# Selective Activation of $\beta$-Arrestin Pathways in AT1R via saRNA Upregulation of GRK2 and GRK5 for Blood Pressure Regulation

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Gene therapy using small activating RNAs (saRNAs) to upregulate GRK2 and GRK5 transcription in neural cells of the rostral ventrolateral medulla (RVLM) presents a plausible strategy for hypertension management. By enhancing the expression of these G protein-coupled receptor kinases (GRKs), this approach aims to bias angiotensin II type 1 receptor (AT1R) signaling towards the $\beta$-arrestin-dependent signaling pathway, which contrasts the inflammatory, vasoconstrictive, and sympathetic nerve activity enhancing effects of G-protein mediated signaling. $\beta$-arrestin signaling mediates anti-inflammatory, vasodilatory, and reducing sympathetic nerve activity response, making it a novel target for antihypertensive therapy. The targeted modulation of the RVLM, a critical brainstem region in cardiovascular control, will allow for central blood pressure regulation. Increasing GRK expression in these neural cells using saRNAs for gene activation is an innovative methodology. This alternative therapeutic strategy prompts research toward the development of antihypertensive treatments that target the underlying mechanisms rather than the symptoms, possibly an approach that will benefit patients with continued uncontrolled blood pressure when medicated. The proposed lipid nanoparticle delivery system via intracerebroventricular (ICV) injections offers a potential method for precise saRNA administration. However, future studies must be conducted to assess the safety, efficiency, and long-term impact of this RNAa gene therapy approach, including potential off-target effects and the consequences of chronic activation of the $\beta$-arrestin pathway in the nervous system.

## Introduction

Gene therapy for hypertension could renovate the current standard of care and help alleviate the economic burden uncontrolled blood pressure has put on the U.S. healthcare system[1]. High blood pressure accounts for an estimated $51 billion of the system's costs. Although 70% of hypertension patients are treated, only half have controlled blood pressure[2].

Studies demonstrate the average efficacy rate is 50% for each common class of blood pressure medication (ACEi, ARBs, $\beta$-Blockers, CCBs, and diuretics)[3].The current trial and error method to prescribe anti-hypertensive medication is economically straining, inconvenient for patient use, and a burden on healthcare services. Physicians layer blood pressure drugs by prescribing multiple classes of drugs to the maximally tolerated dosages to inflict a lowering blood pressure response. If the blood pressure remains uncontrolled, the physician will experiment with a different combination or dosage[4]. These shortcomings in the current standard of care prove there is a need for more effective and sustainable treatment without dependence on adherence to daily medication.

These blood pressure medications have notable limitations. While ACE inhibitors and ARBs target the renin-angiotensin system to reduce vasoconstriction, they can lead to compensatory mechanisms, incomplete blood pressure control, and side effects such as hyperkalemia and renal dysfunction[5]. $\beta$-Blockers lower blood pressure by reducing heart rate and sympathetic activity but can cause fatigue, metabolic disturbances, and reduced exercise tolerance. CCBs lower blood pressure by blocking calcium entry into heart and aterial cells, leading to vasodilation and improved blood flow[6]. However, CCBs can induce edema, dizziness, and reflex tachycardia in many patients. Thiazide diuretics act by blocking the sodium-chloride (Na/Cl) channel in the distal convoluted tubule (DCT) of the kidneys, preventing sodium reabsorption and promoting sodium and water excretion in urine[6]. However, they often cause electrolyte imbalance, dehydration, and metabolic side effects. These limitations emphasize the need for alternative strategies that modulate sympathetic regulation at its central origin rather than acting peripherally. This study proposes the use of small activating RNAs (saRNAs) to modulate the expression of genes such as G protein receptor kinases (GRKs) that affect the signaling pathway of the angiotensin II type 1 receptor (AT1R), which plays a vital role in regulating blood pressure8. This approach may avoid the systemic compensatory effects seen with conventional treatments. By directly modulating central blood pressure control, this approach offers a potentially long-lasting, targeted alternative to current therapies for systemic side effects.

G protein-coupled receptors (GPCR), GRKs, and $\beta$-arrestins play key roles in many physiological inter- and intra-cellular

signaling. Accordingly, GPCRs and related protein treatments comprise 35% of FDA-approved targeted drugs[7]. The GPCR signaling pathway involves the binding of a ligand to the G$\alpha$ subunit of the G protein complex, causing the G$\beta\gamma$ subunit to dissociate from the complex to activate the secondary messengers (cAMP, phospholipase C, and Rho/Rae) to reach intracellular nuclear targets. AT1R, a GPCR that plays a vital role in the renin-angiotensin-aldosterone system (RAAS), regulates the response of vascular resistance, cardiac contractility, fluid intake, and sympathetic outflow through the GPCR signaling pathway via ligand Ang II and secondary messenger cAMP[8].

However, if the GRKs phosphorylate the receptor, the $\beta$-arrestin protein will bind to sites in AT1R located in the receptor's C-tail terminus—the GRK phosphorylation will occur at serine/threonine clusters in the intracellular C terminal tail of AT1R that are important for $\beta$-arr engagement with activated GPCRs—will lead to the recruitment of adaptor proteins $\beta$-arrestin 1 and 2, initiating the $\beta$-arrestin coupling pathway and suppressing the G-protein signaling[9]. $\beta$-arrestin binding promotes an internal mechanism of the AT1R called receptor internalization, which is clathrin-dependent endocytosis of the receptor, thus resulting in signal desensitization and $\beta$-arrestin-dependent signaling. These findings support the plausibility of gene therapy to hyperactivate GRKs required to phosphorylate the C terminal tail of GPCRs to recruit $\beta$-arrestins to manipulate biased GPCR signaling[10,11]. Specifically, $\beta$-arrestin-dependent signaling downstream of the AT1R is a pharmacotherapeutic target for treating heart failure, hypertension, and renal disease due to its inhibitory effects on the G-protein receptor's cellular responses[12].

