGNALING IN THE PARS RETICULATA GABA-ERGIC IN THE SUBSTANTIA NIGRA AND ITS IMPLICATIONS IN NIGRAL NEUROTRANSMISSION

Renin-Angiotensin-system is one of the most widely studied hormonal systems in the peripheral system and is primarily associated with the essential function of regulating blood pressure, fluid and electrolyte balance in the body. Most of the drugs used to treat hypertension currently are targeted towards one or more components of the RAS system. However, increasing studies have presented evidence of local RAS in tissues completely independent of the humoral system. In the CNS, in addition to highly vascularized areas in the brain lacking the blood-brain-barrier (BBB) such as the circumventricular organs, all RAS components have also been found in the brain regions inside the BBB and are suspected to be involved in neuronal differentiation, neurotransmission, and learning and memory. Increasing studies have reported the interaction of brain RAS with pathophysiological mechanisms of many neurological and psychiatric illnesses. However, this extrarenal effect of RAS is only beginning to gain some scientific attention, and the underlying mechanisms are far from elucidated.

All the RAS components are strongly expressed in the midbrain, especially the substantia nigra. Accumulating evidence in recent years has implicated Angiotensin-II (Ang-II), the primary effector peptide of RAS, in the selective degeneration of dopaminergic neurons in the substantia nigra compacta (SNc) in animal models of Parkinson's disease. Ang-II is believed to induce G-protein signaling through Ang-II type 1 receptor (AT1-R) and increase cellular oxidative stress, intracellular calcium load and activate apoptotic pathways in SNc dopaminergic

neurons. Interestingly, studies have also shown Ang-II mediated striatal dopamine release in rats. These studies suggest that Ang-II signaling can induce both intracellular effects and influence dopaminergic neuronal output in the midbrain. However, if Ang-II signaling exists in other neuronal cell types in the substantia nigra is not known.

Substantia nigra is comprised of two primary cell types: dopaminergic and GABAergic neurons. The majority of dopaminergic neurons are located in the SNc, and the SNr is comprised of GABAergic projection neurons with few interspersed dopaminergic neurons. Besides being one of the major output neurons of basal ganglia, SNr GABAergic projection neurons also provide significant inhibitory input to the neighboring SNc dopaminergic neurons, not through a direct axonal projection like its other target areas but via its extensive network of axon collaterals. Inhibitory input from the SNr GABAergic neurons contributes to the essential balance between afferent excitatory and inhibitory inputs to SNc dopaminergic neurons that tightly regulates their cellular activity and output. Indeed, SNr GABAergic neurons are necessary for the voluntary control of movement and are implicated in basal ganglia dysfunctions associated with movement disorders such as Parkinson's disease. RAS components are also expressed in the SNr GABAergic neurons, but it is not known if Ang-II signaling exists in these cells and what effects it may have on intranigral neurotransmission and dopaminergic cell activity.

Here we used a combination of electrophysiology, imaging, and optogenetics to characterize and investigate the role of Ang-II in local neurotransmission in the substantia nigra. We found a heterogeneous effect of Ang-II in the nigral dopaminergic and GABAergic neurons. Ang-II suppressed both electrically and light-evoked activity of SNr GABAergic neurons through a combination of mechanisms: enhancement of postsynaptic GABAa receptors and

increasing the action potential duration. On the contrary, Ang-II had no noticeable direct effect on the activity of SNc dopaminergic neurons and its GABAa receptors. This provides the first evidence of novel Ang-II signaling in SNr GABAergic neurons and its heterogeneous effect in the two nigral cell types. Interestingly, in contrast to observed suppression of SNr GABAergic neuronal activity by Ang-II, under phasic photoactivation of SNr GABAergic neurons, Ang-II enhanced the feedforward inhibitory input to SNc dopaminergic neurons. This shows a non-linear effect of Ang-II on population output of nigral GABAergic neurons and may indicate the involvement of an intricate intranigral network formed by the axon collaterals of SNr GABAergic neurons that can further modulate its effect on postsynaptic targets.

Taken together, studies in this dissertation provide the first evidence of novel Ang-II signaling in the nigral GABAergic neurons, that is heterogeneous in effect than the neighboring nigral dopaminergic neurons and regulates intranigral neurotransmission crucial in normal physiology and behavior.

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BRAIN ANGIOTENSIN-II IN CARDIOVASCULAR HOMEOSTASIS, NEURODEGENERATIVE DISORDERS, AND NEUROTRANSMISSION

1.1 Introduction

Renin-angiotensin system (1) is one of the most widely studied humoral systems and its importance in cardiovascular physiology in normal health and disease is well established. However, with the discovery of extrarenal local RAS in different tissues, including the brain, it is now accepted that local RAS is independent of the circulating hormonal system and is involved in the central control of fluid, electrolyte, and cardiovascular homeostasis. Furthermore, numerous studies have indicated that brain RAS may have pleiotropic effects outside of its classical role in electrolyte homeostasis, body fluid volume regulation, and control of cardiovascular physiology in the peripheral circulation.

In the brain, RAS components are expressed both in the circumventricular organs lacking the blood-brain-barrier (BBB) and in brain regions guarded by the BBB, suggesting an autonomous RAS system independent of the circulation. This widespread expression indicates that central RAS may have a more complicated and heterogeneous effect in the brain that is site-specific and dependent on selective receptors' interaction. Not surprisingly, accumulating evidence has suggested a myriad of Angiotensin-II (Ang-II) effects, the primary effector peptide of RAS, in the brain ranging from neuronal degradation, neuroprotection, neuronal differentiation, neurotransmission to learning & memory. Interestingly, many studies in recent years have demonstrated the role of Ang-II in neurodegenerative disorders such as Alzheimer’s disease, Huntington’s disease, multiple sclerosis, and Parkinson’s disease (PD). Ang-II mediates

its neurodegenerative effects through various mechanisms that included increasing cellular oxidative stress, triggering inflammatory pathways through its actions on glia and astrocytes, and activating apoptotic pathways that collectively contribute to neurodegeneration.

However, as mentioned above, many studies have shown that Ang-II also plays a role in neurotransmission in different neurons, but it is not clear how this effect of Ang-II correlates with its neurodegenerative effects. The first chapter of this dissertation provides a brief introduction of Ang-II's role in cardiovascular physiology in survival and aging and its potential role in age-related cognitive decline. Then a concise review of the role of Ang-II/RAS in different neurodegenerative disorders is provided. Furthermore, this chapter discusses Ang-II's effect in modulating the excitability and activity of neurons in different brain regions and its subsequent physiological response. Lastly, the chapter concludes with specific hypotheses that is addressed in the following chapters.

1.2 Humoral Angiotensin-II and Cardiovascular Aging.

RAS is an endocrine system that regulates cardiovascular homeostasis essential for survival. In response to reduced renal blood flow, the kidney secretes renin which proteolytically degrades angiotensinogen to a precursor protein Angiotensin I. Angiotensin I is subsequently converted to peptide hormone Angiotensin-II (Ang-II), which is the primary effector peptide of RAS. Physiologically, through its action on Ang-II type-1 receptors (AT1-R), Ang-II stimulates retention of salt and water and maintains contraction of blood vessels to regulate peripheral blood pressure. This role of Ang-II has been critical in the survival of our ancestors, who, according to historical and archaeological accounts, engaged in long periods of organized

hunting, which enhanced the risk of dehydration and low blood pressure. Not surprisingly, this essential function of Ang-II has made it and the associated proteins the primary target of most antihypertensive therapy in modern medicine (2, 3).

However, this essential function of Ang-II also poses a challenge in terms of healthy aging. Unlike our ancestors, the life span of homo sapiens has more than doubled. The influence of essential RAS, designed to support healthy cardiovascular function for a much shorter period, now has to function optimally for additional 40-50 more years and support cardiovascular homeostasis throughout aging. Through substantive clinical evidence over the years, it is now well accepted that RAS is more detrimental in old age, and as a result, cardiovascular disease is one of the leading causes of mortality and morbidity in modern society. In support of this observation, accumulating evidence from both clinical and experimental studies has shown Ang-II via AT1-R mediated acceleration of biological aging (4).

Consequently, Angiotensin converting enzyme (ACE)inhibitors and AT1-R blockers have been clinically effective in slowing the development of age-related cardiovascular and metabolic diseases. Interestingly, AT1-R blockers also seem to be protective against age-related brain disorders such as dementia, Alzheimer's, and Parkinson's disease and improve cognitive function (5-13). This dual effect of antagonism of Ang-II in cardiovascular physiology and cognition suggests that extra-renal brain RAS's pathological axis may share a common mechanism with the peripheral effects of circulating RAS. However, Ang-II's central effects are still poorly understood, and its effects in neurons where RAS components are expressed remain to be clarified.

1.3 Brain Ang-II and Neurodegenerative Disorders.

1.3a Parkinson's Disease

RAS components are expressed in the basal ganglia, especially in the nigrostriatal system of rodents and primates, including humans. The primary receptors of Ang-II, AT1-R and AT2-R, are expressed not only in the nigral neurons but also in the glia and astrocytes (14). Furthermore, other precursor proteins for Ang-II production, such as angiotensinogen and prorenin, have been located in the neurons and glia/astrocytes of the midbrain (15). Studies have shown that Ang-II through its inflammatory receptor AT1-R activates NADPH oxidase, located in the dopaminergic neurons as well as in the surrounding glia, and increases cellular oxidative stress and neuroinflammation. In MPTP and 6-OHDA models of Parkinson's disease, enhanced activity of AT1-R activity is suggested to exacerbate dopaminergic cell degeneration through an increase in multiple cellular factors such as increased intracellular and mitochondrial reactive oxygen species (ROS) production, increased tumor necrosis factor-α activity and microglial activation, which leads to increased cellular oxidative stress and cell death. Interestingly, NADPH oxidase inhibitors, ACE inhibitors and AT1-R blockers showed a protective effect on dopaminergic neurons by significantly reducing protein oxidation and peroxidation induced by the neurotoxins (8, 16, 17). Figure-1 below lists some of the key pathological and preclinical studies that show the role of RAS components in the pathophysiology of PD and the protective role of its blockers in both animals and humans. This suggests that Ang-II/AT1-R/NADPH oxidase axis is likely a major factor that contributes dopaminergic cell vulnerability to degeneration, which is similar in mechanism observed in cellular senescence in the peripheral tissues. However, it is not clear whether direct neuroinflammatory effects of Ang-II on dopaminergic neurons or Ang-II mediated microglia and astrocyte activation have a more degenerative influence on the

dopaminergic neurons. In addition to microglia and astrocytes, Ang-II receptors are also expressed in other neurons in the substantia nigra, such as the GABAergic projection neurons in the substantia nigra pars reticulata (SNr), however, the nature of Ang-II signaling in these nigral neurons and its effect on intranigral neurotransmission were not addressed by previous studies.

Study	Sample	Design	Results
Pathophysiology studies			
Ge 1996 [19]	10 PD and 10 controls	Post-mortem binding study	Reduced expression of AT1 receptors in the striatum and SN. AT2 were reduced only in the caudate nucleus
Zawada 2015 [64]	5 PD, 3 presympto-matic PD, 7 controls	Post-mortem binding and neurochemical study	Reduced AT1 membrane immunoreactivity in PD. Increased nuclear AT1 and NADPH oxidase expression
Pessoa Rocha 2016 [65]	30 PD and 20 controls	Cross-sectional comparison	Lower Ang plasmatic levels in PD, which correlated with depression
Konings 1994 [67]	106 PD and 20 controls	Cross-sectional comparison	ACE activity was increased only in PD under dopaminergic treatments.
Lin 2002 [68]	127 PD and 198 controls	Case-control study	Genetic polymorphism of the ACE gene has been associated with increased risk of PD
Mellick 1999 [69]	178 PD and 192 controls	Case-control study	No association between ACE gene mutations and PD risk
Symptomatic effects			
Reardon 2000 [71]	7 modera-tely affected PD	Double-blind, crossover trial with perindopril	Increased response to levodopa and reduction in latency to motor response
PD risk studies			
Becker 2008 [72]	3637 PD and 3637 controls	Case-control study	Similar exposure to ACE inhibitors and AT1 antagonists in PD and controls
Lee 2014 [75]	65000 hypertensive patients	Cohort study	A mild albeit significant reduction in PD risk among users of ACE inhibitors

	PD model	RAS treatments	Results of RAS intervention
ACE inhibitors			
Lopez-Real 2005 [48]	6OHDA / rat	Captopril 55 mg/kg s.c. before and after 6OHDA	Reduced neuronal loss and oxidative damage
Muñoz 2014 [37]	MPTP / mouse	Captopril 20 mg/kg s.c before MPTP	Reduced neuronal loss and oxidative damage
Sonsalla 2013 [50]	MPTP / mouse	Captopril 20 mg/kg s.c before MPTP	Reduced neuronal loss and increased DA striatal content
	MPP+ chronic infusion / rat	Captopril 7.5 mg/kg/day s.c during MPP+	Reduced neuronal loss and increased DA striatal content
Kurosaki 2004 & 2005 [51, 52]	MPTP / mouse	Perindopril 0.2-1 mg/kg before and after MPTP	Reduced neuronal loss and increased DA striatal content. Reduced glial inflammatory reaction and SOD activity
Jenkins 1999 [53]	MPTP / mouse	Perindopril 5 mg/kg/day 1 or 14 days after MPTP	Increased DA content and reduced D2 receptor overexpression when administered 14 days after MPTP
AT1 antagonists			
Grammatopoulos 2007 [33]	MPTP / mouse	Losartan 90 mg/kg before and after MPTP	Reduced neuronal loss
Joglar 2009 [35]	MPTP / mouse	Candesartan 0.5 mg/kg/day s.c before and after MPTP	Reduced neuronal loss, glial inflammatory reaction and NADPH oxidase activity
Mertens 2011 [54]	6OHDA / rat	Candesartan 3 mg/kg/day s.c before or after 6OHDA.	Reduced neuronal loss and increased DA striatal content

Figure 1.1. Key pathological and preclinical studies suggesting the role of RAS components in the pathophysiology of PD and the protective role of its blockers in both animals and humans.

The effect of Ang-II in the nigrostriatal system is not only limited to inducing oxidative and neuroinflammatory stress in dopaminergic neurons. In rats, acute administration of Ang-II in the striatum caused an increase in extracellular levels of dopamine, which was blocked by AT1-R antagonists (18). This suggests that Ang-II via AT1R facilitates dopamine release and could potentially modulate neurotransmission in the striatonigral system. Also, several studies suggest crosstalk between Ang-II and dopaminergic neurotransmission in the nigrostriatal system. In dopamine denervated animals, dopamine depletion leads to a compensatory increase in AT1-R, AT2-R, and NADPH oxidase expression and activity in the nigrostriatal system (5). In dopamine D1 and D2 receptor deficient young mice and rats, a compensatory overexpression of AT1-R and AT2-R was observed. In normal aged rats, dopamine receptors were found to be downregulated, and overexpression of AT1-R was observed (5). This suggests a counter-regulatory mechanism between nigral RAS and dopaminergic system. Taken together, in recent year's intrinsic brain RAS has attracted more attention due to its role in dopaminergic cell vulnerability and has emerged as a potential therapeutic target for Parkinson's disease. However, a comprehensive picture of intranigral RAS signaling and its role in midbrain activity is still lacking and demands further investigation.

1.3b Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by Aβ plaques and neurofibrillary tangles that leads to selective neuron loss. Currently, all the available therapy is merely ameliorative and no disease modifying therapy exists. Age is a primary risk factor for AD and has a strong genetic component (~80%), yet the ensuing mechanism is still not clear. Genome wide association studies (GWAS) and whole genome sequencing (WGS)

approaches have identified several genes that are associated with AD, and some are debated as potential biomarkers. Two recent studies utilizing large AD GWAS meta-analyses and WGS, identified ACE coding variants that can potentially increase the risk of late-onset, sporadic AD (19, 20). ACE encodes for angiotensin converting enzyme 1 (*ACE1*) that cleaves Ang I to Ang II. Because of its widespread distribution, diverse substrate specificity, and role in intrinsic brain RAS, ACE1 has many proposed complex physiological functions outside of its known role in cardiovascular homeostasis, such as memory & cognition. Inhibition of Ang II signaling through ACE1 inhibition has been shown to decrease memory deficit in rodent models of AD and decrease AD incidence in humans (21). Angiotensin receptor blockers (ARBs), meant to reduce peripheral blood pressure, also seem to reduce the incidence of AD (22). However, ACEI's and ARBs are able to enhance memory function in AD independent of their peripheral effect of reducing blood pressure.

ACE1 and other RAS components are altered in the brain of AD patients. ACE1 expression has been found to correlate with tau, phosphorylated tau and Aβ in the cerebrospinal fluid (CSF) of AD patients. In postmortem sporadic AD brains, ACE1 expression was increased in cortical regions. Though there are studies that suggest Aβ increases ACE1 expression or vice versa, the exact reason for this is still not known. Aging also strongly correlates with increased expression of ACE1, and since aging is considered the biggest risk factor of AD, it is believed that ACE1 activity and overall RAS function is also altered in the aging process (7, 19). Therefore, all the studies so far show clear evidence that ACE activity and Ang-II signaling can influence cognitive process in AD patients and contribute to the pathogenesis of this neurodegenerative disease, however, the exact mechanism is still not well understood and is

most likely complex & multifaceted. Therefore, future studies are paramount in determining the relationship between ACE1 genetic variants, aging and AD pathogenesis.

1.3c Multiple Sclerosis

Multiple sclerosis is a chronic inflammatory disease of the central nervous system characterized by injury to the myelin sheaths surrounding the nerve axons which eventually leads to neurological complications due to disrupted communication within the CNS and between the brain and the body. Approximately 2.5 million people is affected by MS worldwide. Similar to parkinson's and Alzheimers disease, the etiology of MS is not well understood. The symptoms typically recur at intervals which are often unpredictable and are termed as 'acute attacks' or 'flares'. Commonly experienced symptoms include muscle weakness, muscle spasms, visual impairments including double vision, incoordination, gait abnormalities and bladder dysfunctions etc. MS is primarily mediated by immune-mediated mechanisms and is considered an autoimmune disorder. Though the oligodendrocytes (myelin sheaths) are primarily affected and the nerve axons and gray matter are though to be spared, recent clinical evidence have also underscored the importance of axonal injury especially in the later stages of MS (23)

As in the peripheral system, central RAS signaling, is also known to activate central immune cells such as glia, astrocytes, macrophages and T-cells in the brain, which is believed to be one of the major factors underlying the promotion of neurodegeneration by RAS in PD, AD and MS. Though, in case of PD and AD, the ACE/Ang-II/AT1-R signaling axis seem to also directly affect the neurons involved in the pathophysiology. However, so far evidence suggests that the effect of central RAS in the pathophysiology of MS is primarily mediated through

activation of the central immune system. Ang-II and AT1-R expression are upregulated in the tissues from MS patients and also in animal models of MS-like disease. Increased ACE and ACE activity are also detected in the CSF of MS patients. In addition, AT1R expression is also upregulated on cells central to MS pathology such as foamy macrophages, T-cells, astrocytes and also in some neurons. Furthermore, in animal models of demyelinating disease, such as experimental autoimmune encephalomyelitis (EAE), AT1-R expression was found to be upregulated in the brain, spinal cord and spleen of the animal when compared to control non-diseased mice.

To further support this, RAS inhibitors such as ACEIs or AT1-R blockers seem to help treat the symptoms and block inflammation associated with EAE. Recent studies have also shed attention on the protective arm of the RAS system that counteracts the inflammatory effects of the pathological arm (ACE, Ang-II and AT1-R) of RAS in the pathophysiology of MS (24). The components of the protective arm of RAS includes Ang (1-7), which is a cleaved product of Ang-II, mas receptor (MasR), ACE2 (angiotensin converting enzyme 2) and angiotensin II type 2 receptor (AT2-R). Some or all of these components are also found to be upregulated in animal models of MS and EAE. Therapeutic use of the Ang (1-7) peptide was found to have significant benefit in reducing the pathology and progression of disease in all animal models of EAE, suggesting that the protective arm of the RAS system counteracts the inflammatory ACE/Ang-II/AT1-R axes and can be exploited as a novel target for treatment of MS.