## Results

### Brain Renin-Angiotensin System

AT1R-mediated effects of angiotensin II (Ang II) in the central nervous system are fundamental to neurogenic hypertension. GRKs associated with AT1R in the brain renin-angiotensin system (RAS) can serve as targets for saRNA-mediated overexpression, promoting blood pressure-lowering signaling effects via phosphorylation of neuronal AT1R. RAS-mediated actions in the central nervous system (CNS) include Ang II augmentation of thirst, sympathetic nerve activity, and vasopressin secretion[13]. Ang II in the brain RAS also induces vasoconstriction and interferes with neuro-vascular coupling[14].

AT1R is localized to cardiovascular control centers in the brain that regulate blood pressure. The hypothalamus's paraventricular nucleus (PVN) is an endocrine, hormonal, and neural control center. The PVN is a divider of neural cell groups; the parvocellular subgroup projects to the rostral ventrolateral medulla (RVLM) regulating the sympathetic nervous system, and the magnocellular neuron group projects to the posterior pituitary to secrete vasopressin. Neurons from RVLM are responsible for blood pressure homeostasis, baroreflex function, and control of sympathetic tone. AT1R expression is upregulated in RVLM neuronal cells and is associated with renal sympathetic nerve activity, blood pressure, and heart rate. AT1R is commonly localized to neural cardiovascular control centers such as RVLM, which regulate blood pressure and fluid balance[15,16].

RVLM neurons regulate the peripheral sympathetic nervous system. Abnormally increased sympathetic nervous system activity heightens the risk of cardiovascular disease events. The sympathetic nervous system and RAS are correlated so that the sympathetic system can be regulated through the modulation of RAS to protect against cardiovascular disease. RVLM neurons are critical in blood pressure regulation. Moreover, stimulation of AT1R potentiates the activity of RVLM neurons. As proof of RVLM acting as a primary control region of hypertension in the body, AT1R blockers (ARB) have been shown to reduce RVLM activity, resulting in antihypertensive effects in essential and experimental hypertension patients[17,18].

The RVLM is a brainstem region critical in cardiovascular regulation; therefore, inhibiting the RVLM outflow suppresses sympathetic nervous system activity and arterial blood pressure, thus demonstrating its significance to hypertension treatment[19]. Consequently, AT1R localized in RVLM neurons is a therapeutic target for cardiovascular diseases like hypertension. saRNA modulation can be implemented in the brain RAS to target GRKs that phosphorylate AT1R in the RVLM to hyperactivate $\beta$-arrestin signaling to stimulate blood pressure control.

The RVLM is an optimal target for saRNA therapy due to its critical role in blood pressure regulation and its relatively high expression of AT1Rs. While other cardiovascular control centers, such as the PVN of the hypothalamus and the dorsal motor nucleus of the vagus (DMV), contribute to cardiovascular regulation, they are not as directly involved in blood pressure modulation as the RVLM. The PVN influences cardiovascular function primarily through autonomic and hormonal mechanisms, regulating the release of vasopressin and activating sympathetic output indirectly. While important, its role is more endocrine and autonomic rather than a direct modulator of blood pressure through neuronal control[20,21]. The DMV, on the other hand, is predominantly involved in the parasympathetic regulation of the heart, mainly affecting heart rate rather than systemic blood pressure, making it a less effective target for saRNA-based intervention.

Among the central cardiovascular control regions—the medulla oblongata(which houses the RVLM and nucleus tractus solitarius (NTS)) and the hypothalamus—the RVLM plays the most direct role in blood pressure regulation, functioning as the major excitatory output center for sympathetic control[22]. While the NTS and the RVLM are key regulators, angiotensin signaling is significantly more active in the RVLM due to its

localization within the Blood-Brain Barrier (BBB ), where it receives locally produced Ang II. The combination of a high local Ang II concentration and increased AT1R expression makes the RVLM the most responsive region to angiotensin-related interventions[23].

Since the RVLM consists of heterogeneous neuronal and glial populations, different cell types may respond differently to GRK2/GRK5 upregulation, influencing therapeutic outcomes. AT1R is highly expressed in C1 catecholaminergic neurons, which drive sympathetic outflow and vasoconstriction[24]. GRK2 is predominantly expressed in cholinergic interneurons, which modulate autonomic functions and cardiovascular regulation, while GRK5 is found in both neurons and glial cells, where it influences long-term hypertrophic and inflammatory signaling in the brain stem[25,26].

To maximize therapeutic benefits and minimize off-target effects, saRNA delivery should be restricted to target neurons involved in blood pressure regulation. This can be achieved through surface ligand functionalization of LNPs, enhancing targeting to C1 neurons, where AT1R is highly expressed. A study using RVG29-Fuctionalized LNPs successfully targeted the BBB and enhanced neuroprotective properties for delivering quercetin to the brain in Alzheimer's disease. The LNP functionalization enabled targeted brain delivery by utilizing and nicotinic acetylcholine receptors for neuronal uptake. The RVG29 peptide could be similarly employed to target C1 neurons for saRN-mediated GRK2/GRK5 upregulation[27].

Additionally, charge-based targeting using cationic lipids can enhance neuronal uptake and avoid glial toxicity. Unintended GRK5 upregulation in glial cells could lead to astrocytic overexpression, disrupting neuroinflammatory balance or microglial activation, contributing to oxidative stress rather than alleviating hypertension. Another potential risk is the accidental spread of saRNA molecules to the NTS, another cardiovascular control center in the brain stem, which could interfere with blood pressure homeostasis, potentially leading to synergistic or antagonistic effects[28]. This study will aim to mitigate non-specific cellular targeting with dual saRNA LNP systems with distinct promoters to fine-tune neuronal specificity and minimize nonspecifc cellular targeting.

### $\beta$-arrestin Signaling in AT1R Pathways

$\beta$-arrestin signaling and biased ligands regulate AT1R pathways. It was found that AT1Rs have multiple active forms, allowing for the binding of Ang II and $\beta$-arrestin-biased agonists. Biased agonists (TRV120027, TRV027, [Sar1,lle4,lle8]-Ang II (SII-AngII)) are different than balanced agonists like Ang II because of their role as receptor ligands that can selectively signal either G protein or $\beta$-arrestin pathways[29]. A study found beneficial effects of the $\beta$-arrestin-dependent AT1R pathway by performing biased agonist TRV0217 infusion into the intracerebroventricular portion of the central nervous system in spontaneously hypertensive rats. The study found that chronic activation of $\beta$-arrestin signaling in the RVLM had significant effects on multiple organ systems. In the heart, it increased cardiac contractility and output while reducing cardiac hypertrophy but also exacerbated aldosterone-dependent heart failure and load-induced hypertrophy. In the kidneys, it lowers blood pressure, and reduces kidney damage and preeclampsia, but negatively contributes to renal fibrosis and decreases sodium reabsorption. In blood vessels, it decreases vascular resistance, endothelial hypertrophy, and blood pressure while also reducing preeclampsia risk, but it increases inflammatory responses and susceptibility to aortic aneurysms. In the brain, it lowers vascular resistance, blood pressure, salt intake, and sympathetic outflow. Thus, chronic $\beta$-arrestin activation in the brain exerts both beneficial and detrimental peripheral effects[29]. Clinical study shows the possibility of saRNA treatment to increase $\beta$-arrestin-dependent AT1R signaling pathways as a successful approach to treat hypertension. This approach surpasses current clinical strategies targeting $\beta$-arrestin signaling, as biased agonists typically have a short half-life and limited specificity for AT1R. Unlike biased agonists that directly activate $\beta$-arrestin at the receptor level, this method enhances $\beta$-arrestin signaling upstream by upregulating GRKs, providing a more sustained and targeted effect. While existing therapies rely on $\beta$-arrestin biased AT1R ligands, this approach offers broader modulation of $\beta$-arrestin pathways, potentially improving efficacy.

Additionally, AT1R $\beta$-arrestin activation causes beneficial peripheral neural effects, including reduced sympathetic nervous system activity, improved baroreflex sensitivity, inhibition of hypertensive vascular reactivity to Ang II, and vasodilation via an increased sensitivity to acetylcholine-mediated relaxation[30]. Another study investigating $\beta$-arrestin signaling in neuronal AT1R found that overexpression of $\beta$-arrestsin1 in the RVLM located in the brainstem of hypertensive rats had a lowering blood pressure effect without affecting normotensive controls. Thus, different nuclei in the brainstem that are the center for producing, regulating, and activating RAAS components are suggested as potential therapeutic targets[31].

Instead of completely blocking both G protein and $\beta$-arrestin signaling pathways of AT1R like the first-line antihypertensive medication therapy ARBs, the inhibition of G protein signaling pathways that cause hypertensive effects can be focused upon[32]. This selectivity can be practiced by Ang II competitors that lower blood pressure by binding to the active site of AT1R, preventing Ang II-mediated vasoconstriction. Although ARBs result in antihypertensive effects, the selective activation of AT1R $\beta$-arrestin signaling could provide protective cardiovascular and antihypertensive effects. Using saRNAs to upregulate the expression of GRKs leads to increased AT1R phosphorylation, thus overstimulating the $\beta$-arrestin dependent AT1R signaling pathway and blocking the G protein pathway, which leads to

reduced blood pressure.

### GRKs Role in $\beta$-arrestin Signaling

GRK phosphorylation of neuronal AT1R promotes the $\beta$-arrestin signaling pathway, which stimulates antihypertensive effects. Therefore, upregulating GRKs via saRNA treatment is a potential way to control blood pressure. The GRK family chemical structure comprises a conserved central catalytic domain, an N-terminal domain that plays a role in kinase localization to the cell membrane, and a variable C-terminal domain that mediates translocation. The serine/threonine phosphorylation site clusters in C-terminal tails are essential for the formulation of stable GPCR-B arrestin complexes[33,34].

A mutational and truncation analysis of AT1R found that Thr332-Lys333-Met334-Ser335-Thr336-Leu337-Ser338 amino acids on the C-terminus are essential for receptor internalization following GRK-dependent phosphorylation, especially the Ser335-Thr336-Leu337 motif. The serine and threonine amino acids have multiple functionalities in $\beta$-arrestin-dependent signaling, including regulation of receptor endocytosis and maintaining stable interactions between AT1R and $\beta$-arrestins after Ang II stimulation[35].

Additionally, $\beta$-arrestin1 has lysin K11, and $\beta$-arrestin 2 has lysin K12, which are critical for interaction with phosphate-binding residues(serine/threonine-rich regions) to form a stable AT1R-B arrestin complex, for proper scaffolding of the signal complex, and activation of the Erk MAPK cascade.

Accordingly, phosphorylation patterns on the receptor C-terminus are indicative of GRK selectivity (interaction stability and arrestin activation). Variation in the phosphorylation patterns causes a differential response in internalization, signaling, and activated $\beta$-arrestin conformations. Certain classifications have been identified, such as Class B receptors form stable arrestin complexes, whereas Type A receptors loosely interact with $\beta$-arrestin. A study conducted targeted knockdown of each GRK by RNAi identified differential responses in AT1R depending on the GRK family. For instance, GRK2/3 phosphorylation of AT1R is associated with receptor endocytosis, and GRK5/6 is linked with Erk1/2 activation but minimal $\beta$-arrestin binding receptor internalization[36].