Interestingly, as described above, the components of protective arms of RAS is found to be upregulated in the MS lesions throughout the disease course in mouse models of MS, EAE and also in human MS postmortem tissues. Since the pathological axes of RAS is also upregulated in MS, this means that both the pathological and protective arm of RAS is activated

in MS. Nevertheless, it is not known if the two counteracting arms of RAS are activated at the same time during the disease course or one is activated prior or later than the other. Moreover, if Ang-II's inflammatory effects are dominant to the neuroprotective effects of Ang (1-7) in MS or vice versa is not known. Clearly, central RAS's role in MS is clinically significant but not well understood and, therefore, needs to be further explored to understand their direct role in MS and derive a potential relationship with its role in other neurodegenerative disorders described above.

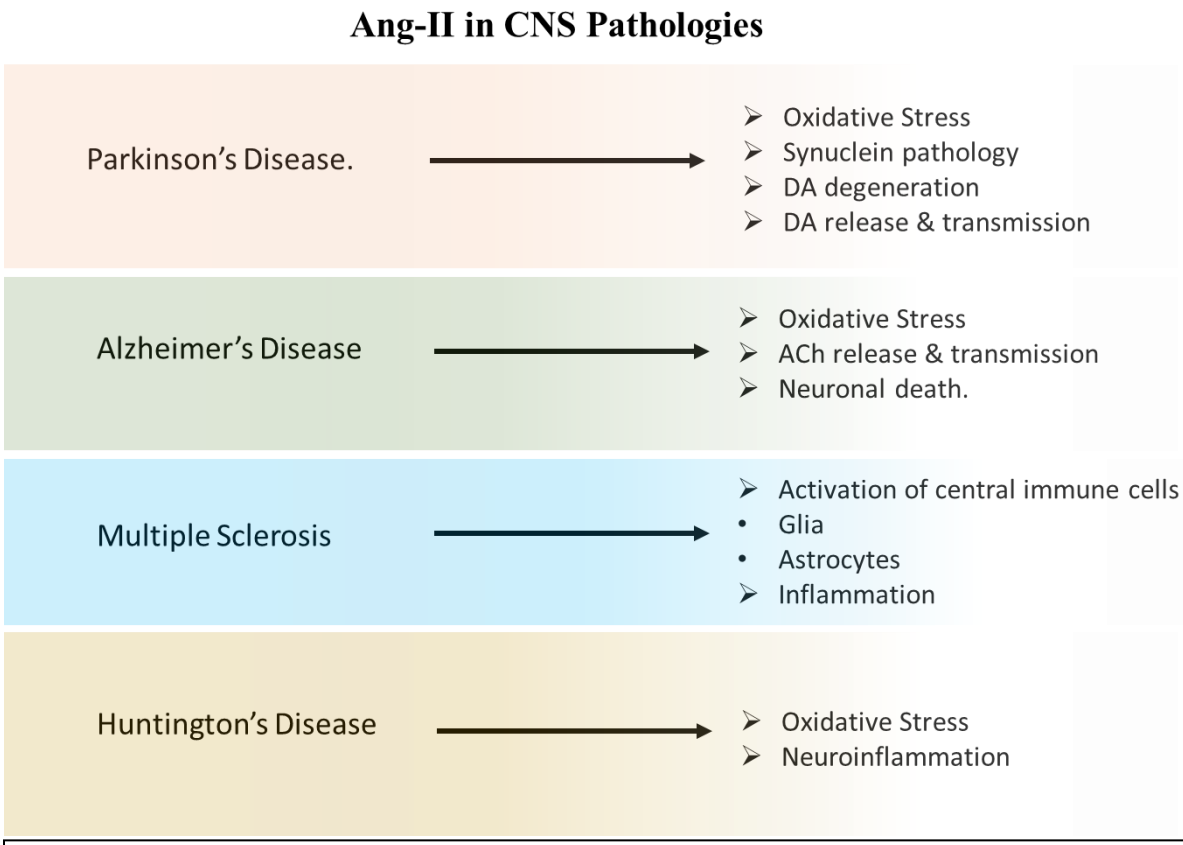

Figure 1.2. Summary of the effect of AT1 receptor mediated Ang-II activity in neurodegenerative disorders.

1.4 Brain Ang-II in Neuronal Excitability and Transmission

The first evidence of renin and angiotensin like activity in the brain was reported in 1971 (25). Since then enormous amount of study has shown evidence of expression of intrinsic brain RAS in various brain tissues, which cannot be possibly achieved by circulating RAS, and have unsurfaced its involvement in various physiological activities ranging from central control of cardiovascular homeostasis to cognition and learning. As described in the previous sections, accumulating evidence over the years has also strongly suggested its involvement in various neurological and neurodegenerative disorders.

However, ever since the early reports of biochemical evidence of RAS components in the brain, uncertainty about whether there are angiotensinergic versus reninergic neurons or a complete functioning RAS exists in every brain tissue still persists. Even though it is well accepted that RAS components such as angiotensinogen and ACE are present in the CSF/interstitial fluid and the precursor protein angiotensinogen is produced in glia and some neurons, the doubt over whether brain RAS is completely independent still lingers. This is primarily due to the evidence that prorenin or active renin can be sequestered in the brain from circulation (26), even though there is unequivocal evidence of renin immunoreactivity colocalizing with neurons within the pituitary, chloroid plexus, medulla oblongata and hypothalamus. Renin immunoreactivity is evident also in glial cells in the medulla oblongata and subfornical organs. In addition, presence of renin mRNA in the brain suggests a mechanism of local synthesis in the expressing brain tissues. Finally, in spite of these lingering doubts over processing of angiotensinogen by renin or other similar proteolytic enzyme for that matter or origins of angiotensin peptides, accumulating evidence in last few decades have provided overwhelming evidence of role of angiotensin-II in neuronal excitability and neurotransmission

in various brain regions. In this section of the chapter, a summary of the role of Ang-II as a neurotransmitter that can regulate excitability and neurotransmission is provided.

1.4a Sympathetic Neurons

The role of Ang-II in regulating fluid and electrolyte balance and cardiovascular function is widely believed to be mediated by peripheral Ang-II but there is evidence suggesting the role of centrally derived Ang-II actions. The centrally derived Ang-II through its actions on cerebral brain regions known to influence the central regulation of fluid and electrolyte balance, alongside with circulating Ang-II via its actions blood-brain barrier lacking circumventricular organs is suggested to regulate fluid & electrolyte balance and cardiovascular function (27-32). Central Ang-II promotes these functions through regulation of body water balance, vasopressin release, blood pressure maintenance, and sympathetic nervous system activity causing adrenaline release (33-35).

The arterial actions of Ang-II in increasing blood pressure is also mediated through its central actions via its actions on sympathetic nerve activity. Accumulating evidence suggests that central Ang-II increases central sympathetic nerve activity and has a pathogenic role in sustaining essential hypertensive state. It was shown that chronic subcutaneous infusion of Ang II caused rapid and marked neuronal activation in circumventricular organs, such as subfornical organ, the nucleus of the solitary tract, and in hypothalamus nuclei such as paraventricular nucleus, and supraoptic nucleus (36). In an in-vitro study, Ang-II facilitated noradrenaline (NA/NE) release in a dose-dependent manner in the rabbit hypothalamus through a potassium dependent mechanism (37). These effects of Ang-II were also replicated in rats. Microdialysis

study showed that intracerebroventricular administration of 100 ng of Ang II increased NA release in anterior hypothalamus of conscious rats and increased blood pressure, which was antagonized by the Ang II receptor blocker (38, 39). Ang-II mediated facilitation of NA release seemed to be pronounced in the hypothalamus of spontaneously hypertensive rats compared with normotensive rats. These studies indicated that the regulatory effect of Ang-II on NA release was mediated via activation of presynaptic AT1-R in the noradrenergic nerve terminals.

In humans, circulating adrenaline is believed to have no direct effect on the regulation of blood pressure. However, studies have indicated that adrenaline can influence blood pressure control indirectly through regulating noradrenaline release. A small amount of adrenaline secreted by the adrenal medulla is transported and accumulated inside the sympathetic nerve endings and activation of these sympathetic neurons can co-release adrenaline along with noradrenaline and activate presynaptic beta-receptors to further facilitate noradrenaline release (40, 41). Elevated secretion of adrenaline from the adrenal medulla in this fashion could therefore lead indirectly to a sustained increase in neuronal release of NA and increase blood pressure. Intracerebroventricular (i.c.v) injection of Ang-II has been shown to increase secretion of adrenaline from rat adrenal medulla and elevate the plasma level of adrenaline (35). This effect of Ang-II was blocked by centrally administered pretreatment with selective AT1-R antagonist, valsartan but not when it was peripherally administered. Pretreatment with selective AT2-R blocker, PD123319 had no effect on the Ang-II induced response. These studies suggest that centrally acting Ang-II and brain AT1 receptors are involved in Ang-II-induced secretion of adrenaline from the rat adrenal medulla. Similar to the facilitation of NA release in the anterior hypothalamus by Ang-II, these studies also suggested the involvement of AT1-R signaling in the brainstem autonomic center projecting paraventricular nucleus (PVN) neurons in the

hypothalamus behind the effect of Ang-II on sympathetic neuronal activity. However, the exact mechanism is still not clear.

1.4b Neuroendocrine Neurons in the PVN.

The paraventricular nucleus of the hypothalamus (PVN) is considered one of the most important autonomic control centers in the brain. Anatomical studies over the years have identified a complex milieu of CNS neurons comprising of those playing essential neuroendocrine roles in controlling hypothalamic-pituatary-adrenal (HPA) axis through corticotropin releasing hormone neurons, the reproductive axis through dopamine and oxytocin neurons, the thyroid axis through thyrotropin releasing hormones, growth & development through somatostatin neurons, regulation of fluid and electrolytes through vasopressin and oxytocin neurons, as well as gastrointestinal and cardiovascular functions through neurons projecting to caudal medullary and spinal autonomic control centers (42). Though the PVN was initially broadly characterized as only containing magnocellular and parvocellular neurosecretory neurons, in recent year's studies have suggested the PVN to contain integrative interneurons of glutamatergic and GABAergic nature. Also, afferent inputs from many integrative centers of the hypothalamus and medulla have been identified, which along with the complex mix of intranuclear neurons and circuitry, controls these seemingly diverse physiological functions of the PVN (42).

In mammals, maintaining a constant osmolality of extracellular fluid (ECF) is paramount to avoid traumatic consequences of brain swelling or shrinking due to acute osmotic stress. Central homeostatic regulation of salt and water balance involves osmosensory neurons that detect small osmotic perturbations and drive homeostatic responses through downstream effector

neurons. Neurons in the organum vasculosum laminae terminalis (OVLT) are the primary osmosensory neurons in the brain and the downstream efferent neurons that are involved in the regulation of ECF osmolality includes oxytocin (OT) and vasopressin (VP) neurons in the supraoptic nucleus (SON) of the hypothalamus. In rats, during normal physiology, maintaining ECF osmolality requires a parallel and proportional excitation of vasopressin and oxytocin neurons by the osmoreceptor afferents, which leads to urinary water retention and sodium excretion. However, during decreased blood volume (hypovolemia) there can be an antiparallel effect on oxytocin and vasopressin neurons, where there is VP release from the secretory neurons but OT secretion is negligible.

Ang-II is known to mediate both peripheral and central homeostatic responses during hypovolemia (43) and is enriched in the subfornical organ neurons that project to the VP and OT neurons in the SON (44). Further, experiments *in-vivo* have shown that subfornical organ neurons are excited by hypovolemia (45). Consequently, a recent study showed that centrally released Ang-II in the SON promotes this state-dependent switching from parallel to antiparallel control of VP and OT neurons (46). Ang-II executes these opposite regulatory effects in vasopressin and oxytocin neurons in the SON through opposite effects on the presynaptic glutamate release, mediated via a retrograde signaling mechanism involving different messenger systems.

In the VP neurons, Ang-II increased presynaptic glutamate release through Ang-II mediated nitric oxide (NO) release from the VP neurons. Bath application of nitric oxide synthase did not block Ang-II's effect but only when applied in the postsynaptic VP neurons using a recording pipette. This suggests that Ang-II signaling causes NO production in the VP neurons which acts as a retrograde messenger and increases presynaptic glutamate release from

the afferent neurons (subfornical organs). Ang-II mediated NO release is well documented in the peripheral circulation as well as in other CNS neurons (47-49) and, more importantly, studies have indicated NO-mediated facilitation of glutamate and GABA release in the SON through presynaptic activation. In the OT neurons, Ang-II had the opposite effect and decreased presynaptic glutamate release by activating retrograde signaling via CB1 receptors. Similar to VP neurons, only local application of CB1 receptor antagonist in the postsynaptic OT neurons blocked the effect of Ang-II but not bath application, suggesting a retrograde mechanism. These findings indicate that Ang-II causes a cell-specific as well as synapse-specific modulation of synaptic glutamate transmission and circuit activity in VP and OT neurons, respectively.

Similar to magnocellular neurons like vasopressin and oxytocin releasing neurons in the PVN, Ang-II also increases the excitability of the parvocellular neuroendocrine cells (putative corticotropin releasing hormone and thyrotropin releasing hormones) of the PVN (50, 51) through a mechanism involving inhibition of delayed rectifier potassium conductance IK and non specific cation conductance (52).

1.4c Afferent Inputs to Hypothalamic Nuclei: SFO & MnPO Neurons

As discussed in the previous section, Ang-II modulates the activity of various neuroendocrine neurons located in the PVN and SON of the hypothalamus and regulated the release of vasopressin, Oxytocin, corticotropin releasing hormone (CRH) and thyrotropin releasing hormone (TRH) etc. In addition to its local postsynaptic signaling within the PVN and SON, Ang-II also regulates the activity of the neurons in these hypothalamic nuclei through afferent projections from subfornical organs (SFO) and median preoptic nucleus (MnPO).

Neurons in both of these nuclei send efferent projections to the PVN and SON and play a pivotal role in the homeostatic regulation of fluid, electrolyte, sleep, and cardiovascular physiology.

Ang-II, angiotensinogen (AGT), and primary Ang-II receptors (AT1-R & AT2-R) are highly expressed in the SFO. SFO is one of the many circumventricular organs that don't have a blood-brain-barrier and is highly vascularized. Therefore, SFO is viewed as one of the several portals whereby circulating Ang-II can centrally influence neurohormonal release, drinking behavior, and cardiovascular physiology. Studies have shown that Ang-II is released as a neurotransmitter upon antidromic excitation of SFO (44), which suggests that Ang-II is either taken up from the circulation or the cerebrospinal fluid. However, knowing the fact that AGT is also abundantly expressed in the SFO, local production of Ang-II may take place within the SFO and then be released as a neurotransmitter from its nerve terminals upon excitation. Interestingly, a recent study showed local production of Ang-II in the SFO of a double transgenic mice expressing human renin (*h*REN) and human AGT (*h*AGT) and regulated drinking behavior (53). The same study showed that cell-specific deletion of *h*AGT gene in the SFO significantly decreased behavior in mice. This suggests that centrally produced Ang-II plays an integral part in the regulation of fluid homeostasis.

Electrophysiological recordings from the efferent magnocellular neurons in the PVN and SON showed prolonged excitability of these neurons in response to a single stimulation of SFO. The increase in the spontaneous activity of PVN and SON neurons were blocked by Saralasin (partial agonist/antagonist of Ang-II) suggesting that Ang-II released from the SFO mediates the facilitatory excitatory inputs to these neurons. Furthermore, SFO neurons themselves are sensitive to Ang-II and are found to be more sensitive than neurons in the PVN and SON but it is suggested that the excitatory effects on SFO neurons were mediated by circulating Ang-II (54).

Increase in Ang-II mediated excitability of SFO neurons were suggested to be due to inhibition of A-type K+ currents (I_A) and opening of non-selective cation channels.

The MnPO lies within the forebrain median terminalis and also plays an important role in cardiovascular physiology and body fluid homeostasis. MnPO sends afferent projections to the PVN and SON magnocellular and parvocellular neurons through which it largely mediates its actions. Lesions to regions containing the MnPO have shown to attenuate thrist and pressor responses induced by Ang-II, hyperosmolality and hypovolemia (1, 55-57). The specific population of PVN projecting MnPO neurons is believed to be central to many of the roles of these neurons as PVN is well positioned to coordinate neuroendocrine and autonomic responses through the mangnocellular and magnocellular neurons described earlier. Not all but majority of MnPO-PVN neurons are sensitive to Ang-II and Ang-II significantly increases the excitability of the MnPO-PVN neurons (28). The MnPO-PVN category of neurons are also presynaptically modulated by Ang-II through afferent projections from SFO. SFO is one of the nuclei in the circumventricular organs that heavily innervate MnPO. Stimulation of SFO results in both excitation and inhibition of MnPO-PVN neurons (58). However, only the neurons that are excited by SFO stimulation are also depolarized by microiontophoretically applied Ang-II and blocked by saralasin (58). Collectively, these studies show Ang-II acting as a neurotransmitter in the anterior hypothalamus and its role in diverse neuroendocrine functions.

1.4d Brain Ang-II Modulates Cholinergic Neurotransmission

The interaction between cholinergic system and central RAS is well known. Activation of central muscarinic receptors by direct receptor agonists induces pressor responses and this

activity is blocked by cholinergic receptor antagonists or blockers of acetylcholine synthesis (59). Also, decrease in acetylcholine content in the hypothalamus, brainstem, and striatum due to blockade of synthesis by hemicholinium-3 was associated with reduction in blood pressure in spontaneously hypertensive rats (60). Alternatively, pressor responses induced by intracerebroventricular injection of Ang-II is blocked by inhibitors of Ach synthesis (61). Additionally, blockers of ACE such as captopril have shown to inhibit stimulation evoked acetylcholine release in the striatum (62). In the human temporal cortex and rat entorhinal cortex Ang-II has been shown to inhibit acetylcholine release (63, 64). Therefore, these studies demonstrate central Ang-II modulating acetylcholine neurotransmission in different brain regions and demonstrates an interaction between central RAS and cholinergic system.

This crosstalk between central RAS and cholinergic system is also suggested to be involved in the pathophysiology of Alzheimer's disease (AD). Ang-II is reported to inhibit acetylcholine release in various animal and brain tissue studies (7, 9, 64). Preclinical studies also reported protective effects of AT1R blocker and ACE inhibitors in cholinergic transmission dependent deficits in animal models of spatial and short-term memory deficits (65, 66). This suggests that targeting Ang-II and associated RAS components could enhance cholinergic release and transmission and could be used as a therapeutic strategy for treatment of AD.

1.4e Brain Ang-II Modulates Dopaminergic Neurotransmission

As described in the sections above, the role of brain Ang-II in the pathophysiology of Parkinson's disease has gained a lot of scientific interest over the years. In addition, increasing evidence over the years has also suggested the role of central dopaminergic activity in regulating

peripheral blood pressure through its interaction with central Ang-II and other RAS components. This suggests a functional interaction between brain RAS and dopaminergic pathways in the central nervous system. However, the role of central RAS in regulating hypertension through central dopaminergic transmission is still controversial.

Ang-II and other RAS components are expressed throughout the brain regions associated with dopaminergic transmission, especially in the substantia nigra and striatum in the midbrain (13, 15, 17, 67-70). Ang-II is known to induce dopamine release in the striatum of rats through an AT1-R dependent mechanism, and the nigrostriatal dopaminergic system is proposed to be involved in baroreflex sensitivity in rats as lesions of the substantia nigra and the nigrostriatal dopaminergic pathways have shown to attenuate baroreflex responses in rats and development of hypertension in spontaneously hypertensive rats (18, 71, 72). Also, ACE inhibitors and AT1-R blockers blocks Ang-II mediated dopaminergic transmission in the nigrostriatal dopaminergic pathways (18, 62) indicating a functional coupling between Ang-II and central dopaminergic system which can have pathophysiological effects as observed in Parkinson's disease but also in physiological regulation of blood pressure. However, the relationship between the two diverse effects of Ang-II through its action on dopaminergic transmission is not clear and demands further studies.