GRK2's potential for pharmaceutical therapy and role in the pathologies of different diseases make it a distinct kinase from the GRK family. Structures specific to GRK2 include an amino-terminal RGS homology domain for interaction with G$\alpha$q and G$\alpha$11 family members, a pleckstrin homology domain for mediating the binding of phospholipids to the G$\beta\gamma$ subunit, and a variant C-terminal domain[37,38]. Additionally, GRK2 is cytosolic, allowing it easier access to GPCRs embedded in membranous microdomains[39]. The upregulation of GRK2 by therapeutic drugs, such as the proposed saRNA treatment, can effectively impact AT1R activity.

While GRK2 is a part of the $\beta$-androgenic receptor kinase subfamily due to its role in canonical agonist-induced desensitization pathways, GRK5 is in the GRK4 subfamily for its role in ligand-independent GRK phosphorylation[40]. Structures specific to GRK5 include a nuclear localization motif that promotes the kinase's translocation to the nucleus, a nuclear export sequence, and a phosphatidylinositol bisphosphate (PIP2) binding domain. GRK5 is a membrane-associated kinase expressed in heart tissue, lung tissue, smooth muscle, skeletal muscle, and the brain. GRK5 was identified as a GPCR-associated signaling factor that can bridge the pathophysiology of cardiovascular and neurodegenerative disorders[41].

A study assessed GRK5's AT1R functionality using immunoprecipitation with an anti-AT1R antibody and immunoblotting using an anti-phosphothreonine antibody (a site of GRK5 action). A 100% increase in phosphorylated AT1R was discovered as a result of GRK5 upregulation[42]. Ang II upregulates both neuronal AT1R and GRK5, but GRK5 overexpression or knockdown also depends on changes in AT1R expression. This is known since GRK5 regulates AT1R and p65 NF-kb (an inhibitory transcription factor of AT1R) post-Ang II- stimulation. Therefore, neuronal GRK5 knockdown using siRNAs results in a greater increase in AT1R and p65 NF-kb after Ang II stimulation[24]. Hence, overexpression of GRK5 via saRNA treatment will result in fewer AT1R, thus reducing its hypertensive effect in the neuronal RAS system. So by upregulating GRK5 via saRNA modulation, not only will $\beta$-arrestin dependent AT1R signaling pathways be activated, but less AT1R will be expressed in neural cells overall, significantly reducing any hypertensive effects from AT1R signaling.

However, the combined overexpression of both GRK2 and GRK5 may not necessarily yield synergistic antihypertensive effects and could potentially lead to adverse outcomes. GRK2 primarily facilitates AT1R internalization through receptor endocytosis; it is cytosolic and acts rapidly following receptor activation. In contrast, GRK5 plays a dual role—it phosphorylates AT1R but also regulates gene transcription via nuclear signaling. Overexpression of GRK5 can lead to a reduction in overall AT1R expression, contributing to a long-term regulation[15,43].

Targeting both GRKs could theoretically enhance antihypertensive effects through immediate desensitization (GRK2) and sustained receptor downregulation (GRK5). However focusing on the upregulation of a single GRK may offer a more controlled therapeutic strategy. Given the potential adverse effects associated with GRK5 overexpression, including interference with adaptive signaling mechanisms in the cardiovascular system, targeting GRK2 alone may be a more favorable approach to minimize off-target effects[15].

This study will investigate the dual modulation of GRK2 and GRK5. Their potential synergistic effects stem from both contributing to $\beta$-arrestin-mediated AT1R desensitization, which

could enhance suppression of excessive sympathetic activity—a potentially beneficial outcome in heart failure. However, antagonistic effects may arise due to their distinct cellular roles: GRK2 is primarily cytoplasmic and regulates acute AT1R desensitization, while GRK5 has nuclear effects, modulating gene transcription (ex. hypertrophy-related pathways)[44]. This suggests differential timing in their actions, where GRK2 exerts rapid effects, while GRK5 has longer-term regulatory functions that could either complement or counteract GRK2 activity.

### Biased-Agonist Independent versus Ang II-induced Pathways of $\beta$-arrestin Signaling

Although both GRK2 and GRK5 can mediate receptor-$\beta$-arrestin interaction—confirmed by a single $\beta$-arretsin2 conformational change sensor recording similar conformational changes for both GRK2 and GRK5 as a result of $\beta$-arrestin 2 attaching to phosphorylated intracellular receptor domains—selectivity of the GRK is dependent upon the configuration of the GPCR, availability of phosphorylation sites, and proximity to other intracellular domains[45]. Findings from a receptor panel for GRK-specificity of $\beta$-arrestin1 and 2 recruitment to different GPCRs confirm GRK selectivity dependency on GRK-specific GPCR phosphorylation patterns.

Ang II-induced $\beta$-arrestin1 and 2 recruitment to AT1R via GRK2 phosphorylation differs from the ligand-independent recruitment of $\beta$-arrestins due to GRK5 phosphorylations. Recorded BRET data subjugated to statistical analysis found increased basal molecular interactions in ligand-independent recruitment of $\beta$-arrestins associated with GRK5, whereas Ang II-induced $\beta$-arrestin recruitment was associated with GRK2[46]. To further assess GRK2 functionality, a different study utilizing dynamic mass distribution (DMR) to assess AT1R activity found that GRK2 overexpression is strongly associated with Ang II-dependent internalization, thus dampening cellular signaling of AT1R[47].

Hence, synthetic ligands like TRV07 are unnecessary for GRK5 and GRK2 saRNA overexpression therapy due to the biased-agonist independent and Ang II-induced pathways of $\beta$-arrestin signaling exhibited when phosphorylated by these kinases, respectively[48,49]. This allows blood pressure control from saRNA overexpressed GRK2 and GRK5 hyperactivating $\beta$-arrestin signaling pathways independent of biased ligands and in the presence of Ang II.

### Off-Target Effects of GRK5 and GRK2 Phosphorylation

Other GPCRs, specifically neurotransmitter receptors, are regulated by GRK2 and GRK5 in the cardiovascular control centers in the brain. Therefore, overexpression of these GRKs from saRNA treatment to promote $\beta$-arrestin antihypertensive signaling pathways may have potential off-target effects. Such that saRNA treatment may cause increased $\beta$-arrestin signaling in other nearby neurotransmitters, including $\alpha$-Adrenergic Receptors that are involved in the sympathetic control of blood pressure, Beta-Adrenergic Receptors ($\beta$AR) that play a role in heart rate and contractility regulation, and Dopamine Receptors that influence various neurological processes, including motor control and reward pathways[50].

#### Adrenergic Neuroreceptors

Adrenergic neuroreceptors bind catecholamines, epinephrine, and norepinephrine. These are components of the sympathetic nervous system. They have an alpha and $\beta$ subfamily, with the alpha group having the $\alpha$1 ($\alpha$1A, $\alpha$1B, $\alpha$1D) and $\alpha$2 ($\alpha$2A, $\alpha$2B, $\alpha$2C) subtypes and the $\beta$ group having the $\beta$1, $\beta$2, and $\beta$3 subtypes.

GRK2 phosphorylates the $\alpha$2a neuroreceptor, which can cause rapid desensitization, whereas the $\alpha$2c receptor is resistant to desensitization due to its specific receptor conformation shape induced by agonists in the $\alpha$2c receptor is not accessible for GRK binding and phosphorylation[51]. $\beta$-arrestin signaling in the $\alpha$2 adrenergic receptor ($\alpha$2AR)subtype results in the blockade of vasoconstriction[52]. Therefore, upregulating GRKs, which causes an increase in $\beta$-arrestin signaling of $\alpha$2AR, will be a positive off-target effect for hypertension treatment.

Meanwhile, $\alpha$1-adrenergic neuroreceptors ($\alpha$1AR) are involved in neurotransmission and regulation of the sympathetic nervous system by binding neurotransmitters norepinephrine and epinephrine. $\beta$-arrestin-dependent signaling of the $\alpha$1AR has anti-oxidative, anti-inflammatory, and anti-proliferative properties that benefit hypertensive patients. Therefore, upregulation of GRKs via saRNA overexpression will cause an increase in $\beta$-arrestin signaling of $\alpha$AR, resulting as a positive off-target effect for hypertension treatment[36,53].

Beta-Adrenergic antagonists are a widely prescribed hypertension medication. The antihypertensive effect of $\beta$-Blockers comes from competitive binding to the $\beta$1 and $\beta$2 receptors. This inhibits $\beta$1 receptors from inducing renin secretion, therefore decreasing blood pressure. The binding to the $\beta$2 receptor (which has a role in cardiac function, metabolism, and vascular tone) inhibits peripheral vasoconstriction and bronchoconstriction. As a result, the drugs lower blood pressure[54].

Beta-Adrenergic Receptor 1( $\beta$1AR) and Beta-Adrenergic Receptor 2 ($\beta$2AR) recruitment of arrestins occurs as a result of GRK phosphorylation, but Beta-Adrenergic Receptor 3 ($\beta$3AR) has a short C-terminal tail; therefore, it lacks GRK phosphorylation sites so upregulation of GRK will not affect $\beta$3AR. GRK-mediated receptor phosphorylation of $\beta$1AR and $\beta$2AR causes $\beta$-arrestin recruitment, sequential receptor internalization, and Erk activation. Therefore, it appears as a better pharmacological option to $\beta$-Blockers. Although $\beta$-Blockers prevent excessive catecholamine stimulation of $\beta$ARs, decrease cellular apoptotic signaling, and normalize $\beta$AR expression, reducing cardiac re-

modeling and mortality. The effects of $\beta$- Blockers are not long-lasting; instead, $\beta$-arrestin-dependent signaling activated by GRKs overexpressed by saRNAs could stimulate cardioprotective signaling and have inotropic effects via $\beta$ARs. This will have longer-lasting antihypertensive effects than $\beta$-Blockers[55].

Therapeutically selected $\beta$-arrestin signaling can counteract the hypertensive effects from catecholamine-induced G-protein signaling, including vasoconstriction, endothelial dysfunction, sodium reabsorption, and retention actions that increase blood pressure[56]. So, overexpression of GRKs via saRNAs can have beneficial off-target effects for $\beta$1AR and $\beta$2AR.

However, the $\beta$2AR-$\beta$-arrestin-1 signaling pathway has been associated with the accumulation of DNA damage in the stress response pathways. As a result, DNA damage may be an unwanted off-target effect of saRNA-mediated GRK upregulation[57,58].

**Other Neurotransmitter Receptors Affected by Overexpression of GRKs**

Dopamine 1 and Dopamine 2 receptors are regulated by desensitization mechanisms controlled by GRKs. Ligand-activated dopamine receptors are bound by multifunctional scaffolding arrestin proteins following GRK phosphorylation, terminating the G protein pathway. Dopamine receptor internalization or downregulation are the results of GRK phosphorylation[59]. The functional difference of $\beta$-arrestin1 and $\beta$-arrestin2 is that $\beta$-arrestin1 prefers recruitment to dopamine receptor 2 following GRK phosphorylation, whereas $\beta$-arrestin 2 prefers dopamine receptor 1. This results in dopamine receptor 1 complexes with Gs to stimulate adenyl cyclase activity and dopamine 2 receptor complexes with Gi to inhibit adenyl cyclase activity[60]. Demonstrating the stimulatory and inhibitory effects of dopamine receptors from $\beta$-arrestin-mediated signaling that will possibly be induced by saRNA upregulation of GRK2 and GRK5.

Dopamine receptor $\beta$-arrestin2 signaling is a treatment for schizophrenia and related disorders due to its pathway in reducing psychotic response in the brain. However, dopamine receptor 1 and dopamine receptor 2 receptor internalization will have side effects of increased risk of substance abuse due to dopamine receptors' primary role in drug reward and the pathogenesis of addiction[61]. So saRNA activation of GRKs will potentially have both positive and negative outcomes in neuropathologies related to dopamine receptors.