Ang-II mediated regulation of dopaminergic transmission is found in almost all the dopamine containing neurons in different brain regions. In some cases, Ang-II mediated modulation of the dopaminergic transmission is through modulation of afferent inputs onto dopaminergic neurons. As discussed previously, Ang-II mediated effects on midbrain dopaminergic neurons is suggested to be either primarily through its direct signaling in the dopaminergic neurons or through inducing reactive glia or astrocytes. In our study, discussed in

detail in chapter 3, we show the first evidence of Ang-II mediated modulation of presynaptic GABAergic neurons in the substantia nigra pars reticulata (SNr) and altered GABAergic input onto dopaminergic neurons in the SNc. Therefore, our study suggest that Ang-II, in addition to its direct modulatory effects on dopaminergic neurons in the SNc, can also regulate their activity through modulation of afferent GABAergic input. Moreover, Ang-II signaling is known to alter GABAergic neurotransmission in many other brain regions and is discussed in detail in chapter 2. Accordingly, this indicates that Ang-II signaling is complex and has modulatory effects on different neurotransmitter systems in different brain regions, and the full extent of how Ang-II can regulate the activity of dopaminergic neurons in the brain is far from being understood.

1.5 Conclusion

In conclusion, this chapter highlights the dual and complex role of Ang-II as a circulating hormone in the periphery and as a neurotransmitter in the central nervous system. For a long time, Ang-II and the associated RAS system were believed to be a circulating hormonal system necessary for regulating fluid and electrolyte balance and cardiovascular homeostasis. However, numerous studies over the last few decades demonstrated the existence of an independent RAS within the CNS and its role in centrally regulating the physiological functions of Ang-II in the periphery. Numerous studies have demonstrated the expression of Ang-II and its associated RAS components in different brain regions, including many that are separated from the circulating RAS by the blood-brain barrier. Consequently, studies over the years showed the involvement of brain Ang-II in various neurodegenerative disorders such as Alzheimer’s disease, multiple sclerosis, Huntington's disease, and Parkinson's disease and expanded the understanding of

extrarenal local RAS in the brain. Depending on the type of receptor involved Ang-II can have a pathological or protective effect in the central nervous system.

Ang-II mediates its effect in the CNS through its action on different ion channels, receptors, transcription factors, and proteins that modulates neurotransmitter release and neuronal activity. As discussed in this chapter, Ang-II modulates the activity of cholinergic, glutamatergic, dopaminergic, and GABAergic neurotransmission in different neurons. Of primary interest is its effect on dopaminergic neurons and its role in exacerbating the degeneration of dopaminergic neurons in rodent models of Parkinson's disease and also its altered expression in post mortem brain tissues from patients. This suggests a key role of brain RAS in the pathophysiology of Parkinson’s disease. However, Ang-II and other RAS components are expressed throughout the substantia nigra, but its role in other intranigral neurons such as GABAergic neurons in the pars reticulata is not known. Interestingly, Ang-II is found to regulate GABAergic neurons and neurotransmission in different brain regions, such as the central nucleus of amygdala, paraventricular nucleus of the hypothalamus, median preoptic nucleus, subfornical organ etc. which are key brain region in regulating cardiovascular homeostasis, fear associated anxiety and physiological responses, emotions, and the neuroendocrine system. Therefore, it is likely that Ang-II signaling also exists in the nigral GABAergic neurons, which can further modulate dopaminergic neuronal activity and output. Interestingly, more than 70% of synapses made on SNc dopaminergic neurons are GABAergic in nature, and pars reticulata GABAergic neurons provide one of the major GABAergic inputs to SNc dopaminergic neurons.

In conclusion, this dissertation addresses my overall hypotheses that Ang-II signaling exists in the GABAergic neurons of substantia nigra reticulata (SNr) and modulates GABAergic input onto SNc dopaminergic neurons. Furthermore, my proposal focuses on mechanistic components

involved in novel Ang-II signaling in the SNr GABAergic neurons and how it can regulate their activity and the activity of downstream SNc dopaminergic neurons. Specifically, this includes two specific mechanistic hypotheses: 1) Ang-II signaling modulates the firing of SNr GABAergic neurons through AT1-R signaling and 2) Ang-II mediated modulation of SNr GABAergic neuronal activity further affects feed-forward inhibitory input to SNc dopaminergic neurons. In the next chapter, we discuss the extent of GABAergic control of dopaminergic neurons, its role in regulating dopaminergic cell activity, and how GABAergic neurotransmission in different brain regions is regulated by Ang-II. In the final chapter, my experimental studies address the hypotheses described above and provide the first evidence of Ang-II signaling mediated modulation of GABAergic neurotransmission in the substantia nigra.

CHAPTER 2

GABA-ERGIC CONTROL OF DOPAMINERGIC NEURONS AND ANG-II MEDIATED REGULATION OF GABA-ERGIC TRANSMISSION IN THE CNS.

2.1 Introduction

Dopaminergic neurons in the midbrain innervated primarily by other nuclei of the basal ganglia form a network that generates neural signals crucial for motivated behavior than any other brain region. This network is comprised of dopaminergic neurons in the ventral tegmental area (VTA), substantia nigra pars compacta (SNc) and dopaminergic neurons in the retrorubral field (RRF). Firing pattern of these dopaminergic neurons encode for reward prediction error, which is a critical mediator of reinforcement learning (73-76). Disruption of these dopaminergic neurons is central to many psychiatric disorders, including drug addiction and also in motor and psychological deficits associated with degeneration of dopaminergic neurons in Parkinson's disease. Remarkably, these ventral dopamine neurons generate signals for reward, aversion, and associative learning which requires the integration of a vast array of sensory, motor, and cognitive information (77, 78). This is made possible by the ability of dopaminergic neurons to integrate their intrinsic firing with a variety of inhibitory and excitatory inputs it receives from its afferent projections.

Ventral midbrain dopaminergic neurons receive inputs from all the major neurotransmitters in the CNS: glutamate, GABA, acetylcholine, dopamine and norepinephrine. It is widely accepted that the intrinsic property of dopaminergic neurons to fire in a tonic single-spike activity, pause, and then fire in phasic bursts underlies its ability to integrate signals from different afferent inputs and encode for reward prediction error. A host of evidence both *in vitro* and *in vivo* demonstrates that electrical stimulation and agonist application can induce

spontaneous burst firing in dopaminergic neurons which is suggested to be dependent on NMDA receptor activation. However, NMDA receptor activation cannot be the only mechanism behind burst generation as it cannot explain how glutamatergic inputs are generated at the right times which control the timing of burst firings (77). This is demonstrated by suppression of a reward response when a previous stimulus predicts an identical reward, which suggests that this suppression must occur on a timescale on the order of hundreds of milliseconds (74, 79). One explanation for this precise regulation of dopaminergic burst firings is suggested to be due to heavy GABAergic innervation of dopaminergic neurons that prevents DA burst firing by a disinhibitory mechanism (80-83).

It is suggested that more than 70% of the synapses made onto substantia nigra dopaminergic neurons are GABAergic, although the proportion of GABAergic synapses seems to be lower in the VTA (80, 84, 85). Both GABAa and GABAb receptors mediate the inhibitory actions of GABA in dopaminergic neurons and the extent and nature of inhibition depends on the location and density of these receptors (81). Both GABAa and GABAb receptors are also suggested to suppress burst firings of midbrain dopaminergic neurons (81, 86)

In this chapter, an overview of the GABAergic control of dopaminergic control is provided, especially dopaminergic neurons in the substantia nigra pars compacta (SNc). All the known afferent GABAergic inputs to dopaminergic neurons and their physiological roles are discussed. More importantly, how these GABAergic afferents can regulate burst generation in dopaminergic neurons through a disinhibitory mechanism is discussed. More focus is provided on the afferent non-direct intranigral GABAergic input from the neighboring substantia nigra reticulata (SNr), which is believed to be one of the dominant inhibitory sources to SNc dopaminergic neurons due to its proximity and nature of synaptic connections with the

dopaminergic neurons. Also, in this chapter, a comprehensive review of studies showing evidence of Ang-II mediated regulation of GABAergic neurotransmission in different brain regions and its underlying mechanism is provided. Lastly, information on the expression of RAS components within the nigral GABAergic and dopaminergic neurons is provided and the chapter concludes with a hypothesis that is addressed experimentally in the following chapters.

2.2 GABAergic Control of VTA Dopaminergic Neurons

The VTA dopaminergic neurons plays a significant role in reward, motivation, cognition and aversive behaviors. Though the majority of neurons in the VTA is dopaminergic (~65%), VTA is a heterogeneous structure comprised of neurons of Glutamate and GABA origin and as well as neurons that exhibit a combinatorial nature that can corelease dopamine and glutamate, dopamine and GABA and glutamate and GABA.

The tonic and phasic firing of VTA dopaminergic neurons are tightly regulated by the glutamate, cholinergic and GABAergic afferent inputs from different brain areas. VTA dopaminergic neurons are strongly innervated by GABAergic inputs originating from nucleus accumbens shell, ventral pallidum, rostral tegmental nucleus (RMTg) and GABAergic neurons within the VTA which are known to control VTA dopamine neuronal activity i*n vivo*. Eliminating GABAergic tone from ventral pallidum GABAergic afferents was found to increase asynchronous firing of VTA dopaminergic neurons. In recent years, VTA GABAergic neurons has emerged as a significant modulator of VTA dopaminergic activity. Ethanol is suggested to significantly modulate VTA dopaminergic neurons through its actions on both VTA GABAergic neurons and other GABAergic afferents. VTA GABAergic neurons are suggested to tonically suppress VTA dopaminergic neurons but when they themselves are inhibited by distal

projections their suppression on VTA dopaminergic neurons is relieved which in turn disinhibits them. In support of this, studies have shown inhibition of VTA GABA neurons resulting in pro-reward behavior, increased social interaction, anxiety reduction, and increased explorative behavior. This suggests that VTA dopaminergic neurons are modulated directly by GABAergic afferents from distant brain areas as well as indirectly by VTA GABAergic neurons which themselves are regulated by distal inputs of both excitatory and inhibitory nature from different brain areas. However, the relative contribution of specific GABAergic afferents to VTA dopaminergic neurons is still not well understood.

2.3 GABAergic Control of Nigrostriatal Dopaminergic Neurons.

The vast majority of synapses made to dopaminergic neurons in the substantia nigra, somewhere around 70%, is GABAergic (80). These GABAergic afferents primarily arise from nuclei within the basal ganglia and the densest projections originate from the neostriatum, the globus pallidus, and the GABAergic neurons in the substantia nigra reticulata (SNr). The overwhelming majority of nigral dopaminergic neurons are located in the substantia nigra pars compacta (SNc) with few scattered group of cells also resides in the SNr.

Identification of afferents to SNc dopaminergic neurons is challenging due to their cellular morphology and also the anatomical organization of the nucleus. The pars compacta is a relatively thin, flattened, disk-shaped nucleus of densely packed cells sitting dorsal and superior to the larger GABAergic neurons containing pars reticulata. The cell bodies of the majority of dopaminergic neurons reside in the pars compacta and extend their dendrites to the neuropil of the pars compacta. Also, all dopaminergic neurons send one or every so often two dendrites, up to a millimeter in length, ventrally and perpendicular to the pars compacta, deep into the pars

reticulata. These dendrites are often the largest emitted by a neuron in the CNS and traverse the entire extent of the pars reticulata. The proximal ends of these dendrites are closely intermingled with the soma and dendrites of the GABAergic neurons in the pars reticulata. This dendritic organization of dopaminergic neurons often makes mapping the afferent inputs hard because of the blurry distinction between afferents to the dopaminergic and those to the GABAergic neurons. However, the known GABAergic afferents to dopaminergic neurons are well accepted.

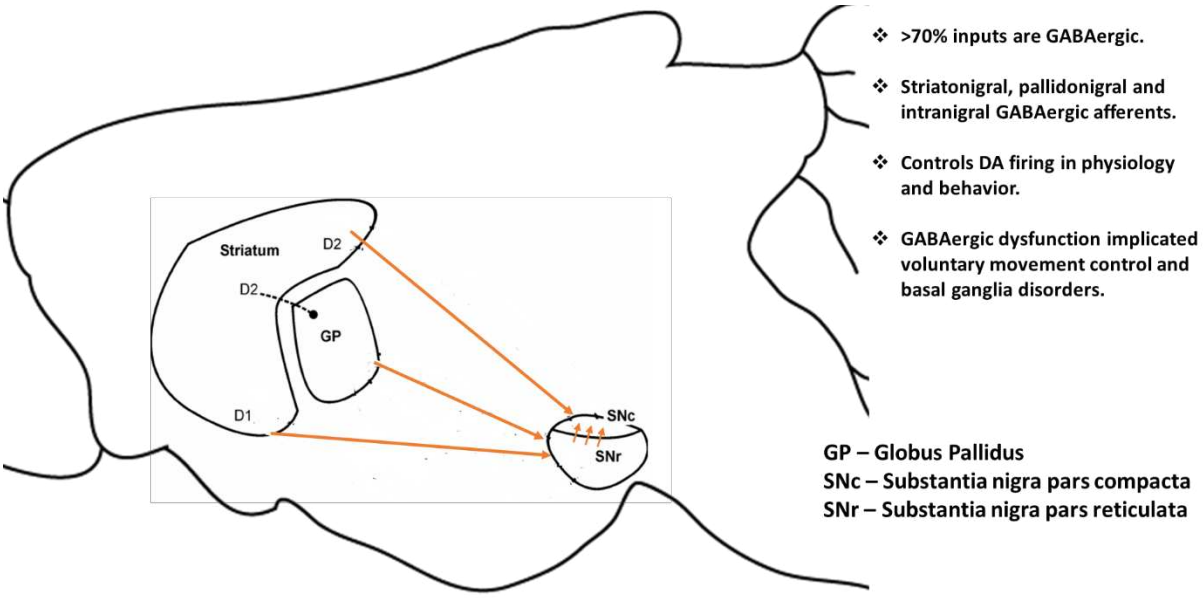

Figure 2.1. GABAergic afferents to SNc dopaminergic neurons.

2.4 GABAergic Afferents to Nigrostriatal Dopaminergic Neurons

2.4a Striatonigral Afferents

The striatonigral pathway is called the direct pathway emanates from the spiny projection neurons in the striatum, as shown in figure 2a. These comprise about 50% of the spiny cell efferents and the remainder 50% projects to the globus pallidus. These striatonigral neurons

selectively express dopamine receptor subtype D1 and colocalize with substance P, dynorphin and GABA immunolabeling. The direct striatonigral pathways, not only project to dopaminergic neurons in the SNc but also the GABAergic neurons in the SNr. However, these projections don't carry the same information, as the striatonigral neurons that projects to SNc dopaminergic neurons are from the patch compartment in the striatum while for the SNr GABAergic neurons the striatonigral inputs originate in the matrix compartment in the striatum. The striatonigral projection is slow conducting compared to other GABAergic afferents to SNc dopaminergic neurons and also by far the slowest of all the long-range projecting GABAergic neurons within the basal ganglia. The mean firing rate of these neurons is very low (<1 Hz) and the membrane potential oscillates between a hyperpolarized state with little to no firing and a depolarized state in response to excitatory inputs from the cortex.

Stimulation of striatonigral projections produced long latency monosynaptic hyperpolarizing IPSPs that were mediated by GABA-a receptor dependent chloride currents (87). Application of bicuculline or picrotoxin, but not GABA-b antagonist CGP 55845A or saclofen, completely blocked these IPSPs suggesting the inhibitory effect of striatal stimulation on SNc dopaminergic neurons is predominantly or exclusively mediated by GABAa receptors (81, 88). In some cases application of GABA-b antagonists slightly augmented the inhibition seen in SNc dopaminergic neurons in response to stimulation of striatonigral pathway, which is suggested to be due to blockade of presynaptic GABA-b receptors (81). In addition, local application of GABAa receptors also increased the spontaneous firing rate of SNc dopaminergic neurons and shifts their firing pattern to burst mode (81, 88). Similar to its lack of effects on synaptically evoked stimulation of striatonigral projections, local blockade of GABA-b receptor in SNc dopaminergic neurons by selective antagonists did not lead to burst firing. This suggests that

GABAa receptors predominantly underlie the inhibitory effects observed in SNc dopaminergic neurons.

2.4b Pallidonigral Afferents

Globus pallidus (GP) is another basal ganglia nuclei that heavily innervates SNc dopaminergic neurons (Fig 2a). Similar to striatonigral afferents, pallidonigral GABAergic inputs onto dopaminergic neurons also significantly modulates dopaminergic neuronal activity. Stimulation of globus pallidus evokes short latency IPSPs in SNc dopaminergic neurons and often causes complete suppression of spontaneous firing. Lesion of GP or muscimol mediated inhibition of GP neurons can also elicit burst firing in SNc dopaminergic neurons. Pallidonigral inhibition of SNc dopaminergic neurons is primarily mediated by GABAa receptors as picrotoxin blocks the inhibitory effects of pallidal stimulation and increases the rate of spontaneous firing as well as the pattern of firing in SNc dopaminergic neurons.

Pallidonigral projections also project onto GABAergic neurons in the SNr. Pallidal inhibition dramatically increases the firing of SNr GABAergic neurons and pallidal excitation suppress the spontaneous activity of SNr GABAergic neurons. As discussed more in detail in the next section, SNr GABAergic neurons exert robust, fast-acting, transient inhibition of SNc dopaminergic neurons and are significantly regulated by pallidonigral inhibitory inputs, which further modulates the activity of nigral dopaminergic neurons. Therefore, globus pallidus exerts significant control of neuronal activity of SNc dopaminergic neurons directly and through a disynaptic pathway involving pars reticulata GABAergic neurons.

2.4c Intranigral Afferents

Substantia nigra pars reticulata (SNr) is located ventrolateral to the pars compacta and is primarily comprised of GABAergic neurons. SNr is one of the major output nuclei of basal ganglia where the final stage of information processing takes place and conveys information to target nuclei such as the thalamus, brainstem premotor areas, and superior colliculus. Through its innervation on these target areas, GABAergic projection neurons in the SNr provide access to basal ganglia to control motor, cognitive and emotional-motivational information processing (89, 90).

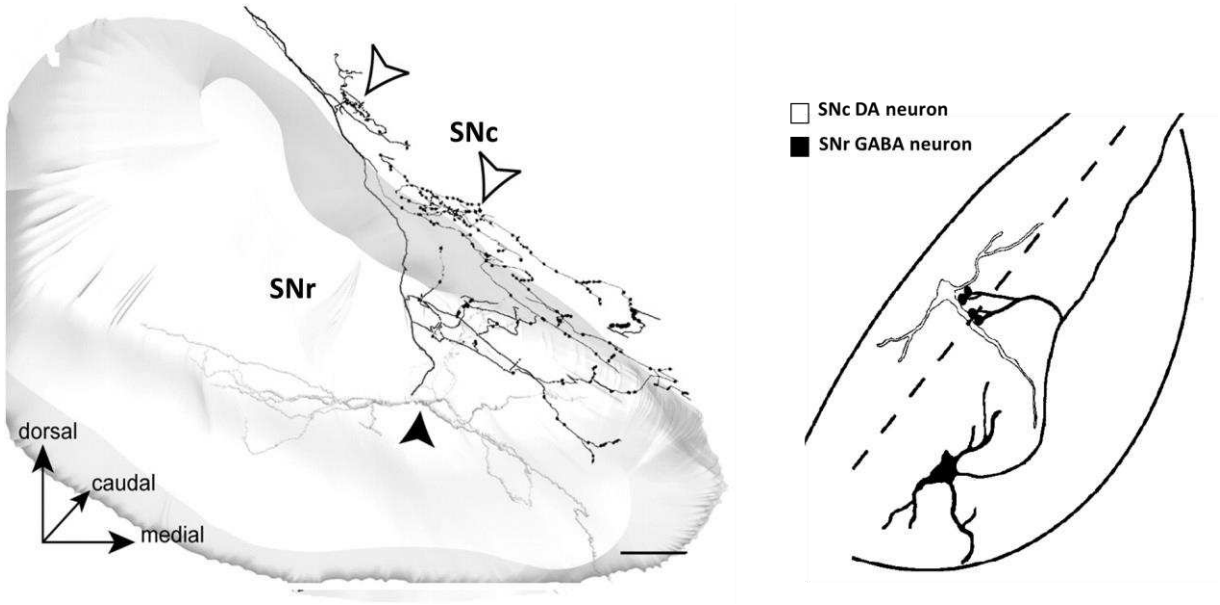

Figure 2.2. Intranigral GABAergic afferents to SNc dopaminergic neurons. SNr GABAergic neurons sends dense network of axon collaterals to SNc and provides robust short-latency inhibitory inputs to dopaminergic neurons. (Adapted with permission from [89] and [88] © The Journal of Neuroscience)

In addition to sending long projections to structures extrinsic to the basal ganglia, the projection neurons of SNr extend a local axon collateral network that innervates both the SNr (Fig 2a & 2b) and SNc (Fig 2a) and locally inhibit both nigral GABAergic and dopaminergic neurons (84, 88-90). These axon collaterals are also suggested to make synapses with

pallidonigral fibers originating from the globus pallidus. A striking neurophysiological feature of SNr GABAergic projection neurons is their short duration high frequency spontaneous spike firing (~25-30 Hz *in vivo*), which is several times higher than that of the low frequency firing of SNc dopaminergic neurons. Through this intrinsic spiking, SNr projection neurons keep a tonic inhibitory control of SNc dopaminergic neurons.