Among the opioid receptors, K-opioid receptors and delta-opioid receptors are highly expressed in the central nervous system and engage in interactions with $\beta$-arrestin following GRK phosphorylation. However, K-opioid receptors have limited phosphorylation sites so GRK-dependent regulation has little influence on the receptor's behavior. Activation of p38 mitogen-activated protein kinase pathway following arrestin recruitment to K-opioid receptors by GRK phosphorylation allows for analgesic effects but with the adversities of sedation, dysphoria, and hallucination[62]. Moreover, the delta-opioid receptor is a therapeutic target for neurologic and neuropsychiatric disorders where GRK phosphorylation is not optimal due to it's recruitment of $\beta$-arrestins. Delta-opioid receptor-agonist induces seizure results from excessive $\beta$-arrestin2 recruitment[63].

GRK2 and GRK5 regulate a variety of GPCRs in the central nervous system that are critical for varying neurological processes. Adrenergic Receptors, Dopamine Receptors, and Opioid receptors play a role in the maintenance of cardiovascular and neural homeostasis, and their dysregulation can contribute to various neurological or cardiovascular disorders. saRNA activation of GRKs can activate $\beta$-arrestin signaling in these receptors and cause the above-mentioned positive or negative off-target effects.

**Mitigating Off-Target Effects**

Optimizng saRNA modifications is crucial to preventing excessive GRK2/5 stimulation of dopamine and opioid pathways while still promoting the desired effects on adrenergic receptors. Overactive GRK phosphorylation of dopamine and opioid receptors has been associated with negative consequences. Therefore, conducting in vitro assays, such as binding affinity studies, is essential to evaluate the likelihood and reccurrnce of off-target effefcts before saRNA administration[64].

Additionally,implementing a monitoring system is critical for assesing both efficacy and potential off-target effects. For example, transcriptome analysis, including RNA sequencing before and after saRNA treatment, can help identify unintended gene expression changes. Similarly, proteomic profiling, which assesses protein expression levels , can detect off-target effects at the translation level[65].

To further enhance specificity, targeted delivery mechanisms should be employed to direct saRNA specifically to cells expressing the intended receptors, minimizing systemic exposure and reducing off-target effects. Utilizing LNPs engineered to recognize and bind to specific cell surface markers can facilitate precise delivery[66].

Moreover, sequence optimization plays a key role in improving specificity. Designing saRNA sequnces to closely match the target mRNA while minimizng homology to non-target sequences can help reduce unintended interactions. Maintaining an optimal GC content(typically around 40% to 60 %) can also influence binding affinity and enhance target specificity[66].

**Neuronal Circuits Affected by GRKs**

The upregulation of GRK2 and GRK5 via saRNA-mediated mechanism is expected to reduce excessive sympathetic outflow, contributing to antihypertensive effects. However, this modulatory may also lead to potential consequences but with the potential consequences such as diminished adaptive autonomic responses, altered baroreflex function, and off-target effects on neurotransmission.

The impact on C1 catecholaminergic neurons (sympathoexcitatory circuit): the C1 neurons in the RVLM play a critical role in AT1R-mediated signaling, regulating sympathetic outflow. GRK2/GRK5 upregulation could attenuate AT1R signaling, reducing excessive sympathetic activity. While this reduction may contribute to antihypertensive effects, it could also blunt adaptive responses to stress and exercise by affecting vasomotor control[67].

The Cholinergic Circuit from the Pednuculpotine Tegmental Nucleus: cholinergic projections to the RVLM facilitates baroreflex control and sympathetic activation. GRK2/GRK5 modulation may alter muscarinic receptor signaling, potentially impairing cardiovascular reflexes. This could potentially impact autonomic balance and reduce exercise induced cardiovascular responses[68].

The GABAergic Inhibitory neurons from the Caudal Ventrolateral Medulla (CVLM): CVLM provides inhibitory GABAergic input to the RVLM, regulating sympathic output. GRK2/GRK5 upregulation in the RVLM may alter GABAargic receptor signaling, modifying the balance between sympathetic excitation and inhibition, which could modify blood pressure regualtion[69].

The Glutamatergic inputs from the PVN: PVN neurons project to the RVLM and regulate sympathetic outflow through AT1R activation. Increased GRK2/GRK5 expression could desensitize AT1R-mediated excitation in the RVLM, potentially reducing stress-induced hypertension and improving cardiovascular regulation[70].

**RNA-Targeted Therapeutics**

RNA-targeted therapeutics are becoming novel pharmacological approaches to hypertension treatment. Clinically approved RNA therapy so far for hypertension treatment includes the implementation of direct antisense oligonucleotides(ASO), a nucleic-acid-based therapeutic, that inhibits liver angiotensinogen mRNA translation, as well as small interfering RNA (siRNA) that suppresses liver angiotensinogen via the RNA interference pathway[71,72]. For example, IONIS-AGT-LRxis a hepatocyte-directed ASO drug in the clinical development process for hypertension and heart failure. It targets the start of the RAS pathway by suppressing the synthesis of liver-deriver angiotensinogen[73]. Whereas, Zilebesiran is the siRNA therapeutic agent that inhibits the synthesis of angiotensinogen by post-transcriptional silencing of the angiotensinogen gene[74].

saRNA presents a promising and practical alternative to RNA interference methods like siRNA and ASO due to its lower dosage requirements and longer-lasting effect, potentially leading to fewer side effects. In terms of efficacy, siRNA and ASOs are potent gene silencing tools, they require continuous administration due to their transient effects. In contrast, saRNA enables prolonged gene activation, reducing the need for repeated dosing and enhancing therapeutic efficiency.