Antidromic activation of SNr GABAergic projections neurons through thalamic stimulation produces a short latency IPSPs in SNc dopaminergic neurons and often results in complete suppression of their firing (91). In contrast to inhibition from striatonigral afferents, SNr GABAergic neurons produces a short-latency and potent inhibition of SNc dopaminergic neurons. This is suggested to be due to the proximity of SNr GABAergic neurons to the nigral dopaminergic neurons, nature of synaptic contacts made between the two nigral cell types, and the difference in relative distribution and expression of GABA-a and GABA-b receptors between afferents from SNr and nigrostriatal pathway. The majority of striatonigral afferents synapse onto relatively distal dendrites of dopaminergic neurons, whereas afferents from SNr makes synapses of large boutons with proximal dendrites of dopaminergic neurons in the SNc. Also, the density of synapses made by SNr projection neurons with SNc dopaminergic neurons is suggested to be significantly higher than other afferent inputs because of the anatomical feature of the local axon collateral network, where an individual axon makes repeated contacts with the nigral dopaminergic neurons, sometimes forming pericellular baskets around the soma of dopaminergic neurons (84, 91, 92). In contrast to striatonigral afferents, inhibition by SNr GABAergic neurons of nigral dopaminergic neurons mostly had a short latency early component and lacked late-inhibitory component. The late inhibitory response seen from striatonigral inputs is attributed to relatively enriched expression of GABA-b receptors in the distal dopaminergic

dendrites where majority of striatonigral inhibitory afferents makes synapses. In addition, afferents from striatum have significantly higher expression of presynaptic GABAb receptors than those from the SNr GABAergic neurons, which accounts for an occasional decrease in dopaminergic cell inhibition on stimulation of the striatum.

Another significant way SNr GABAergic projection neurons modulates dopaminergic cell activity is through disinhibition. As discussed in the previous section, pallidonigral fibers from globus pallidus innervate both SNr GABAergic and SNc dopaminergic neurons. Inhibition of globus pallidus almost doubles the firing rate of SNr GABAergic neurons while stimulation dramatically decreases their firing (93). As a result, lesion of globus pallidus or muscimol induced inhibition of pallidonigral fibers from globus pallidus produces a paradoxical decrease in the firing rate and burst firing of SNc dopaminergic neurons. Alternatively, chemical stimulation of pallidonigral projections by blocking GABA-a receptors using bicuculline produces a paradoxical increase in dopaminergic cell spontaneous and burst firing due to disinhibition of SNr GABAergic neurons. In other words, disinhibition of globus pallidus inhibits SNr GABAergic neurons which in turn disinhibits SNc dopaminergic neurons. These results demonstrate that effect of pallidonigral afferents on SNc dopaminergic neurons are predominantly mediated via preferential inhibition of pars reticulata GABAergic projection neurons. This is consistent with relatively denser projections from the globus pallidus to the SNr when compared to the SNc and also difference in sensitivity to GABA between the two nigral cell types (84, 87, 92).

2.5 Ang-II Modulates GABAergic Neurotransmission in the CNS.

2.5a Periaqueductal Gray (PAG) Neurons.

The periaqueductal gray (PAG) is a nucleus that plays a critical role in the processing of behavioral responses such as fear and anxiety to threatening stimuli, autonomic functions, and also in processing of nociception (94). The major intrinsic circuit within the PAG is a tonic GABAergic network that regulates the different functional components of PAG. Ang-II receptors, AT1-R in particular, is expressed in the PAG and site-specific injection of Ang-II in the PAG increases arterial blood pressure (95). AT1-R blockers blocked the increase in arterial blood pressure but not AT2-R. *In vitro* slice electrophysiology study showed that Ang-II increased the frequency of mIPSC's in the PAG but did not increase the amplitude of mIPSCs. The increase in mIPSC frequency was blocked by both AT1-R blocker (losartan) and GABAa-R blocker (bicuculline), suggesting presynaptic AT1-R mediated increase in GABAergic transmission in the PAG (96).

PAG is also involved in micturition reflex through its regulation of pontine micturition center (97). PAG is controlled by many brain areas such as prefrontal cortex, cerebellum, basal ganglia, and the paraventicular nucleus (PVN) in the hypothalamus. As discussed earlier, Ang-II regulates the activity of paraventricular nucleus (PVN) (28, 39, 48-50, 52, 98-101). Interestingly, Ang-II is shown to regulate the activity of PAG neurons and increase micturition reflex through inhibition of GABAergic transmission in the PAG (102). This was found to be mediated by presynaptic AT1-Rs, presumably in the afferent terminals from PVN, through activation of AT1-R/PLC/PKC/NADPH oxidase/superoxide anion pathway. This suggests that AT1-R modulates synaptic GABAergic input to PAG neurons, as also seen in the Ang-II induced PAG mediated increase in arterial blood pressure (96).

2.5b Central Regulators of Sympathetic Tone: PVN, RVLM, IML.

The paraventricular nucleus (PVN) neurons in the hypothalamus integrate information for the processing of neuronal signals involving physiological functions such as neuroendocrine and cardiovascular functions. In the brain, it is one of the five major areas for sympathetic premotor control and studies have suggested its role as an important source of excitatory drive for sympathetic vasomotor tone (103-106). PVN parvocellular neurons mainly project to rostral ventrolateral medulla (RVLM) and the presympathetic neurons in the intermediolateral (IML) cell column in the spinal cord. As discussed in previous sections, Ang-II, its receptors, and other RAS components are expressed in the PVN. Circulating Ang-II can affect the activity of PVN neurons through its action on circumventricular organs that project to PVN or as neurotransmitter through local RAS activity. IML projecting PVN neurons are innervated by subfornical organ (SFO) neurons in the circumventricular organs. Ang-II is also known to regulate the activity of SFO.

In the PVN neurons, Ang-II is shown to have an excitatory effect through attenuation of synaptic GABA input (99, 107). In the spinally (IML) projecting PVN neurons, Ang-II attenuated GABAergic input through AT1-R mediated production of reactive oxygen species, superoxide anions in particular (101). The effect of Ang-II was blocked by pertussis toxin (PTX), a blocker of $G_{i/o}$ protein, suggesting a G-protein coupled ROS production. However, the increase in ROS did not affect voltage-gated Ca^{+2} channel which is widely accepted as one of the common downstream targets of Ang-II mediated ROS release in different tissue types and also a regulator of neurotransmitter release (108-111). It is suggested that Ang-II mediated increased ROS could negatively regulate vesicular release and/or affect proteins associated with

neurotransmitter release in the presynaptic membrane, such as SNAP-25. However, the exact mechanism is still not known.

As mentioned above, PVN neurons also project to neurons in the RVLM and indirectly modulates sympathetic outflow through neurons in the RVLM. Ang-II is shown to excite glutamatergic neurons in the RVLM through AT1-R and increase arterial blood pressure (112, 113). However, Ang-II through postsynaptic AT2-R in the RVLM is shown to increase synaptic GABA input and lower blood pressure in normotensive rats (114). Specific AT2-R agonist C21 increase GABA levels and selective AT2-R antagonist PD123319 attenuated the effect, confirming the role of AT2-R. Ang-II mediated this effect through decreasing synaptic release from GABAergic afferents to RVLM, and required a functioning central nitric-oxide pathway. However, the exact source of the GABAergic afferent to RVLM modulated by Ang-II is still not known. In summary, these studies provide evidence of Ang-II receptor subtype dependent differential regulation of GABAergic neurotransmission in neurons responsible for central regulation of blood pressure and sympathetic tone.

2.5c Amygdala

Amygdala is a structure in the forebrain with different subnuclei. It plays a central role in stress response and emotional learning, especially from the context of fear. Numerous studies over the years in rodents, primates, and humans have demonstrated the involvement of the central nucleus of amygdala (CeA) in mediating the different physiological aspects associated with fear and anxiety. The CeA neurons exert its control through sending projections to sites responsible for mediating different aspects of the stress response such as hypothalamus, brainstem, and basal forebrain, and also receives inputs in return from these structures (115-118).

Thirst and sodium peptide is one of the physiological aspects of stress response since it maintains the balance of fluid and electrolyte levels in the body which further regulates peripheral blood pressure. The CeA neurons are reciprocally connected to regions involved in sodium appetite such as the PVN, nucleus of the solitary tract (NTS), lateral parabrachial nucleus (LPBN), and the median preoptic nucleus (MnPO). Ang-II is an important regulator of sodium intake and all the other associated RAS components necessary for the production of Ang-II have been reported in the CeA. Similar to other areas in the brain reported in the previous sections, such as PVN, PAG and RVLM, Ang-II is found to regulate GABAergic neurotransmission in the CeA neurons. Ang-II increased the amplitude of mIPSCs in CeA neurons without affecting the frequency of mIPSCs, suggesting a postsynaptic mechanism. This effect was blocked by selective AT1-R blocker losartan and G-protein inhibitor (GDP-β), indicating a G-protein coupled AT1-R signaling mechanism. Ang-II mediated increase in mIPSCs lead to a decrease in spontaneous activity of CeA neurons which was also blocked by losartan and GDP-β. However, Ang-II failed to inhibit CeA neurons in the presence of selective GABAa-R blocker bicuculline, indicating that GABAa-R lies downstream of AT1-R signaling and underlies the facilitation of synaptic GABAergic input by Ang-II in the CeA neurons.

In addition to its role in regulating sodium intake through regulation of GABAergic transmission in CeA neurons, Ang-II via AT2-R has been reported to also influence fear conditioning. Selective activation of AT2-R GABAergic neurons in the CeA by C21 decreased fear-associated freezing in mice (119). In another report, Ang-II via AT1-R regulated the anxiety state induced by stress in the fear-potentiated plus-maze behavior (120). Consistent with the effect of Ang-II in regulating fear and stress response in CeA neurons, upregulation of ACE2 enzyme, which breaks down Ang-II to Ang-(1-7), decreased anxiety-like behavior in mice

through activation of Mas receptors (MasR) in the GABAergic neurons in basolateral amygdala (BLA). ACE2 overexpression increased the frequency of spontaneous IPSCs onto BLA GABAergic pyramidal neurons, indicative of increased presynaptic GABA release (121). Therefore, these studies demonstrate Ang-II as a key modulator of GABAergic neurotransmission in the neurons in the amygdala and its role in fear-associated physiological effects in emotion and behavior.

2.5d Median Preoptic Nucleus.

The median preoptic nucleus (MnPO) is a structure located in the anterior hypothalamus and dorsal to other nuclei in the preoptic area. It is considered a pivotal brain region for osmoregulation, thermoregulation, and homeostasis. In the context of maintaining osmotic balance, changes in osmolality due to Na^+ levels have been shown to alter firing activity of MnPO neurons. In the Na^+ responsive neurons of MnPO endogenous Ang-II is found to tonically control the afferent GABAergic transmission through a postsynaptic mechanism. Blocking of AT1-R activity by Saralasin and losartan significantly decreased the amplitude of inhibitory postsynaptic currents (IPSCs) and exogenously applied Ang-II increased the IPSC amplitudes in the Na^+ responsive MnPO neurons. This facilitation of IPSC was GABAa -R mediated and suggest a postsynaptic mechanism involving crosstalk between AT1-R and GABAa-R. Interestingly, Ang-II mediated facilitation of GABAergic transmission was only limited to Na^+ sensitive MnPO and in the non-Na^+ responsive MnPO neurons Ang-II depolarized the neurons and increased their cellular activity (122). The afferent inhibitory GABAergic input onto Na^+ responsive MnPO neurons is suggested to be from neurons in the subfornical organ (SFO). As discussed in chapter 1, circulating Ang-II regulates the activity of SFO neurons. However, Ang-

II is also released as a neurotransmitter upon activation of SFO neurons and it is suggested that *de novo* synthesis of Ang-II can take place within the SFO (44). These studies demonstrate the role of Ang-II in modulating the excitability of MnPO neurons through regulating GABAergic transmission.

2.5e Dorsomedial Hypothalamus

The dorsomedial hypothalamus (DMH) is a key brain region in mediating panic-like responses. It coordinates signals from neuroendocrine, autonomic, and behavioral inputs associated with emotional stress. Activation of neurons in the DMH in rats induces physiological responses associated with panic responses such as increased heart rate, anxiety, increased blood pressure, and flight behaviors. Ang-II receptors, AT1-R and AT2-R, are expressed in the DMH and neurons in the DMH are also innervated by SFO that are further modulated by Ang-II and also release Ang-II as a neurotransmitter (44, 123). Blocking postsynaptic AT1-R in the DMH by selective blockers attenuates stress induced cardioexcitatory response, suggesting that postsynaptic AT1-R in DMH may be involved in panic-responses. In one study, rats with disrupted GABAergic transmission in the DMH blocking AT1-R in the DMH by either saralasin and/or losartan attenuated panic response induced by panic inducing agents such as sodium lactate and noradrenergic agent yohimbine (123). This suggests that Ang-II via AT1-R induces panic responses by either modulating GABAergic neurotransmission in the DMH or potentiating AT1-R mediated DMH excitation in the absence of GABAergic input. The exact mechanism remains to be seen.

2.6 Conclusion

In conclusion, this chapter provides a summary of interplay between Ang-II signaling and GABAergic neurotransmission in different neuronal types in the CNS. Depending on the neuronal type, receptor subtype, and presynaptic or postsynaptic expression of receptors, Ang-II can either have a facilitatory or inhibitory effect on GABAergic neurotransmission. Though, Ang-II can activate a vast array of signaling factors through both G-protein dependent and independent signaling pathways, the exact mechanism underlying functional coupling of Ang-II and GABAergic transmission is not clear.

GABA is the primary inhibitory neurotransmitter in the CNS and is present in ~40% of all synapses. It is present in every brain region and fine-tunes neurotransmission. Dysfunction in GABAergic neurotransmission is involved in several brain disorders in different stages of life such as ADHD, epilepsy, autism spectrum disorder, depression, anxiety, and neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. In Parkinson's disease, one way to clinically diagnose and confirm the disease is by detecting specific changes in GABAa receptor subunits in the substantia nigra and caudate nucleus in the post-mortem brain of PD patients (124). α4 containing GABAa-Rs are reported to be increased by 22 fold in these brain regions. Also, GABA dysfunctions are implicated in PD associated non-motor symptoms such as depression and mood swings (125). All these studies suggest altered GABAergic neurotransmission in the pathophysiology of PD.

All the primary GABAergic afferents to pars compacta dopaminergic neurons is also summarized in this chapter. More than ~70% synapses made onto SNc dopaminergic neurons are believed to be GABAergic. GABAergic neurons in the SNr is one of the primary sources that provides fast and dense tonic inhibitory input onto SNc dopaminergic neurons through its axon

collaterals. Ang-II receptors, ACE, and other RAS components are reported to be expressed in both the SNc dopaminergic and SNr GABAergic neurons. Ang-II signaling has been demonstrated by numerous studies where it is suggested to have neurodegenerative effects on these neurons. However, it is not known if Ang-II signaling exists in the SNr GABAergic neurons. Therefore, we hypothesize that that Ang-II signaling can modulate the activity of SNr GABAergic neurons and regulate GABAergic neurotransmission in the substantia nigra, which can further alter dopaminergic cell activity and output. In the next chapter, we test our hypothesis and provide the first evidence of novel Ang-II signaling in the SNr GABAergic neurons, which involves AT1-R mediated facilitation of GABAa receptors and modulation of GABAergic neurotransmission in the substantia nigra. Our study provides a compelling evidence-based rationale to further explore and re-evaluate brain RAS's role in basal ganglia and disorders associated with it.

CHAPTER 3

ANGIOTENSIN II MODULATES GABA-ERGIC NEUROTRANSMISSION IN THE MOUSE SUBSTANTIA NIGRA

3.1 Overview

Angiotensin-II in the brain is known to affect cellular pathways that renders dopaminergic cells in the substantia nigra compacta (SNc) selectively vulnerable to degeneration in Parkinson's disease (PD). However, if Ang-II signaling can condition intranigral neurotransmission through afferent inputs to dopaminergic neurons is not known. The neighboring GABAergic projections neurons of the substantia nigra reticulata (SNr), through their extensive network of dendritic arbors and axon collaterals, provides major inhibitory input to SNc dopaminergic neurons. Herein, through use of transgenic mice, electrophysiology and optogenetics we provide the first evidence of novel AT1 receptor mediated Ang-II signaling in SNr GABAergic neurons, which resulted in suppression of their electrically evoked neuronal output via facilitation of postsynaptic $GABA_A$ receptor (GABAa-R) activity and prolongation of action potential duration. In contrast, Ang-II had no noticeable effect on the electrical properties of SNc dopaminergic neurons. Paradoxically, in response to phasic photoactivation of the population of SNr GABAergic neurons, Ang-II enhanced feedforward inhibitory input to SNc dopaminergic neurons, suggesting a non-linear relationship between cellular activity and neuronal output. Thus, our data shows a heterogenous effect of Ang-II on the activity of the two nigral cell types and reveals a complex non-linear effect of Ang-II on intranigral GABAergic neurotransmission. This novel angiotensinergic signaling in the SNr GABAergic neurons implies a role of brain renin-angiotensin-system in basal ganglia activity in normal health and disease and demands further inquiry.

3.2 Introduction

Renin-angiotensin-system (1) is usually associated with the peripheral cardiovascular system, however, numerous reports describe a fully-formed and independent central RAS ((14, 69, 126-128). Increasing evidence suggests that the peptide hormone angiotensin II (Ang-II), a primary RAS effector, contributes to neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (14, 69, 126). In animal models of PD, Ang-II-dependent activation of AT_1 receptors promotes dopaminergic neuronal cell loss in the substantia nigra compacta (SNc) (8, 17). Evidence also suggests that Ang-II evokes dopamine release in the rat striatum, and several studies report altered Ang-II receptor expression levels in tissue samples from Parkinson's patients (6, 15, 18, 129, 130). However, Ang-II receptors are widely expressed in both dopaminergic and GABAergic neurons in the SN of rodents and primates, including humans (15). Nonetheless, if Ang-II signaling can affect afferent GABAergic inputs to SNc dopaminergic neurons is not known.

More than 70% of synapses made on SNc dopaminergic neurons are GABAergic and these inhibitory inputs strongly regulates their activity and global output (80, 84). GABAergic projection neurons in the substantia nigra reticulata (SNr), located ventrolateral to the SNc, through their axon collaterals provides a major inhibitory GABAergic input to SNc dopaminergic neurons (84, 89, 131, 132). This intranigral inhibitory circuit is found to be involved in regulating phasic dopaminergic output during behaviors such as reward extinction and conditioned negative association (133, 134). Indeed, inhibition of SNr GABAergic neurons causes disinhibition bursting of SNc dopamine neurons and increases dopamine levels in the striatum as well as within the basal ganglia (83, 84, 88). Modulation of intra-basal ganglia DA neurotransmission via SNr GABAergic neurons is well established and contributes to basal

ganglia circuit dysfunction (90, 135). Interestingly, prior investigations show that Ang-II via activation of AT1 receptors regulates GABAergic neurotransmission in the anterior hypothalamus, amygdala, and median preoptic nucleus (96, 122, 136). Considering the strong expression of Ang-II receptors throughout SN and a predominantly GABAergic control of dopaminergic neurons, Ang-II signaling in the SNr GABAergic neurons is conceivable and could potentially modulate intranigral GABAergic and DAergic neurotransmission.

Herein, we tested the hypothesis that Ang-II signaling modulates the activity of GABAergic neurons in the mouse SNr and regulates GABAergic neurotransmission in SNc dopaminergic neurons. Using a combination of *ex vivo* brain slice electrophysiology and optogenetics on two lines of transgenic mice, one with fluorescent tagged dopaminergic neurons and other with cell-specific expression of channelrhodopsin 2 (ChR2) in SNr GABAergic neurons, we show that Ang-II via AT1 receptor-dependent mechanism acutely suppresses electrically evoked action potential (AP) firing of SNr GABAergic neurons. This effect was dependent on postsynaptic GABAa-receptors activation and prolonged action potential duration (APD) by Ang-II. Paradoxically, upon phasic photoactivation of SNr GABAergic neurons, Ang-II mediated suppression did not result in decreased GABA output, but rather enhanced feedforward inhibition of SNc dopaminergic neurons. This suggests that under synchronous activity Ang-II has a nonlinear effect on GABAergic neurotransmission in the postsynaptic dopaminergic neurons in the SNc. These studies provide evidence of angiotensinergic signaling in nigral GABAergic neurons as well its heterogenous effect on the two dominant cell types in the nigra and, also reveals a potential role of the intranigral microcircuitry which may further modulate the effect of Ang-II on GABAergic neurotransmission in the substantia nigra and the output of basal ganglia as a whole in health and disease.

3.3 Materials and Methods

Animals

We bred the following two mouse strains from The Jackson Laboratory to generate a tdTomato reporter mice for dopaminergic neurons (Fig. 3.1*A*): Cre-dependent tdTomato reporter mice [B6.Cg-*Gt(10)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J, stock #007909], and tyrosine hydroxylase (TH) promotor-driven Cre expression [B6.Cg-*7630403G23Rik*$^{Tg(Th\text{-}cre)1Tmd}$/J, stock #008601]. In order to focally stimulate SNr GABAergic neurons in *ex-vivo* slice we used a transgenic mice expressing ChR2 fused to yellow fluorescent protein under the control of mouse thymus cell antigen 1 (*Thy 1*) promoter (stock #007612, Jackson laboratory), which specifically expresses ChR2 in SNr GABA neurons, but not in SN dopamine neurons. All mice used for the study were between 4-8 weeks old. To detect transgene and floxed alleles, we sent samples to an automated genotyping service (Transnetyx) for standard polymerase chain reaction analysis.

Animal care and euthanasia

Mice received *ad libitum* access to standard chow and tap water while housed individually or in groups of less than four in a temperature and humidity-controlled room set on a 12-h light/dark cycle. On the day of experimentation, adult male and female mice were deeply anesthetized under isoflurane, decapitated, and the brains were removed and placed into ice-cold artificial cerebral spinal fluid (aCSF) containing (mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.4 NaH_2PO_4, 25 $NaHCO_3$, 11 D-Glucose, 2.4 $CaCl_2$, pH = 7.4, osmolarity = 310 mOsm) bubbled with 95% O_2/5% CO_2. All procedures, including euthanasia, were performed in strict accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee of Colorado State University.

Slice electrophysiology

Coronal slices (≈ 240 μM thick) containing the substantia nigra reticulata (SNr) and compacta (SNc) were cut (Leica VT1200S; Leica Microsystems), transferred to a holding chamber, and incubated for 30-60 min at 35°C in aCSF supplemented with MK-801 (100 μM), and stored at 21°C until used for experimentation. To record from cells, we transferred individual slices to a chamber continuously perfused with 21-22°C aCSF at a flow rate of 2-3 ml/min. Nigral GABAergic and dopaminergic neurons were identified and differentiated based on their well-established electrophysiological features (described below) as well as expressed fluorescence in the respective transgenic lines: TdTomato for dopaminergic neurons (TH-Cre-TdTomato) and YFP for GABAergic neurons (Thy1-ChR2-YFP).

Borosilicate glass pipettes, with resistances of 3-5 MΩ, were fabricated on a laser micropipette puller (Model P-2000; Sutter Instrument). Whole-cell current-clamp recordings of evoked spike activity used a potassium gluconate-based intracellular solution composed of (mM): 120 K-gluconate, 20 KCl, 10 HEPES, 0.2 EGTA, 2 $MgCl_2$, 10 phosphocreatine, 2 Mg-ATP, 0.3 Na-GTP, pH = 7.5 adjusted with KOH, osmolarity = 290-295 mOsm). Pipettes for recording spontaneous postsynaptic currents (PSCs) and miniature inhibitory PSCs (mIPSCs) contained a high chloride potassium methylsulfate-based solution composed of (mM): 57.5 K-methyl sulfate, 57.5 KCl, 20 NaCl, 1.5 $MgCl_2$, 5 HEPES, 0.1 EGTA, 10 phosphocreatine, 2 Mg-ATP, 0.3 Na-GTP, pH = 7.5 adjusted with KOH, osmolarity = 290-295 mOsm).

We visualized cells with a 40X water immersion objective on an upright microscope (Zeiss) equipped with Dodt gradient contrast and collected data with an EPC-10 USB patch-clamp amplifier controlled with PatchMaster software (v.2.30; HEKA Electronik). Data were low-pass filtered at 10 kHz and sampled at 50 kHz. We compensated for fast and slow capacitive

transients, series resistance and only used recordings with stable series resistances < 20 MΩ. To ensure the fidelity of our measurements, we continuously monitored electrophysiological parameters, including series resistance, leak, and membrane voltage. Cells with unstable recording parameters, such as leak change of > 20% and high series resistance, were flagged and not used. GABAergic SNr neurons were electrophysiologically identified and distinguished from dopaminergic neurons by their well-characterized electrophysiological profile (134, 137-139).

After gaining whole-cell access, cells were held at -65 mV (corrected for a liquid junction potential of 5 mV (140)). Following stabilization, cells were hyperpolarized stepwise from -65 mV to -140 mV to measure I_h and provide an initial characterization of the cell as either GABAergic or dopaminergic. Evoked APs were then recorded in response to 2s long current injections, increasing stepwise from -50 pA to 150 pA, for at least 5 mins. After obtaining a series of stable recordings with aCSF (control), we superfused cells with aCSF supplemented with Ang-II (500 nM) for a minimum of 5 mins and recorded evoked APs for ≥ 15 mins. To conclude the experiment, Ang-II was washed out by superfusion with standard aCSF for at least 5 mins before any more recordings were made. For experiments with the AT_1 receptor antagonist losartan (1 μM) and the $GABA_AR$ antagonist picrotoxin (1 μM), each drug was superfused alone or with Ang-II. For these recordings, we used the same experimental design as used for Ang-II alone.

We recorded spontaneous whole-cell outward PSCs with a high chloride (57.5 mM) internal solution (described above) at a liquid junction potential-corrected setting of -70 mV. We did not correct for leak and discarded any cell with leak > ± 100 pA or a change in leak > ± 50 pA during the length of the recording. Ang-II was either bath perfused for 3-5 mins or puffed using a 1-3 MΩ glass pipette positioned ahead of the recording pipette and in the direction of

laminar flow with Picospritzer III (Parker, Cleveland, OH). To isolate and record spontaneous mIPSCs at a holding potential of -70 mV, we blocked excitatory synaptic transmission using a cocktail of drugs: the voltage-dependent sodium blocker tetrodotoxin (TTX; 500 nM), the AMPA/kainate receptor antagonist CNQX (1 µM), the NMDA receptor antagonist MK-801 (1 µM), and the nicotinic receptor antagonist hexamethonium bromide (100 µM). IPSC's were confirmed to be mediated by $GABA_A$ receptors with picrotoxin (100 µM).

Photostimulation and Slice Electrophysiology

Light evoked EPSPs from ChR2 expressing SNr GABA neurons were activated by giving three 100-ms long light pulse with an interstimulus interval of 2-s using a 470 nM LED (Thorlabs) driven by LEDD1B driver (Thorlabs). Intrabusrt EPSPs or spikes from three light-evoked responses were averaged as one recording and at least 3-5 recordings were used from each group to get a final average. K-Gluconate based internal solution (described above) was used for recordings of both light evoked EPSPs and IPSCs. IPSCs in SNc dopaminergic neurons were recorded in the presence of excitatory synaptic blockers and were identified as outward current deflections in response to photostimulation of SNr GABA neurons Only SNc dopaminergic neurons showing outward current deflections in response to photostimulation of SNr GABAergic neurons were used for the study. Similarly, neurons showing light evoked excitatory postsynaptic potentials (EPSPs) were considered GABAergic, whereas, neurons showing light evoked IPSPs were considered dopaminergic. EPSPs and IPSPs were recorded in current clamp mode at -65 mV (adjusted for junction potential) using the K-Gluconate based internal solution.

Drugs

We purchased CNQX, MK-801, Ang-II, hexamethonium bromide, losartan, and picrotoxin from Sigma; and TTX from Tocris Biosciences. Drugs were prepared immediately before use in either distilled water (Ang-II, TTX, losartan, hexamethonium bromide) or DMSO (CNQX, MK-801, picrotoxin) and diluted in aCSF to achieve the desired concentration.

Experimental Design and Statistical analysis

Data were analyzed using Axograph X and GraphPad Prism (v.8) software. We used one-way, two-way ANOVA's, mixed-effect analyses and paired student t-test as indicated. Individual points in the figures represent data from a single cell. We performed only one experiment per brain slice and obtained no more than three recordings per mouse. We used male and female mice for all experiments. However, a sex-difference analysis revealed nothing of significance; thus, we pooled our data. Averaged data are presented as the mean ± SEM unless otherwise specified; n = number of cells recorded from and significance was defined as $p < 0.05$ unless otherwise indicated.

3.4 Results

To test our hypothesis that Ang-II regulates the activity of GABAergic projection neurons in the mouse SNr, we formulated the following requisite experimental criteria: 1) exogenous Ang-II must alter the AP firing characteristics of positively identified SNr GABAergic neurons in *ex vivo* brain slices; 2) Ang-II must promote changes in the electrophysiological properties of SNr GABAergic neurons by mechanisms consistent with the observed changes in AP firing behavior; 3) the observed effects of Ang-II must be sensitive to pharmacological blockade of cognate Ang-

II receptors and; 4) Ang-II should suppress light-evoked EPSPs in ChR2 expressing SNr GABAergic neurons and; 5) Ang-II must modulate GABAergic input onto postsynaptic SNc dopaminergic neurons.

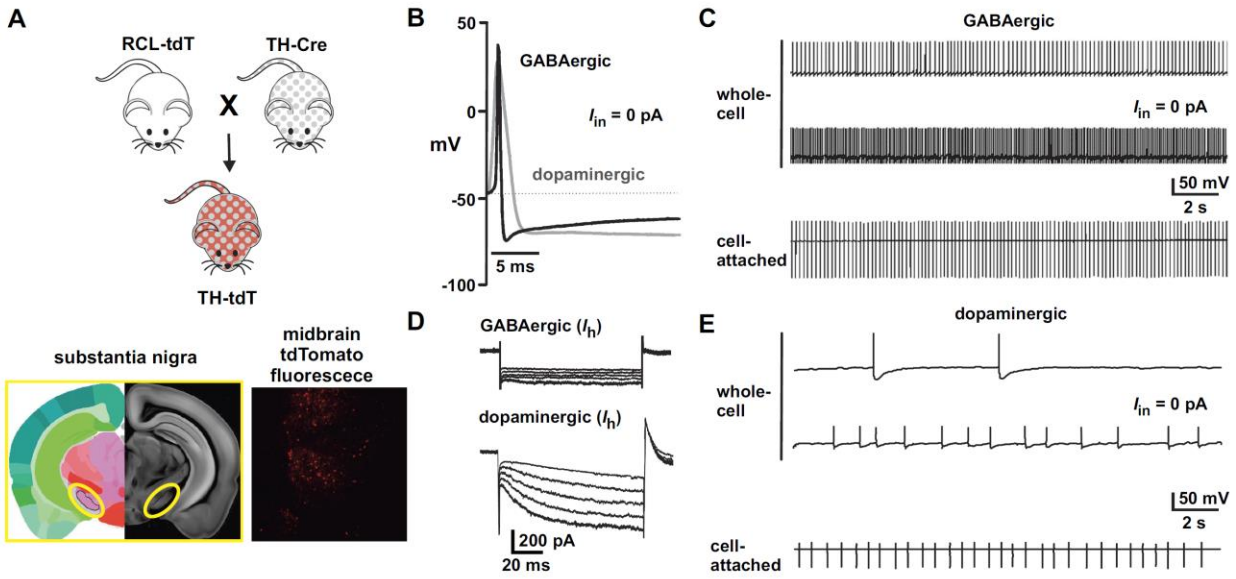

Figure 3.1. Electrophysiological Characteristics of GABAergic and dopaminergic neurons in the mouse substantia nigra. A, RCL-tdT Cre reporter mice crossed with tyrosine hydroxylase (TH) promotor-dependent Cre expressing mice with td-Tomato expression (red fluorescence) restricted to TH expressing dopaminergic neurons. Snr GABAergic neurons were differentiated from SNc dopaminergic neurons by lack of td-Tomato fluorescence and distinct electrophysiological profile. B, Snr GABAergic neurons have a narrower (< 1.5 ms) action potential width than SNc dopaminergic neurons (> 3 ms). C, Representative sustained high-frequency firing of Snr GABAergic neurons recorded in whole-cell (top) and cell attached configuration (bottom). D, Snr GABAergic neuorns, in contrast to SNc dopaminergic neurons, show little to no Ih in response to a series of hyperpolarizing pre pulses (-70 to -140 mV). E, Representative low-frequency, slow, and slow irregular pacemaker-like firing in SNc dopaminergic neurons.

Ang-II Suppresses Evoked Action Potentials in SNr GABAergic Neurons

We performed *ex-vivo* whole-cell electrophysiology on freshly prepared coronal midbrain slices from TdTomato dopaminergic neuron reporter mouse to investigate the effects of Ang-II

on GABAergic projection neurons in the SNr. To begin, we used whole-cell current-clamp to record spontaneous and depolarization-evoked action potentials. As a means to provisionally identify and distinguish GABAergic neurons from dopaminergic neurons, we selected cells by their lack of tdTomato fluorescence and neuroanatomical features (Fig. 3.1*A*; see Materials and Methods). After obtaining electrophysiological access, we confirmed or contested cell identities using the well-characterized electrophysiological profiles of GABAergic and dopaminergic neurons (88, 139, 141, 142). Thus, criteria used to categorize cells as GABAergic included: 1, an apparent absence of tdTomato fluorescence; 2, the presence of sustained high-frequency spontaneous AP firing (> 10 Hz); 3, an action potential width of < 2 ms; 4, little or no hyperpolarization currents; and 5, minimal adaptation to injected depolarizing currents (Fig. 3.1*B,C*, *D*, *& E*). Alternatively, we identified neurons as dopaminergic based on their cell size, anatomical location, detectable tdTomato fluorescence, slow pacemaker-like AP firing, action potential durations > 2 ms, robust hyperpolarization currents, and pronounced adaptation to injected depolarizing currents.

To achieve a relatively controlled measure of AP activity, we used an evoked AP protocol consisting of 2-s current injections increasing incrementally from -50 pA to +150 pA. In cells identified as GABAergic, following control recordings in unsupplemented aCSF, bath-applied Ang-II (0.5 μM) decreased evoked AP firing during current injections of 50, 100, and 150 pA (Fig. 3.2*A* *&* 3.2*B, n* = 17, $p = 0.003$ for 50 pA, $p < 0.001$ for 100 pA and 150 pA; mixed-effect analysis). In 10 out of 17 cells, the effect of Ang-II on evoked AP firing was at least partially reversible upon washout. Illustrating the degree of AP firing suppression, Ang-II increased the mean interspike interval approximately 2 folds (Fig. 3.2*C*; n = 17, $F_{(2,29)} = 5.976$, *p*

= 0.005; Mixed effect analysis). Additionally, Ang-II also increased the variability (i.e., irregularity) of the interspike intervals (Fig. 3.2*D*; $n = 17$, $F_{(2,28)} = 5.488$, $p = 0.01$; Mixed effect

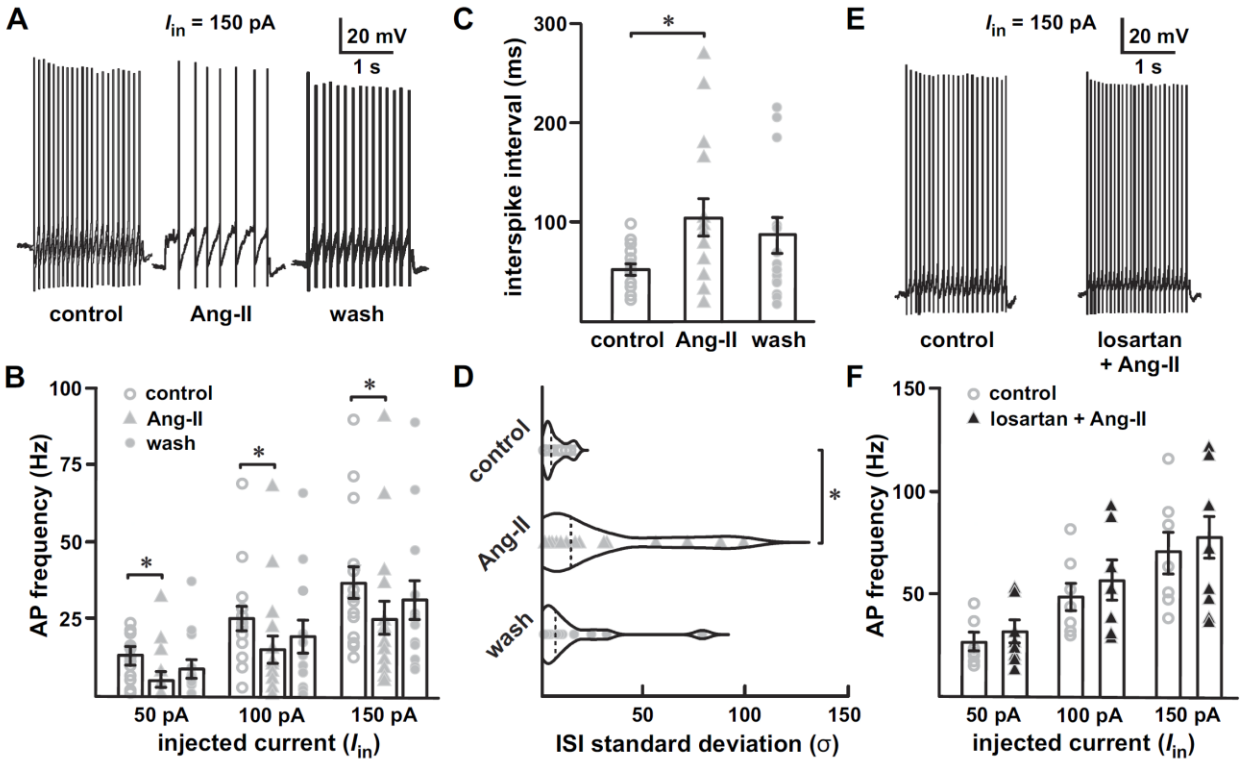

Figure 3.2. Angiotensin-II decreases SNr GABAergic projection neuron spike firing. ***A***, Evoked action potentials in response to 150 pA current injection. ***B,*** Individual scatter plot of evoked spike frequency in the SNr GABAergic projection neurons in response to 50 pA, 100 pA, and 150 pA current injections. Ang-II significantly decreased evoked firing in all stimulus levels, 50 pA-150 pA (*** $p < 0.001$, two-way repeated-measures ANOVA). In 10 out of 17 cells, washout partially reversed the effect of Ang-II. ***C***, Ang-II increased the SNr GABAergic interspike interval (ISI; ** $p = 0.005$, mixed effect analysis) and (***D***) increased the irregularity of firing as quantified by the ISI standard deviation (* $p = 0.01$, mixed effect analysis)***. E & F,*** The AT_1-R specific blocker losartan (1 µM) abolished the suppression of evoked SNr GABAergic spike firing by Ang-II.

analysis). Indicative of AT_1 receptor involvement, preincubation with losartan (1 µM) abolished decreased evoked AP firing following Ang-II application (Fig. 3.2E & 3.2*F*). To test for potential contributions by Ang-II type 2 receptors (AT2Rs), we used the specific AT2R

antagonist PD123319. In contrast to AT_1 receptor blockade with losartan, PD123319 (1 μM) did not alter Ang-II-dependent suppression of SNr GABAergic neuronal activity ($n = 5$, $F_{(1,4)} = 5.896$, $p = 0.072$, Repeated measures Two-way ANOVA; data not shown). From these data, we conclude that Ang-II decreases evoked AP firing in SNr GABAergic neurons via AT_1 receptor signaling. Note that we collected comparable data on identified SNc dopaminergic neurons (data not shown). However, the well-described adaptive responses of dopaminergic neurons to depolarization, and eventual depolarization block, precluded meaningful interpretation of these data (139, 142).