Regarding safety, siRNA and Asos may induce off-target effects or immune responses due to unintended interactions with non-target RNAs. However, saRNA's requirement for promoter-specific targeting may lower these risks. Further studies are needed to fully confirm its specificity and potential immunogenicity. From a practicality standpoint, saRNA's self-amplifying nature allows for lower therapeutic doses compared to siRA, improving its feasibility for clinical practice[75].

Gene overexpression via mRNA, CRISPR activation, ASOs, and saRNAs, are viable options for therapeutic applications. This study utilizes saRNA for gene overexpression due to several advantages. CRISPR activation requires delivery of both a guide RNA and a Cas9 enzyme, making it less practical for clinical use due to the complexity of current delivery systems. The ASOs are designed to inhibit mRNA translation via the upstream open reading frames in the 5'UTR. Also it is challenging to produce mRNA longer than 150 nucleotides in length, yields exponentially decreases as the length increases. The saRNA is the preferred option because of its ease of synthesis and the availability of bioinformatic tools that aid in predicting saRNA sewunces that act on promoters[76].

Following the biology of protein regulation, where distal enhancer elements and active gene promoters interact through chromosomal looping to recruit transcription factors, chromatin modifiers, mediator complex, and RNA polymerase II for transcription activation, the roles of RNA therapeutics in gene expression can be better understood[77].

Nucleic acid-based therapeutics (NBT) are small molecules that can directly interact with RNA and protein to recruit endogenous enzymes to target RNA and upregulate proteins. This category of therapeutics includes ASOs, natural antisense transcript-specific oligonucleotides, saRNAs, and microRNA blockers. To enhance the transfection efficiency of NBTs, scientists often perform the following chemical modifications: backbone modifications, ribose sugar substitutions, nucleobase derivatives, stabilizing or cleavable internucleotide linkages, and functionalization with various conjugates. To design effective NBTs, the polyanionic hydrophilic structure of nucleic acids is crucial— due to its pharmacokinetic and pharmacodynamic properties such that it slows down cellular membrane penetrance, the endosomal escape increases susceptibility to endonuclease degradation and off-target interactions[78,79].

saRNA is a type of small double-stranded RNA (dsRNA) like siRNA and miRNA, but instead of inhibiting expression, it induces gene expression via activation of promoter sequences of targeted genes, this mechanism is termed RNA activation (RNAa). RNAa is dependent on Argonaute proteins (Ago), specifically Ago2, for cytosolic maturation of saRNa. Ago2 is assumed to be an RNA-programmable homology search and binding protein. RNAa pharmacokinetically takes 48 hours for gene activation to be detectable[80,81]. saRNA is an optimal RNA-

targeted therapeutic for the upregulation of GRK2 and GRK5 for increased blood pressure control.

### Neuronal saRNAs

Numerous studies have successfully tested saRNAs that play a role in RNAa for the activation of gene expression in cultured cells. saRNA is a therapeutic agent that influences the development of biotechnological applications that don't require the synthesis of a hazardous construct system with exogenous DNA sequences. Current challenges in therapeutic applications of saRNA therapy are low stability, degradation, opsonization in the bloodstream, and off-target effects[82].

Similar to RNAi (siRNA and miRNA), a posttranscriptional gene silencing mechanism with dsRNAs, saRNAs are dependent on the Ago protein family. Overall, transcriptional and epigenetic modulation of expression dsRNAs requires Ago2. In both the RNA-induced silencing complex and RNA-induced transcriptional activation complex (RITA), Ago2 plays the same role of initial RNA duplex loading and strand processing. Promoter-targeted saRNA loaded on Ago2 serves as a promoter-associated platform for the recruitment of RH9 (nuclear DNA helicase II) and CTR9 (a component of PAF1C response for transcription initiation and elongation) to assemble the (RITA) complex[83].

There are two models of locus-specific gene activation: promoter-targeted duplex RNA activating and natural antisense disruption to change chromatin structure[84,85]. However, numerous studies have shown that endogenous saRNAs act via the first model by selectively activating gene expression in neurons through a promoter-targeted duplex. A study found Ago2-loaded saRNAs successfully associated with the targeted promoter regions and RNAP II to enhance initiation and elongation in transcription—validating the specificity of RNA-guided genome targeting mechanisms. Anyways, both RNAa models occur at the transcriptional level of gene regulation and have an on-target effect[84].

Numerous studies have tested saRNA-mediated locus-specific neural gene activation. One study intended to find an alternative approach to genome engineering of gene deficits or therapeutic stimulation via artificial transactivators for hemizygosity neurological diseases. saRNAs were tested on a haploinsufficient Foxg1 brain patterning gene that is vital to various neurodevelopmental and physiological pathophysiologies. The study selected 8 synthetic saRNAs upregulating Foxg1 in neocortical precursor and limited the RNAa to neural cells that naturally express Foxg1 and don't hide endogenous gene tuning. It was found that the saRNA-mediated gene upregulation involving recruitment of RNA polymerase II was dependent upon Ago2, and the saRNA was able to associate with target chromatin via nascent noncoding RNAs. The saRNAs were delivered into the cells by intracerebroventricular (ICV) injections of recombinant AAV vectors. The saRNa successfully reaching target genes after ICV injection demonstrates the plausibility of saRNA treatment administered via ICV injection for upregulation of certain GRKs to confer a blood pressure-lowering response[85].

Another study successfully used infusion into the cisterna magna in hypertensive rats to inject dsRNAs into the RVLM to silence Noxa1 to alleviate oxidative stress. This demonstrates the plausibility of saRNA treatments directly injected into the brain to bypass the blood-brain barrier to target the cardiovascular center RVLM[86].