Ang-II Prolongs Action Potential Durations in SNr GABAergic Neurons.

Sustained spontaneous high-frequency AP firing (10 – 15 Hz, *in vitro*) is a striking characteristic of SNr GABAergic neurons (139, 142-144). The high-frequency firing in these cells is autonomously generated and involves multiple ion channels from more than five families (143, 145-147). We found that in SNr GABAergic neurons, Ang-II prolonged the duration of high-frequency APs in a largely reversible fashion (Figs. 3.3 & 3.4). Indeed, Ang-II increased the half-width of both evoked (Fig. 3.3*C*; $n = 17$; $F_{(2,29)} = 5.440$; $p = 0.010$; mixed-effect analysis) and spontaneous APs (Fig. 3.4*C*; $n = 5$; $F_{(2,6)} = 5.808$; $p = 0.040$; one-way repeated measures ANOVA). The effects of Ang-II on AP duration were prevented by preincubation with losartan (1 μM; Fig. 3.4*B*).

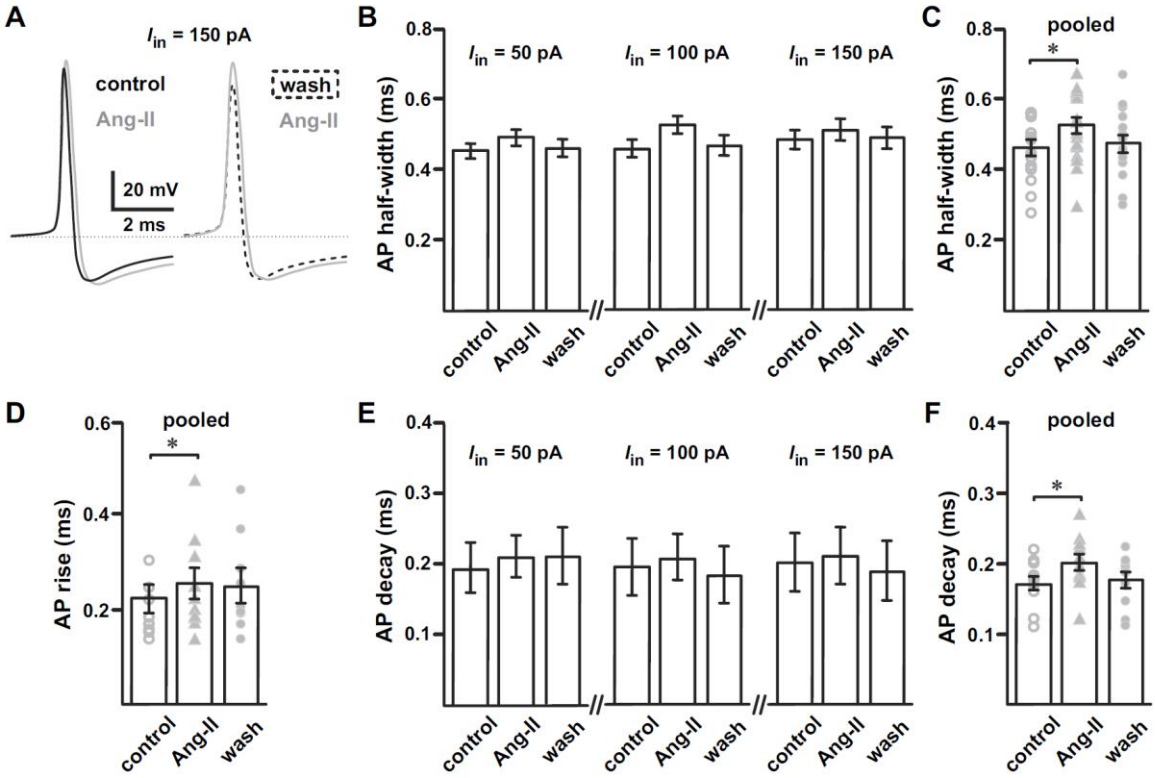

Figure 3.3. Ang-II slows the action potential kinetics of SNr GABAergic neurons. ***A***, Representative AP waveforms of SNr projection neurons showing that Ang-II reversibly slows AP kinetics. ***B & C,*** Ang-II increased the AP half-width of SNr GABAergic neurons (* $p = 0.010$, mixed-effect analysis). ***D***, Ang-II slowed the rise of APs in SNr GABAergic neurons (** $p = 0.005$, repeated measures one-way ANOVA). ***E & F,*** Ang-II slowed the decay of APs in SNr GABAergic neurons ($^{**}p = 0.004$, repeated measure one-way ANOVA).

Further analysis of evoked APs in Ang-II-responsive SNr GABAergic neurons (i.e., those cells showing AP prolongation with Ang-II; 11 of 17 cells), revealed slower AP kinetics after Ang-II application. Specifically, Ang-II slowed evoked AP rise-time (Fig. 3.3*D*; $n = 9$; $F_{(2,20)} = 7.166$; $p = 0.005$; Repeated measures one-way ANOVA) and slowed AP decay (Fig. 3.3*F*; $n = 11$; $F_{(2,20)} = 7.577$; $p = 0.004$; Repeated measure one-way ANOVA). Although no apparent effect on spontaneous AP rise times was evident, Ang-II also slowed the decay of spontaneous APs in GABAergic neurons (Fig. 3.4*C*; $n = 5$; $F_{(2,6)} = 9.207$; $^{*}p = 0.015$; Repeated measures one-way

ANOVA). Interestingly, and contrasting SNr GABAergic neurons, cells identified as dopaminergic had no observable changes in AP duration or kinetics in response to Ang-II (Fig. 3.4*D*). These data suggest that AT_1 receptor activation by Ang-II prolongs AP durations of SNr GABAergic but not SNc dopaminergic neurons. Further, the observed suppression of AP firing of SNr GABAergic neurons following Ang-II administration could arise, at least in part, as a consequence of AP prolongation due to a slowed rise and decay kinetics.

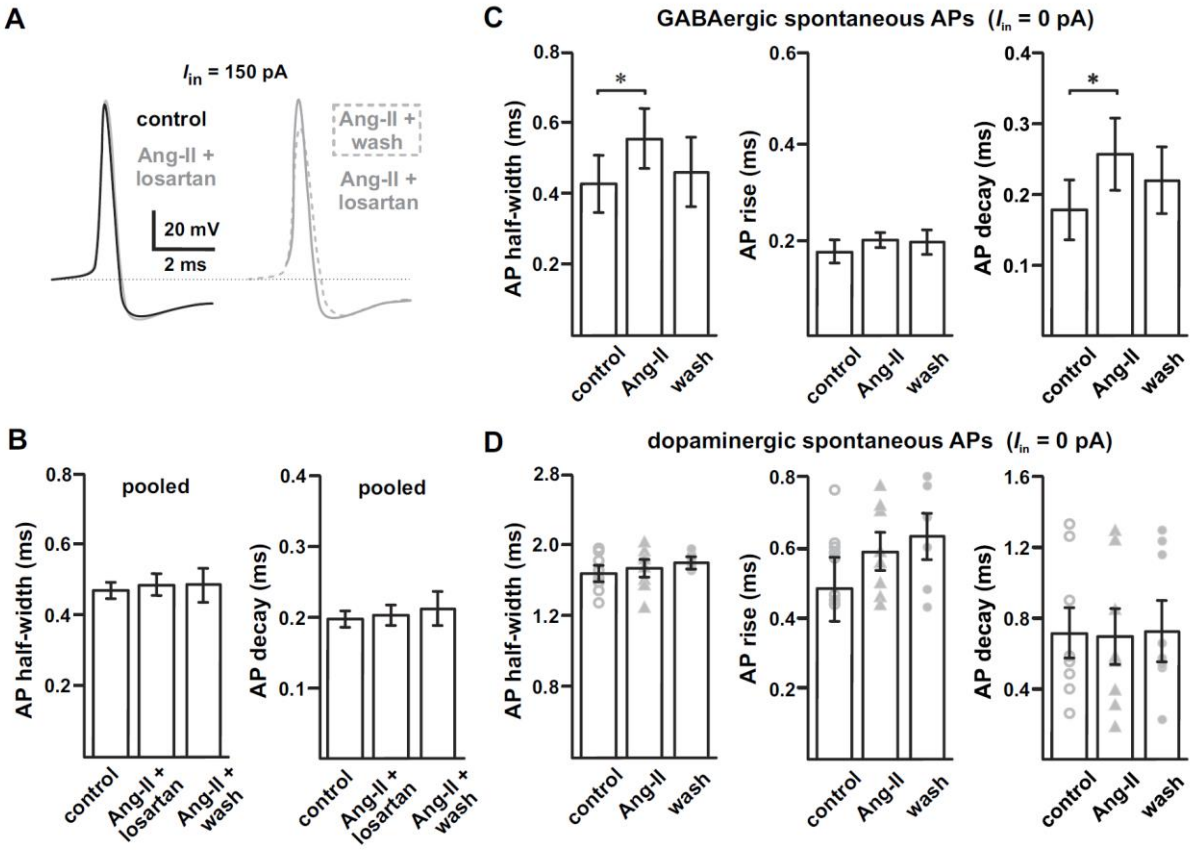

Figure 3.4. Disparate effect of Ang-II on SN GABAergic and dopaminergic neurons. ***A,*** Losartan blocks Ang-II mediated increase in AP duration in SNr GABAergic neurons; losartan washout with continued Ang-II perfusion prolonged the AP duration. ***B***, Summary data showing that losartan (1 μM) blocks Ang-II mediated increase in AP half-width and decay. ***C,*** Ang-II increased the duration of SNr GABAergic neuron spontaneous APs (* p = 0.04, AP half-width; * p = 0.015, AP decay; repeated measures one-way ANOVA). ***D,*** Unlike SNr GABAergic neurons, Ang-II had no noticeable effect on the AP kinetics of SNc dopaminergic neurons.

Ang-II Potentiates Postsynaptic $GABA_A$ Receptors in SNr GABAergic Neurons

Striatal GABAergic medium-sized spiny neurons provide tonic inhibitory input to the SNr GABAergic neurons (80, 148-150). These GABAergic neurons in the SNr express mostly

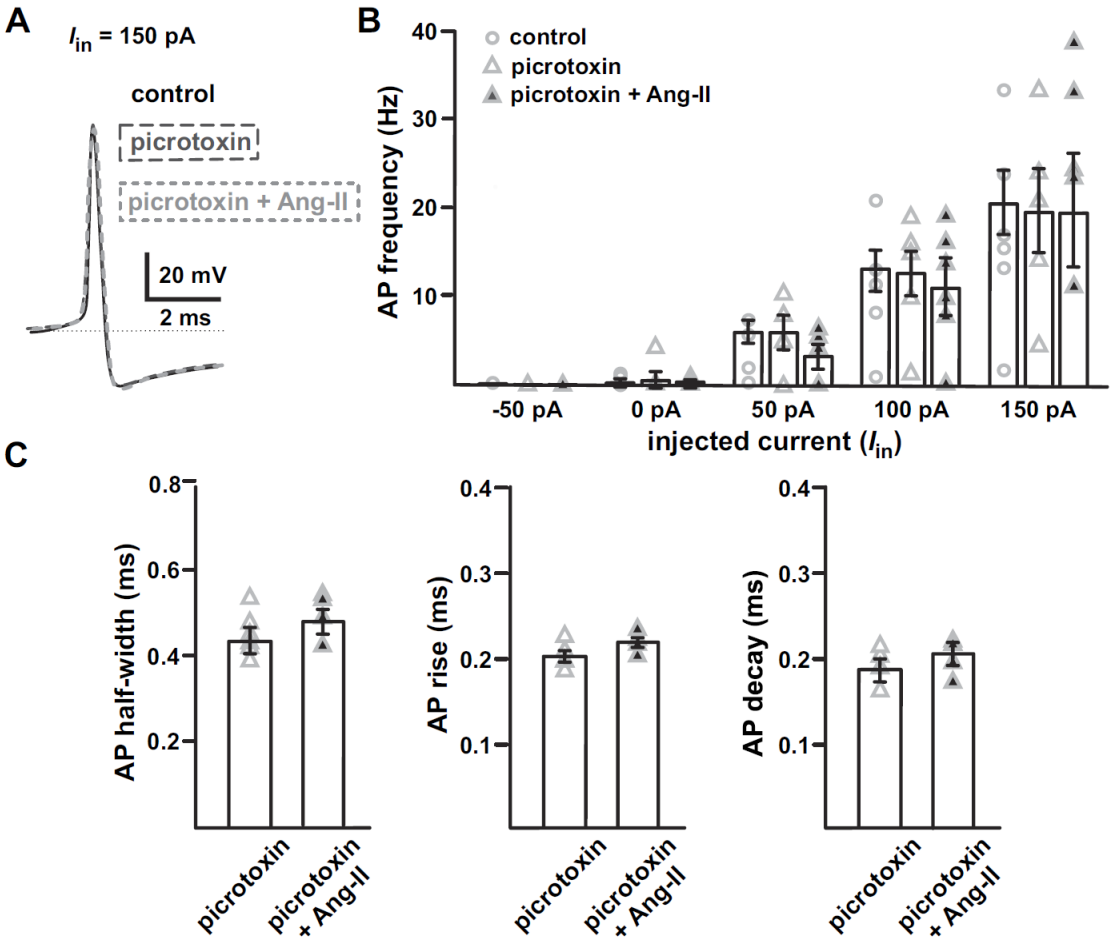

Figure 3.5. $GABA_A$ receptor blockade prevents Ang-II mediated suppression of evoked spike firing in the SNr GABAergic projection neurons. ***A***, Overlaid spike waveforms for control conditions, in the presence of the $GABA_A$ receptor antagonist picrotoxin (1 μM), and in the presence of picrotoxin plus Ang-II. ***B,*** Summary plot showing evoked spike firing of SNr GABAergic neurons under control conditions, in the presence of picrotoxin (1 μM), and in the presence of picrotoxin plus Ang-II. ***C,*** $GABA_A$ receptor blockade with picrotoxin attenuates Ang-II mediated increases in SNr GABAergic neuron AP durations.

$GABA_A$ receptors, but few $GABA_B$ receptors. Indeed, electrical stimulation of the striatum produces short-term inhibition of SNr GABAergic neurons that is blocked by the $GABA_A$ receptor antagonists such as picrotoxin (148, 151, 152). To test if the observed suppression of SNr GABAergic neuronal activity by Ang-II involves $GABA_A$ receptors (see Fig. 3.2), we replicated our evoked AP experiments in the presence of $GABA_A$ receptor antagonist picrotoxin. Suggesting $GABA_A$ receptor involvement, preincubation with picrotoxin (1 μM) partially blocked Ang-II-dependent suppression of GABAergic neuronal firing (Fig. 3.5*B*). Interestingly, picrotoxin partially blocked but did not completely attenuate Ang-II mediated increase in AP half-width (Fig. 3.5C; $n = 5$, $p = 0.119$; Student's paired t test), rise time (Fig. 5C; $n = 5$, $p = 0.103$; Student's paired t test) and decay (Fig. 6*C*; $n = 5$, $p = 0.101$; student's paired t-test) in these cells. These data suggest that Ang-II potentially suppresses the excitability of SNr GABAergic neurons by two independent mechanisms (slowed AP kinetics and potentiation of $GABA_A$ receptors).

To further examine the effects of Ang-II-dependent modulation of $GABA_A$ receptor activity in SNr GABAergic projection neurons, we recorded spontaneous postsynaptic currents (PSCs) (Fig. 3.6). Although Ang II did not significantly alter the frequency or average amplitude of spontaneous PSCs in these cells (Fig. 3.6*B*), we did observe a roughly two-fold increased incidence of high-amplitude spontaneous PSCs, which are 3X the mean amplitude (≥ -150 pA) (Fig. 3.6*B*; $n = 9$; $p = 0.012$; paired Student's *t*-test). Finally, we replicated our spontaneous PSC experiments in the presence of AP and excitatory synaptic blocker cocktail (see Materials and Methods) to confirm that the Ang-II-dependent increases in PSCs are mediated postsynaptically. Synaptic blockade drastically reduced the overall activity in our recordings to reveal the presence of spontaneous miniature IPSCs (mIPSCs; Fig. 3.7*A*). Similar to our experiments in the absence

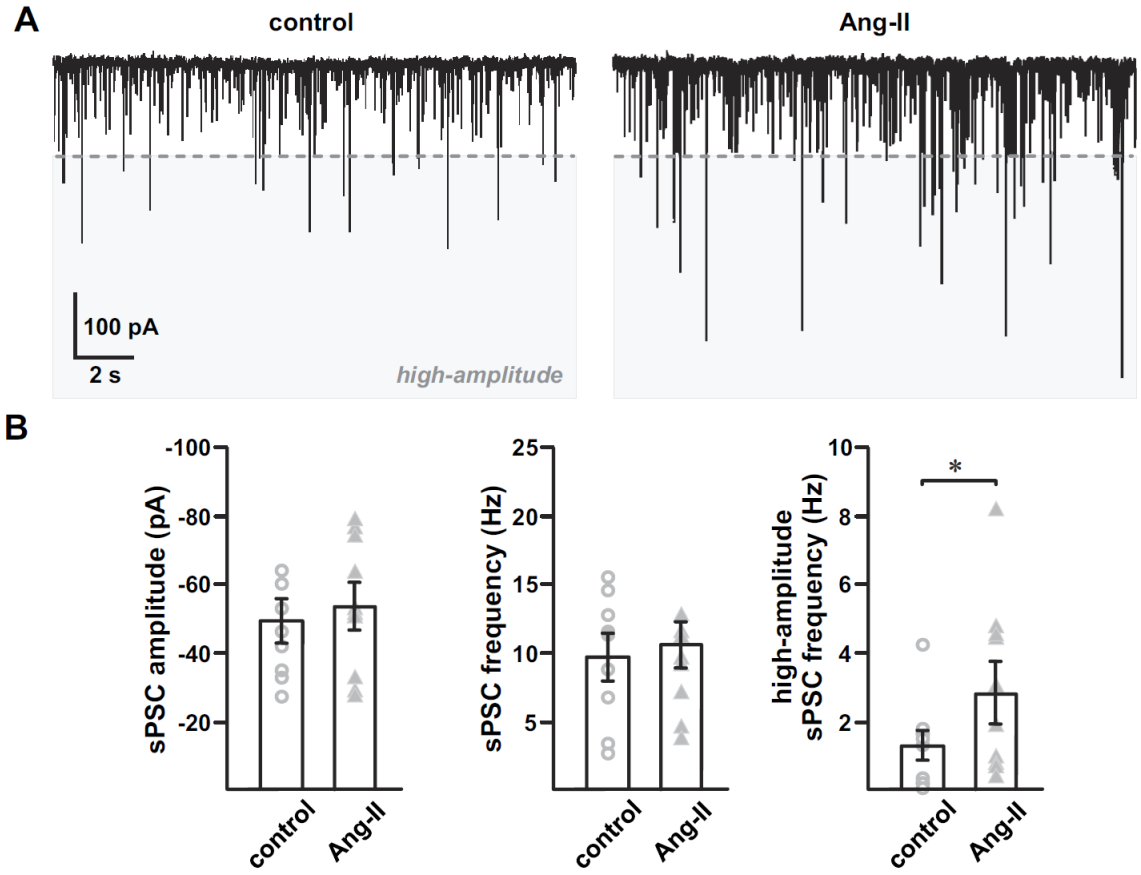

Figure 3.6. Ang-II increases SNr GABAergic neuron spontaneous postsynaptic currents. ***A***, At a holding potential of - 65 mV (junction potential corrected), spontaneous postsynaptic currents (PSCs) were recorded with a high chloride (57.5 nM) internal pipette solution before and after 5 min of Ang-II (500 nM) exposure. ***B***, Ang-II did not change the average PSC amplitude ($p = 0.211$, paired Student's t-test) and frequency ($p = 0.466$, paired Student's t-test) of spontaneous postsynaptic Cl^- currents. However, Ang-II increased the observed incidence of high-amplitude spontaneous postsynaptic currents (*right*; * $p = 0.012$; paired Student's t-test). High-amplitude spontaneous PSCs, demarcated by the dashed line, were defined a priori as currents with an amplitude ≥ three times the mean current amplitude under control conditions.

of high amplitudes, which are ≥ 3X the mean amplitude (≥ -121.86 pA) approximately by 2.5 folds without a significant change in the average mIPSC amplitude or frequency (Fig. 3.7*A*, 3.7*C*, 3.7*D* & 3.7*E*; $n = 12$; $p = 0.001$; paired Student's t-test). Notably, picrotoxin (1 µM) abolished the Ang-II-dependent increase in high-amplitude mIPSCs (Fig. 3.7) in all recorded

cells. We repeated the experiment using local puff of Ang-II using Picospritzer III but, interestingly, did not observe any noticeable increase in high amplitude mIPSC's (data not shown). However, when Ang-II was superfused back in the bath, increase in high amplitude IPSCs were restored (data not shown). This suggests that the GABAa-Rs affected by Ang-II are most likely located in distant dendritic terminals away from the soma and are embedded deep in the slice which cannot be activated by transient topical puff of Ang-II. From these data, we conclude that Ang-II potentiates postsynaptic $GABA_A$ receptors in SNr GABAergic neuron,

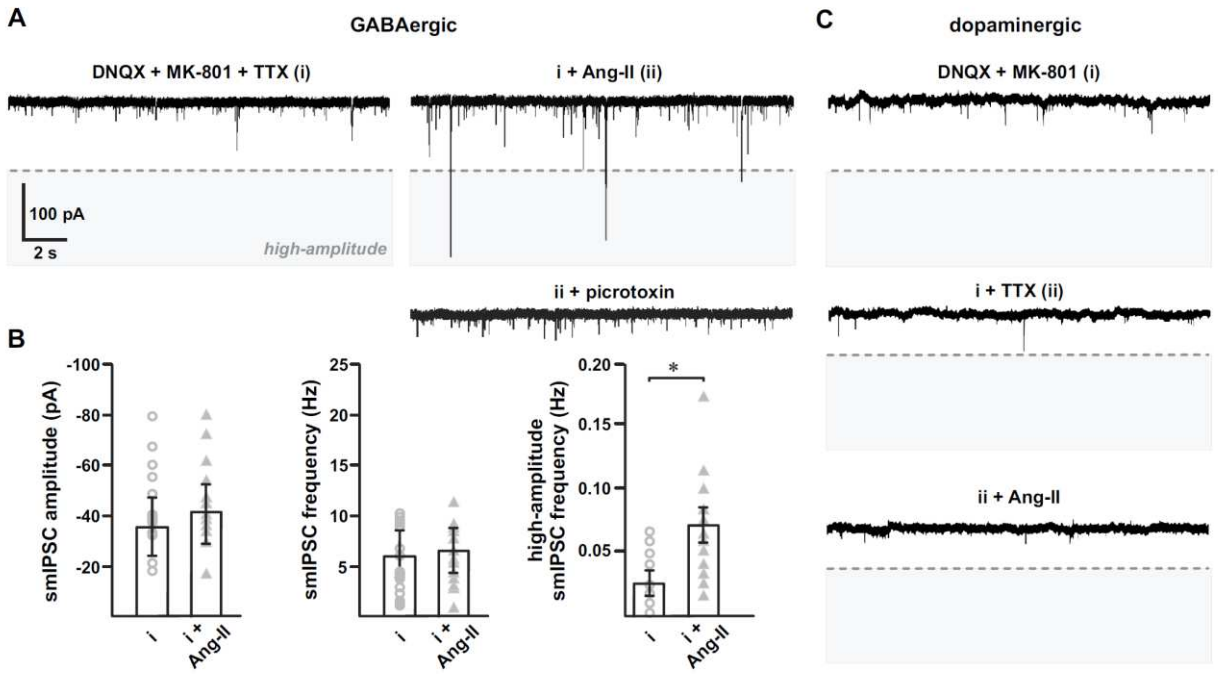

Figure 3.7. Ang-II increases picrotoxin-sensitive spontaneous miniature IPSCs in SNr GABAergic neurons. Spontaneous mIPSCs recorded from SNr GABAergic and SNc dopaminergic neurons in the presence of excitatory synaptic blockers (DNQX, MK-801; 1 μM)) and TTX (500 nM). ***A***, Representative traces showing Ang-II-mediated enhancement of high-amplitude postsynaptic $GABA_A$ receptor currents. High-amplitude spontaneous PSCs, demarcated by the dashed line, were defined a priori as currents with an amplitude ≥ three times the mean current amplitude recorded in the presence of excitatory synaptic blockers and TTX. ***B***, Summary data showing Ang-II-mediated increases in high-amplitude mIPSCs (p = 0.019, one-way repeated measures ANOVA), but not average mIPSC amplitude and frequency. ***C,*** In contrast to SNr GABAergic neurons, Ang-II had no detectable effect on spontaneous mIPSCs in SNc dopaminergic neurons.

which supports our initial observation of suppressed excitability of these nigral neurons by Ang-II (see Fig. 3.2).

GABAa Receptors in SNc Dopaminergic Neurons are Not Affected by Ang-II

We also recorded spontaneous mIPSC's from SNc dopaminergic neurons and found minimal background mIPSC's compared to SNr GABA neurons (Fig 3.7*B*). Ang-II did not cause any detectable change in the spontaneous mIPSC's (Fig 3.7B) of SNc dopamine neurons (data not shown). This suggests that in basal state, in contrast to SNr GABA neurons, Ang-II has very little or no direct postsynaptic effect on the GABAergic activity in SNc dopaminergic neurons.

Ang-II Increases GABAergic Input to SNc Dopaminergic Neurons

In our previous experiments, Ang-II suppressed electrically evoked spike output in individual GABAergic neurons. However, activity of a single neuron is not representative of the population and inputs from individual neurons encodes for and translates into a characteristic population output. In order to induce phasic synchronous neural activity of the SNr GABAergic neuron population we took advantage of Thy1-ChR2-YFP mice which specifically expresses ChR2 in SNr GABAergic neurons but not in the nigral dopaminergic neurons. In order to confirm if Ang-II mediated suppression of electrically evoked firing of individual SNr GABAergic neurons is also sustained under phasic photoactivation of the population, we recorded EPSPs (Fig 8*A*) from SNr GABAergic neurons upon photoactivation by a 100 ms long 470 nm light pulse. Consisitent with our earlier observation, Ang-II (0.5 μM) decreased the number of intraburst spikes in 4 out of 6 recorded SNr GABAergic neurons by approximately

25% (Fig 8*B* & 8*C*). This further supports our preliminary observation of negative modulatory effect of Ang-II on electrically evoked spike output of SNr GABAergic neurons.

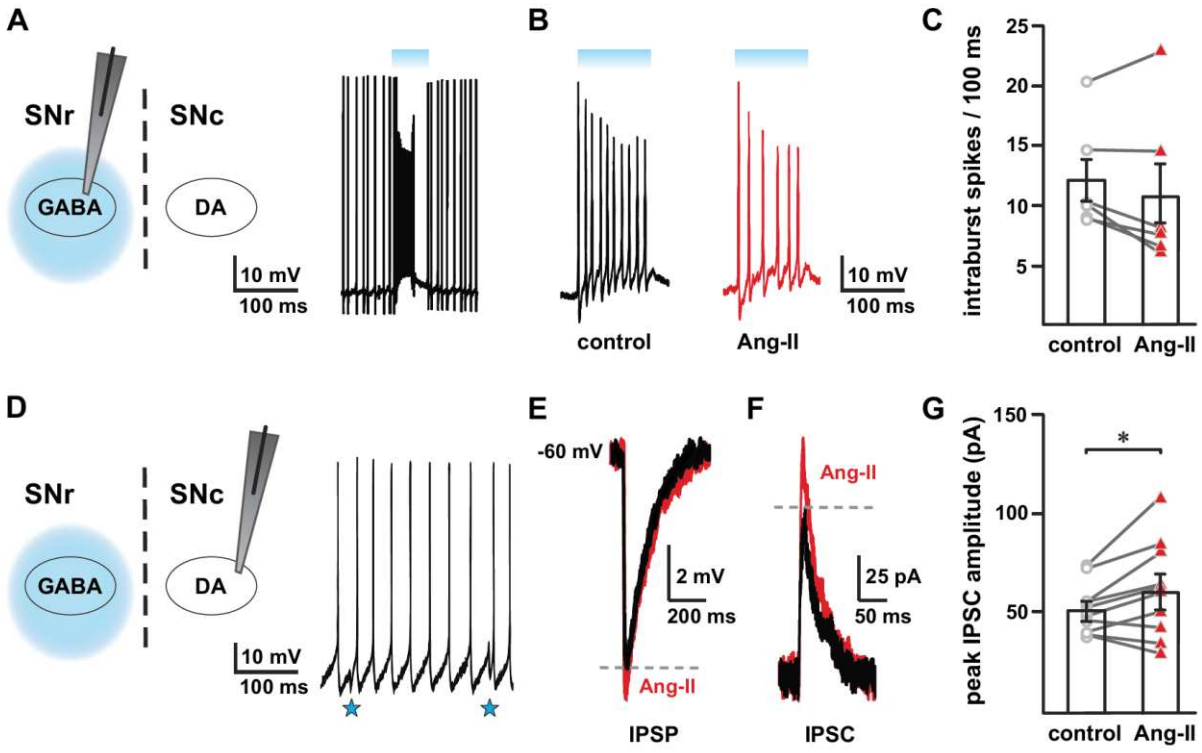

Figure 3.8. Ang-II modulates SN GABAergic neurotransmission. ***A,*** Light-evoked EPSPs (bursts) in SNr GABAergic neurons in response to a 100 ms long light pulse. ***B,*** Ang-II decreased light-evoked intraburst spikes in SNr GABAergic neurons; intraburst spikes shown are enlarged from *panel A*. ***C,*** Summary data showing the effect of Ang-II on intraburst spikes (EPSPs) in SNr GABAergic neurons in response to 100 ms long light pulse. In 4 out of 6 SNr GABAergic neurons, Ang-II decreased intraburst spikes by ~ 25 %. ***D,*** IPSPs recorded from SNc dopaminergic neurons in response to photostimulation of SNr GABAergic neurons, as shown by downward deflection of membrane potential (*blue stars*). Ang-II enhanced light-evoked inhibitory input onto SNc dopaminergic neurons as shown by an increase in the amplitude of light-evoked IPSPs (***E***) and IPSCs (***F & G***; * $p = 0.049$, paired Student's t-test).

SNr GABAergic neurons provides robust, monosynaptic inhibition of SNc dopaminergic neurons (84, 88, 91, 134). Therefore, an alternative way to measure the effect of Ang-II on

population output of SNr GABAergic neurons is to record from postsynaptic dopaminergic neurons in the SNc upon phasic photoactivation of SNr GABAergic neurons. Since Ang-II suppressed both electrically and light evoked activity of SNr GABAergic neurons, we should see decreased GABAergic input and disinhibition of postsynaptic SNc dopaminergic neurons. We recorded IPSCs and IPSPs from SNc dopaminergic neurons in response to light stimulation of SNr GABAergic neurons by 100 ms long light pulse. Paradoxically, we saw ~ 18% increase in the amplitude of light evoked IPSC's in SNc dopaminergic neurons (Fig 8*F* & 8*G*; $n = 10$; $p = 0.049$; paired Student's t-test). Consequently, we observed a proportional increase in the light evoked IPSP amplitude (Fig 8*E*). This shows that Ang-II mediated suppression of SNr GABAergic neurons, as demonstrated in our preceding experiments, does not have a reciprocal disinhibitory effect on the postsynaptic SNc dopaminergic neurons upon synchronous population activity, but strengthens feedforward inhibition of nigral dopaminergic neurons. This suggests a non-linear effect of Ang-II on cellular activity and population output of SNr GABAergic neurons.

3.5 Discussion

In this study, we tested the hypothesis that Ang-II signaling exists in nigral GABAergic neurons, exerts an acute modulatory influence on mouse SNr GABAergic projection neurons, and regulates inhibitory feedforward input to SNc dopaminergic neurons. We utilized whole cell recordings and optogenetics in transgenic mice with markers for nigral GABAergic and dopaminergic neurons and cell specific expression of ChR2 in SNr GABAergic neurons We utilized midbrain coronal slices to minimize the influence of presynaptic connections and carry out electrophysiological recordings in relative isolation. For our experiments, we used 500 nM

Ang-II, a high working concentration for a ligand-receptor pair with a Kd less than 5 nM. However, we opted to use an elevated Ang-II concentration to ensure adequate penetration of bath-applied Ang-II into the slice during our recordings.

Canonical AT_1 Receptor Signaling Acutely and Reversibly Suppresses Evoked Action Potentials in SNr GABAergic Neurons.

In neurons identified as GABAergic, we found that acute application of Ang-II decreased evoked AP firing frequencies. We found that the effects of Ang-II on SNr GABAergic neurons were sensitive to AT_1 receptor blockade by the specific antagonist losartan. This is consistent with AT1R mediated modulation of GABAergic neurotransmission in the neurons of amygdala and hypothalamus. Suggestive of a dynamic regulatory mechanism, Ang-II suppression of SNr GABAergic AP firing was largely reversible upon washout.

In contrast to the blockade of AT_1 receptors with losartan, we observed no attenuation of Ang-II-dependent modulation of SNr GABAergic neuronal APs with the specific AT_2 receptor antagonist PD123319. Neither losartan nor PD123319 alone altered baseline AP firing characteristics in SNr GABAergic neurons. Additional angiotensin-related signaling modalities, such as other angiotensin molecules (e.g., IV and 1-7) and receptors (e.g., AT_4 receptor and *Mas* receptors), could potentially participate in SNr GABAergic neuromodulation. Contributions by these mechanisms are likely minimal at best, given the relative abundance of Ang-II and AT_1 receptors and our results with losartan blockade of AT_1 receptors.

Ang-II Slows Action Potential Kinetics and Increases Firing Variability of SNr GABAergic Neurons.

Waveform analysis of Ang-II-responsive SNr GABAergic neuron APs revealed that Ang-II slows AP decay & rise, prolongs AP duration of both evoked and spontaneous APs (except AP rise-time) and also promoted variability in the pattern of AP firing. This is consistent with reported changes in the electrical activity of many cell types exposed to Ang-II (109, 153, 154). Fast delayed rectifier, Kv3.1 and KV3.4 channels, are primarily responsible for maintaining high sustained AP firing of SNr GABAergic neurons (144, 147, 155). Since AT1-receptors are reported to inhibit delayed rectifier potassium channels in the hypothalamus and brain stem neurons through a Gq-coupled protein kinase-II dependent pathway (156), a similar mechanism may underlie the inhibitory effect of Ang-II on SNr GABAergic neurons. Future investigations are necessary to identify and mechanistically characterize ion channels potentially modulated by AT_1 receptors in these cells.

Ang-II Activates Postsynaptic GABAa-R in SNr GABAergic Neurons.

SNr GABAergic neurons receive robust inhibitory input from the striatum and external globus pallidus (90, 92, 157). Postsynaptic GABAa-R mediate majority of the inhibition in these neurons (151, 158)). Our data show that GABAa-R blockade with picrotoxin prevents Ang-II mediated suppression of evoked firing in SNr GABA neurons. Further, we observed an approximately 2.5-fold increase in picrotoxin-sensitive high-amplitude mIPSCs with Ang-II, but no noticeable change in average frequency and amplitude. This suggests that Ang-II activates postsynaptic GABAa-R in nigral GABAergic neurons. Consistent with this hypothesis, Ang-II

mediated facilitation of $GABA_A$-R activity is reported in neurons of the rat median preoptic nucleus (122).

We also recorded mIPSCs with local puff of Ang-II on SNr GABAergic neurons but found no detectable change in the mIPSCs. However, re-perfusion of Ang-II in the bath restored the increase in mIPSCs. This suggests that GABAa-Rs activated by Ang-II signaling are most likely expressed in distal dendritic terminals away from the soma embedded deep in the slice that cannot be penetrated and stimulated by transient puff of Ang-II. Further investigation is necessary to determine relative localization and the underlying mechanism of functional coupling between AT1-R and GABAa-Rs in SNr GABAergic neurons.

Ang-II Does Not Change the Action Potential Kinetics and Basal IPSCs in SNc Dopaminergic Neurons.

Evidence suggests that Ang-II signaling via AT_1 receptors impacts SNc dopaminergic neuronal function, homeostasis, and viability (8, 15, 17, 69). At present, it is unclear if the effects of Ang-II on SNc dopaminergic neurons are direct, indirect, or a combination of both. We recorded evoked APs, PSCs, and IPSCs from SNc dopaminergic neurons. We could not reliably obtain interpretable AP firing data as a consequence of the well-characterized adaptive response of dopaminergic neurons to depolarizing currents (data not shown). Unlike SNr GABAergic neurons, Ang-II had no noticeable effect on the AP kinetics, PSCs, and IPSCs of SNc dopaminergic neurons, suggesting a heterogeneous effect in the two nigral cell types.

Lack of detectable effect of Ang-II on dopaminergic neurons in our experiments could be due to significantly lower expression of GABAa-Rs, different subunit composition, and lack of canonical K^+-Cl^- co-transporter, KCC2a in nigral dopaminergic (158, 159). SNr GABAergic

projection neurons express KCC2 that actively pumps [Cl^-] outside the cell and maintains hyperpolarized (-71 mV) chloride reversal potential (160). In contrast, chloride reversal potential is set around -63 mV in SNc dopaminergic neurons due to lack of KCC2 (161). Therefore, GABAa receptor activation produces a significantly larger hyperpolarization in SNr GABAergic neurons than SNc dopaminergic neurons that may explain no detectable effect of Ang-II in the dopaminergic neurons.

Ang-II Decreases Light Evoked EPSPs in SNr GABAergic Neurons

Under synchronous activity, output of SNr GABAergic neuron population is controlled by feedback inhibition within the SNr (133). This is mediated through the axon collaterals of SNr GABAergic neurons, which in addition to making synaptic contacts with dopaminergic neurons in the SNc, also extends its collaterals to GABAergic neurons within the nigra and provide a significant gain control of their population output (84, 88, 133). In order to further confirm if the negative modulatory effect of Ang-II on SNr GABAergic neurons can also prevail under phasic synchronous population activity, we used a transgenic mice (THY1-ChR2-YFP) with cell specific expression of ChR2 in the GABAergic neurons but not dopaminergic. Consistent with our observations on electrically evoked activity of SNr GABAergic neurons, Ang-II decreased light evoked EPSPs (intraburst suprathreshold spikes) in 4 out of 6 recorded neurons by aproximately 25%. This further supports the inhibitory effect of Ang-II on SNr GABAergic neurons. However, considering the dense, complex and non-uniform arborizations of SNr axon collaterals, we suspect that the effect of Ang-II may not be homogenous in all SNr GABAergic neurons. Response of an individual nigral GABAergic neuron to Ang-II upon phasic stimuluation of SNr will depend on the extent of local connections it makes with other

neighboring GABAergic neurons. Therefore, intranigral feedback inhibition may further modulate the effect of Ang-II on the population output of SNr GABAergic neurons.

Ang-II Enhances Feedforward Inhibitory Input to SNc Dopaminergic Neurons

SNr GABAergic neurons provides robust, monosynaptic inhibition of SNc dopaminergic neurons (Tepper et al., 1995; Tepper and Lee, 2007; Brazhnik et al., 2008; Pan et al., 2013). An alternative measure of population output of SNr GABAergic neurons is the strength of their feedforward inhibitory input to postsynaptic SNc dopaminergic neurons. Paradoxically, Ang-II increased the amplitude of IPSC and IPSP in SNc dopaminergic neurons in response to photostimulation of SNr GABAergic neurons. This is inconsistent with Ang-II mediated suppression of SNr GABAergic neurons, which we expected to have a reciprocal disinhibitory effect on postsynaptic SNc dopaminergic neurons. This non-linear effect of Ang-II on SNr GABAergic neuronal activity and their output to postsynaptic SNc dopaminergic neurons suggests that there is a more complex system at play here, such as the extensive network of intranigral axon collaterals.

SNr GABAergic projections have both intranigral and extranigral collateralizations, and the non-uniform nature of these branchings can give rise to variability in conduction velocity, excitability, myelination, number of synaptic boutons, distribution of ion channels, and other aspects of signal propagation in neurons within a network (162-165). This would have an effect on the neuronal response properties at the microcircuitry level, such as that within the nigra. Consequently, summed activity arising from such heterogeneous interlinked network could result in a range of synchronous and/or asynchronous population activity and could have a varied effect on the postsynaptic population. Therefore, the paradoxical facilitation of inhibitory input to

nigral dopaminergic neurons by Ang-II upon phasic activation of SNr GABAergic neurons can be partially explained by the sophisticated and assymetrical nature of intranigral microcircuit arising from the intricate network of axon collaterals.

3.6 Conclusions

From these data, we conclude that Ang-II signaling occurs in the SNr GABAergic neurons via a postsynaptic AT_1R-dependent mechanism. Our data shows enhancement of postsynaptic GABAa receptors and slowing of AP kinetics by Ang-II as contributing factors for the suppressive effect of Ang-II in the SNr GABAergic neurons. However, this negative modulatory effect of Ang-II on SNr GABAergic neurons did not translate into disinhibition of postsynaptic SNc dopaminergic neurons, but rather enhanced the inhibitory input. This shows a non-linear effect of Ang-II on GABAergic neurotransmission in the postsynaptic dopaminergic neurons which we suspect is due to the complex and non-uniform intranigral inhibitory microcircuit formed by extensive arborizations of axon collaterals of SNr GABAergic neurons. Further investigation into the microcircuit dynamics, underlying signaling cascades and effector proteins (e.g., ion channels), and the ensuing effects on SNc dopaminergic neurons by Ang-II, is warranted.

Chapter 4

Conclusion

Independent RAS in the brain and its role in different brain tissue is still not well understood. Circumventricular organs in the brain lacking the blood-brain barrier (BBB) are subject to circulating RAS, and Ang-II affects their neuronal activity and their efferent targets in the brain guarded by the BBB. However, de novo synthesis of Ang-II, the primary effector peptide, is demonstrated in other brain areas, and the widespread expression of all RAS components in different brain tissues strongly suggests an extrarenal local RAS system in the CNS. Consequently, numerous studies suggest the role of Ang-II and other RAS peptides in memory, cognition, neuronal differentiation, neuroendocrine function, and neurodegenerative disorders. This indicates that the brain RAS has an intricate role in the brain, and the specific cellular and physiological outcomes of its signaling will depend on the specific brain tissue, neuronal type, and interaction with specific receptors.

In the periphery, Ang-II signaling affects various tissues through many signaling factors such as G-proteins, membrane lipids & proteins, intracellular peptides, kinases, lipases, other enzymes, proinflammatory cytokines & chemokines, reactive oxygen species, transcription factors, and ion channels. Of particular interest is Ang-II's ability to initiate G protein signaling through its receptors, AT1-R and AT2-R. G-protein signaling can initiate a cascade of downstream signaling that can activate various kinases, lipases, peptides, and transcription factors, further activating different second messenger pathways. Moreover, Ang-II can also directly activate receptors and peptides independent of G-protein signaling, such as NADPH oxidase, EGFR, β-arrestins, ERK-1/2 etc. This ability of Ang-II to affect myriad signaling

factors and multiple pathways makes it difficult to determine the precise mechanism behind its various physiological actions. The same is warranted for central RAS in the brain.

In the brain, local central RAS plays an essential role in centrally controlling cardiovascular homeostasis, sympathetic outflow, and neuroendocrine functions. This is primarily mediated through Ang-II's action on different hypothalamic nuclei, its afferent inputs, and the efferent targets. However, Ang-II's role in the brain is not just limited to hypothalamus. It also plays a significant role in many other brain regions involved in the pathology of neurodegenerative disorders such as Alzheimer's disease, multiple sclerosis, Huntington's disease, and Parkinson's disease. Numerous studies over the years have suggested the role of the pathological arm of Ang-II in the vulnerability of SNc dopaminergic to degeneration in Parkinson's disease. Primary Ang-II receptors, AT1-R and AT2-R, have been observed in the basal ganglia of rodents, monkeys, and humans. The findings from research on the potential role of Ang-II in Parkinson's disease's pathophysiology lead to studies of the effect of ACE inhibitors on human patients of PD as well non-patients, where ACE inhibitors decreased the risk of PD.

Various mechanisms underlie Ang-II mediated increase in dopamine cell vulnerability to degeneration. Increased cellular oxidative stress, increased intracellular calcium, mitochondrial dysfunction, and inducing apoptotic pathways are some of the leading mechanisms suggested for Ang-II's effect on dopaminergic neuronal health. In addition, Ang-II and its receptors expressed in microglia and astrocytes are also suggested to contribute to dopaminergic cell degeneration through microglial activation. However, if Ang-II signaling in other neurons may play a role in dopaminergic cell activity or degeneration is not known. Ang-II receptors are expressed throughout the substantia nigra. Dopaminergic neurons are heavily innervated by the neighboring GABAergic neurons in substantia nigra pars reticulata (SNr) and are under tonic inhibition from

these neurons, regulating their activity and cellular output. Nevertheless, if Ang-II signaling exists in these nigral GABAergic neurons is not known.

Experiments in our study demonstrated the evidence of novel Ang-II signaling in the SNr GABAergic neurons. Ang-II suppressed the evoked firing activity of these neurons through an AT1-R dependent mechanism. Interestingly, Ang-II increased the duration of action potential and facilitated postsynaptic GABAa receptors, which may underlie the mechanism behind the suppression of SNr GABAergic neuronal firing. Using transgenic mice, whole-cell electrophysiology, and optogenetics, we showed that Ang-II suppressed both electrically evoked and light-evoked phasic burst firing of SNr GABAergic neurons. However, Ang-II had no noticeable effect on the firing activity, AP kinetics, and GABAergic currents in the SNc dopaminergic neurons. This suggests heterogeneous effect of Ang-II signaling in the two primary nigral cell types. Also, unexpectedly, Ang-II increased the feedforward inhibitory input onto SNc dopaminergic neurons in response to phasic photoactivation of Channelrhodopsin-2 (ChR2) expressing SNr GABAergic neurons. This suggests that Ang-II also has a non-linear effect on GABAergic neurotransmission in the nigral dopaminergic neurons. Though we have not yet investigated the underlying mechanism behind the heterogeneous and non-linear effect of Ang-II on GABAergic neurotransmission in the SNc dopaminergic neurons, we summarize our findings and discuss the probable mechanisms behind the observed effects of Ang-II in these neurons in this concluding chapter.

4.1 Ang-II Suppresses SNr GABAergic Neurons

As discussed in chapter 2, Ang-II signaling is found to affect GABAergic neurotransmission in neurons in different hypothalamic nuclei and in the amygdala. Tonic GABAergic input from SNr GABAergic neurons regulates SNc dopaminergic neurons' activity, and since Ang-II receptors and other RAS components are expressed in the substantia nigra, Ang-II mediated regulation of GABAergic transmission in the nigra is likely. We found that Ang-II significantly decreased the high frequency evoked firing of nigral GABAergic neurons and suppressed their activity. Action potential (AP) waveform analysis revealed that Ang-II increased action potential duration by increasing the AP width, decay and rise time. This suggests that ion channels involved in maintaining AP firing and generation are potentially a downstream target of Ang-II signaling in the SNr GABAergic neurons. Unlike the neighboring SNc dopaminergic neurons that predominantly express Kv4.3 channels, SNr GABAergic neurons express voltage-activated delayed rectifier KV3 channels, which is essential for repolarizing the cell membrane after an AP and resist inactivation for sustained high-frequency firing. Since Ang-II via AT1-receptors are reported to inhibit delayed rectifier potassium channels in the hypothalamus and brain stem neurons through a Gq-coupled protein kinase-II dependent pathway (156), a similar mechanism may underlie the inhibitory effect of Ang-II on SNr GABAergic neurons. Future investigations are necessary to identify and mechanistically characterize the specific ion channels potentially modulated by AT_1 receptors in the SNr GABAergic neurons.

In our study, selective GABAa-R antagonist picrotoxin blocked Ang-II mediated suppression of evoked AP firing, increased AP duration and GABAergic IPSCs in SNr GABAergic neurons, suggesting that GABAa-R is also a downstream target of Ang-II in these

neurons. However, we did not investigate if facilitation of GABAa-R by Ang-II is dependent on AT1-R activation or Ang-II is an allosteric modulator of GABAa-R. Results from our study so far suggest that Ang-II suppresses the firing activity of SNr GABAergic neurons through a combination of mechanisms; a) activation/inhibition of ion channels involved in AP generation and b) facilitation of GABAa-R activity.

4.2 Ang-II Facilitates Postsynaptic GABAa-R

SNr GABAergic neurons are innervated by afferent GABAergic inputs from nuclei within the basal ganglia such as striatum and globus pallidus externus (GPe). Also, GABAa-R's are strongly expressed in SNr GABAergic neurons. Therefore, Ang-II mediated facilitation of GABAa-R could be a presynaptic effect or mediated through postsynaptic GABAa-R or both. In order to severely limit afferent synaptic inputs, we used acute midbrain coronal slices of 240 microns and blocked synaptic transmission by using AP generation blocker TTX and glutamate excitatory synaptic transmission blockers (MK-801 and DNQX) and recorded spontaneous mIPSC's. Ang-II significantly increased the frequency of picrotoxin-sensitive high-amplitude mIPSC's without affecting the average amplitude and average frequency in SNr GABAergic neurons. This suggests that Ang-II mediated facilitation of inhibitory GABAergic currents is primarily mediated postsynaptically and is not due to increased GABA release from presynaptic terminals.

It is important to note that SNr GABAergic neurons also express dopamine receptors (D1, D5) and receive inputs from SNc dopaminergic neurons (144, 166-168). Somatodendritic dopamine release can excite SNr GABAergic neurons through D1R activation. Furthermore,

afferent striatonigral inputs that selectively innervate SNr are known to strongly express D1R. Therefore somatodendritic release of dopamine as a result of its spontaneous pacemaker-like activity can, in theory, activate the afferent striatonigral terminals and increase synaptic release of GABA of studies. Studies have also suggested an interplay between Ang-II signaling and dopaminergic system in the SNc dopaminergic system and renal cells (169-172). However, if a similar interplay exists between Ang-II signaling and dopaminergic transmission in SNr GABAergic neurons is not known. Therefore, Ang-II mediated facilitation of GABAa-R can also result from either activation of presynaptic D1R expressing striatonigral afferents by ambient dopamine and/or Ang-II mediated modulation of dopaminergic transmission in the SNr GABAergic neurons. However, since we did not see any noticeable increase in spontaneous IPSC frequency but only in picrotoxin-sensitive IPSC amplitude in the presence of Ang-II, we can rule out the afferent D1R expressing terminals as a potential mediator of increased IPSCs. On the other hand, if Ang-II signaling modulates dopaminergic transmission in the afferent terminals and in the postsynaptic SNr GABAergic neurons is unknown. Therefore, more studies need to be done to explore the interplay between Ang-II and dopaminergic transmission in the SNr GABAergic projection neurons.

4.3 Ang-II and SNc Dopaminergic Neurons

Ang-II receptors are strongly expressed in the SNc dopaminergic neurons. As discussed in chapter 2 and from our study detailed in chapter 3, Ang-II signaling modulates several neurons' excitability in the brain. To determine if Ang-II signaling affects the dopaminergic cell activity, we recorded evoked firing, spontaneous firing, and postsynaptic currents from identified SNc dopaminergic neurons. Dopaminergic neurons can fire in a pacemaker-like fashion or in a

random manner, and because of this erratic firing behavior *in vitro* we could not conclude if Ang-II has any effect on its spontaneous firing and, therefore, the recording data were not used. Similarly, for evoked firing, we could not conclude the effect of Ang-II because of the well-accepted highly adaptive nature of dopaminergic neurons that leads to depolarization block in response to current input. However, we analyzed the AP waveform of SNc dopaminergic neurons and, unlike SNr GABAergic neurons, Ang-II had no noticeable effect on the AP kinetics in these neurons.

We also recorded spontaneous EPSCs and IPSCs from SNc dopaminergic neurons, which were significantly lower compared to SNr GABAergic neurons. This could be partly due to inherently slow spontaneous activity of SNc dopaminergic neurons and severed afferent inputs in our *ex vivo* midbrain coronal slice preparation. It is known that dopaminergic neurons exhibit high-frequency burst firing and relatively higher spontaneous firing frequency in vivo, which is likely due to its different excitatory afferents and is not usually seen *in vitro*. Ang-II had no detectable change in SNc dopaminergic IPSCs, whereas in SNr GABAergic neurons, spontaneous mIPSC amplitude increased by more than two-fold. Markedly lower IPSCs in SNc dopaminergic neurons than SNr GABAergic neurons can be explained by significantly lower expression of GABAa-Rs, different subunit composition, and lack of canonical K^+-Cl^- co-transporter, KCC2a in nigral dopaminergic (158, 159).

On the other hand, SNr GABAergic projection neurons express KCC2 that actively pumps [Cl^-] outside the cell and maintains hyperpolarized (-71 mV) chloride reversal potential (160). In contrast, in SNc dopaminergic neurons, the chloride reversal potential is set around -63 mV due to lack of KCC2 (161). Therefore, GABAa receptor activation produces a significantly

lower hyperpolarization in SNc dopaminergic neurons than the neighboring SNr GABAergic neurons.

4.4 Ang-II and Phasic Activity of SNr GABAergic Neurons

Ang-II suppressed the spike firing of electrically stimulated single SNr GABAergic neurons. However, SNr is a highly interconnected and collaterized network due to highly arborized axon collaterals of SNr GABAergic neurons. These axon collaterals synapse onto GABAergic cells within the nigra and exhibit feedback inhibition within the network. Therefore, the activity of a single SNr GABAergic neuron cannot represent the population output. To activate synchronous population activity of SNr GABAergic neurons, we utilized a transgenic mouse line with ChR2 selectively expressed on SNr GABAergic neurons but not in the SNc dopaminergic neurons. We recorded EPSPs from SNr GABAergic neurons in response to 100 ms long 470 nm light pulse. In response to photostimulation, SNr GABAergic neurons exhibited burst-like firing. In some cases, as previously described by Brown et al.(133), SNr GABAergic neurons exhibited feedback inhibition seen as a progressive decrease in intraburst spike frequency resulting in a complete cessation of spikes. However, in most recorded SNr GABAergic neurons, we observed typical burst firing without any feedback inhibition in response to photostimulation and only used these neurons for data.

Consistent with findings from electrical stimulation, Ang-II decreased the intraburst spike frequency in SNr GABAergic neurons in response to photostimulation. This suggests that Ang-II signaling also suppresses the synchronous spike activity of SNr GABAergic neurons. However, as we mentioned above, we only included neurons that did not exhibit feedback inhibition as

described by other studies. Therefore, it could be argued that Ang-II mediated inhibition of SNr GABAergic neurons may further inhibit the feedback inhibitory microcircuit and result in an overall increase rather than a decrease in the population activity. Nonetheless, the degree of feedback inhibition is dependent mainly on the extent of parallel inhibitory input from neighboring GABAergic neurons in the SNr, and it is likely that parallel input is not homogenous throughout the inhibitory microcircuit. Furthermore, light evoked stimulus is still recorded from a single SNr GABAergic, and it is limited in its ability to represent the population output under synchronous activity. Nonetheless, it is beyond the scope of our study to determine the consequences of Ang-II signaling within the inhibitory microcircuit and its effect on the overall population output of Snr GABAergic neurons. One way to determine population output is to examine the feedforward inhibitory input on the downstream dopaminergic neurons in response to phasic photostimulation of SNr GABAergic neurons, which is discussed in the next section.

4.5 Ang-II and GABAergic Neurotransmission Onto SNc Dopaminergic Neurons

SNr GABAergic neurons exert robust tonic inhibitory input on the SNc dopaminergic neurons and regulate their activity. As discussed in the section above, one way to determine Ang-II's effect on the population output of SNr GABAergic neurons is to examine feedforward inhibitory input onto the downstream SNc dopaminergic neurons. We recorded IPSCs and IPSPs from the nigral dopaminergic neurons in response to photostimulation of SNr GABAergic neurons. Since Ang-II suppresses electrically and optically evoked activity of SNr GABAergic neurons, we expected a decrease in light evoked IPSC and IPSP amplitude in SNc dopaminergic neurons. However, we observed a paradoxical increase in the amplitude of IPSCs and IPSPs in SNc dopaminergic neurons. This suggests Ang-II mediated strengthening of

feedforward inhibitory input onto SNc dopaminergic neurons. This non-linear effect of Ang-II on SNr GABAergic neuronal activity and their inhibitory output onto postsynaptic SNc dopaminergic neurons suggests the role of a complex intranigral microcircuitry arising from extensive network of axon collaterals.

SNr GABAergic projections have both intranigral and extranigral collateralizations that are non-uniform in nature and can give rise to variability in conduction velocity, excitability, myelination, density of synaptic boutons, distribution of ion channels, and other aspects of signal propagation in neurons within a network (162-165). This would have an effect on the neuronal response properties within the intranigral inhibitory microcircuitry. Consequently, summed activity arising from such heterogenous interlinked network could result in a range of synchronous and/or asynchronous population activity and could have a varied effect on the postsynaptic population. Therefore, the paradoxical facilitation of inhibitory input to nigral dopaminergic neurons by Ang-II upon phasic activation of SNr GABAergic neurons may result from the sophisticated and assymetrical nature of intranigral microcircuitry.

However, it is important to note that other factors may also play a role in the paradoxical strengthening of inhibitory input onto SNc dopaminergic neurons, such as high expression of endocannabinoid receptors in the nigral GABAergic neurons but not in the dopaminergic neurons (173), crosstalk between CB1 receptor signaling and GABAa receptors in the SNr (152, 174), the potential interplay between Ang-II signaling and CB1 receptor activation as seen in other cell types (175, 176), Ang-II's effect on intranigral dopaminergic neurotransmission and reactive oxygen species signaling to name a few. Our study did not investigate the role of these signaling factors in intranigral neurotransmission. Future studies are needed to determine the

contribution of these factors and intranigral microcircuitry in Ang-II mediated effects in neurotransmission within the substantia nigra.

4.6 Final Remarks

Ang-II signaling in the substantia nigra, though indicated to exacerbate degeneration of dopaminergic neurons in animal models of Parkinson's disease, is still not well understood, partly due to a wide variety of signaling molecules and downstream messengers that can be activated by AT1-R, AT2-R, and other receptors of Ang-II such as AT4. In addition, nigral dopaminergic neurons are heavily innervated by efferent inputs from both extranigral and intranigral nuclei, which further complicates the effect of Ang-II signaling in these neurons. Ang-II and its receptors are highly expressed in the neurons in these nuclei. However, Ang-II signaling in these presynaptic neurons and its effect on the postsynaptic nigral dopaminergic neurons is not known. The neighboring SNr GABAergic neurons provide one of the most potent inhibitory inputs to the nigral dopaminergic neurons, and Ang-II receptors, ACE, and other RAS components are well expressed in these cells. Nevertheless, studies so far have overlooked Ang-II signaling in the nigral GABAergic neurons and its role in GABAergic and dopaminergic transmission in the substantia nigra.

In this study, we demonstrated novel Ang-II signaling in the SNr GABAergic neurons resulting in suppression of their spike firing activity through GABAa-R activation and prolongation of action potential duration. We also show a heterogeneous effect of Ang-II in the nigral dopaminergic neurons, indicating the importance of cell type on Ang-II's effect in the substantia nigra. Interestingly, we observed a paradoxical increase in the light-evoked IPSCs in most of the recordings from postsynaptic nigral dopaminergic neurons, suggesting that the negative

regulatory effect of Ang-II on the SNr GABAergic neurons does not translate into a weakened feedforward input onto the dopaminergic neurons. More studies need to be done to identify the mechanism behind the paradoxical strengthening of inhibitory input onto SNc dopaminergic neurons upon phasic population activity of SNr GABAergic neurons. Therefore, our study strongly suggests that Ang-II signaling in the presynaptic inputs, such as originating from the nigral GABAergic neurons and other basal ganglia nuclei, can potentially modulate the effect of Ang-II on the nigral dopaminergic neurons and may play an important role in the intra & extra-nigral neurotransmission in health and disease.

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