The invasiveness of a direct injection into the brain is a pitfall to using ICV for saRNA treatment, but peripheral RNA injections have difficulty passing the blood-brain barrier and would prove to be an inefficient method. But continued research in RNA delivery systems could identify a repetitively effective delivery system that passes the blood-brain barrier. A study showed promising results using Molecular Trojan Horses combined with Trojan Horse Liposome technology to deliver RNA or DNA-based therapeutics across the blood-brain barrier via intravenous administration (IV). In this approach, formulation techniques like advin-biotin technology are used to attach the dsRNA to the molecular trojan horse—stabilizing the system for in vivo delivery. This peripheral injection method could replace the need for invasive procedures like ICV injection for the delivery of RNA therapeutics in brain regions[87].

To test the specificity of saRNA-guided genome activation, a study performed chromatin isolation by biotinylated RNA Pull-down (CHIbRP) to biotinylate a saRNA duplex at the $3'$ end of one of the strands of the targeted chromatin associated with saRNA. Then the duplexes were transfected into cells. Results observed from RNAa activity of the $3'$-biotinylated saRNA duplexes reveal elements of the Ago2-loading profile that specifically influence targeted gene expression of mRNA and protein levels. The on-target effect of the Ago2-loaded saRNA guide and the results of the statistically significant enrichment of target promoter DNA confirmed sequence-specific binding at targeted gene promoters[88]. Therefore, off-target effects can be minimized by the specificity of saRNA-guided genome activation of GRK2 and GRK5 to increase $\beta$-arrestin-dependent signaling of AT1R for increased blood pressure control.

Different biochemical tools are used to assess saRNA activity. For instance, a nuclear run-on (NRO) assay is used to determine the transcription rate of saRNA-inducible genes. Chromatin immunoprecipitation (CHIp) analyses are used as proof of enrichment of RNAa, confirming the transcriptional mechanisms of saRNA-driven activation. These biochemical tools can be used to test the efficacy of saRNA-mediated upregulation of GRK2 and GRK5 before administration in vivo[89].

### Delivery of the saRNAs

#### Liposome-based Lipofectamine RNAiMAX

Liposome-based Lipofectamine RNAiMAX is a common

transfection agent because it applies to various cells. Central to the lipofectamine system are the cationic lipids, composed of a phospholipid bilayer with a positively charged phosphate head and one or two hydrocarbon tails. The cationic lipids interact with the saRNA via electrostatic attractions of the inner part of the polar head group with the phosphate backbone of nucleic acids. The liposome's positive charge attracts the cell membrane's negative charge, allowing for the endocytosis of saRNA into the cell. However, the chemical structure of the phospholipid bilayer affects the transfection efficiency: weakly charged heads and longer alkyl chains have been associated with high transfection efficiency. But the non-neutrality of the liposomes poses a toxicity threat to the saRNAs[90]. A study intended to identify a lipofection protocol for upregulating mir-21 expression levels in neurons. Successfully reported the use of lipofection protocol to deliver microRNA, a type of dsRNA, into targeted neurons in vitro with minimal off-target effects. Proof of concept for precise lipofection delivery of saRNAs for upregulation of GRKS into the RVLM[91].

**Lipidoid nanoparticles for saRNA delivery to neural cells**

Lipid nanoparticle systems (LNPs) may be a better alternative to lipofectamine for the therapeutic targeting of neurons. Although cellular uptake is comparable in neural cells, tested siRNA-loaded LNPs remain stable in neural cells for seven days, while lipofectamine-facilitated delivery of siRNAs was not well tolerated by cells and appeared to have higher toxicity and immunogenicity in neuronal models[92]. The outcome of lipofectamine delivery of siRNAs could potentially have a comparable outcome for saRNA delivery. Therefore, LNP delivery of saRNAs into the RVLM via ICV injection is optimal to confer a stable and specific association with GRK2 and GRK5 genes.

LNP characteristics can be manipulated for improved neural cell delivery. The LNP system consists of cationic lipids and other helper lipids like distearoylphosphatidylcholine (DSPC), polyethylene glycol-dimyristoyl glycerol (PEG-DMG), and cholesterol. The optimal characteristics for neural cells include high encapsulation efficiency and enhanced intracellular release via effective endosomal escape by cationic lipids. The efficiency of the endosomal escape is contributed to the acidic microenvironment of endosomes that gives LNPs a positive charge to cause endosomal destabilization when the negative anionic endosomal lipids associate with the positive LNPs. This leads to effective saRNA release in the cytoplasm. Furthermore, ionizable cationic lipids have a pka value below 7 for optimal oligonucleotide encapsulation, and the lipids at the surface are relatively neutral and around the physiological pH to minimize charge on LNP systems, reducing toxic side effects and allowing rapid clearance from circulation, the saRNA treatment can quickly and safely increase gene expression of GRKs to manipulate the signaling pathway of AT1R[93].

C12-200 lipidoid has the highest delivery efficacies due to its high cell uptake in vitro. Therefore, saRNA-LNPs using C12-200 lipidoid would be optimal for maximizing saRNA uptake in neural cells of the RVLM[94].

**Designs of LNP Drugs**

LNP technology for genetic drugs requires efficient encapsulation of RNA polymers into LNP systems. Ionizable cationic lipids are preferred for the high loading efficiency of genetic drugs in an LNP system with a diameter of less than 100 nm and a low surface charge. This prevents issues with instability, positive surface charge, and dose-limiting toxic side effects.

So far, LNP technology for in vivo delivery of siRNA for therapeutic purposes has been used to silence targeted genes from IV injection for the treatment of cardiovascular disease, liver disease, and cancer. Onpattro is the first FDA-approved LNP-based RNAi drug for the treatment of polyneuropathy[95].

The ethanol loading approach to encapsulating oligonucleotides is preferable for siRNA LNP systems due to its meeting of kinetic, reproducible, and scalable standards for an in vivo delivery system for genetic drugs. Formulating LNP siRNA systems requires rapid microfluidic mixing of solutions with cationic lipids and siRNA. Microfluid mixing with the staggered herringbone micromixer (SHM) offers high siRNA encapsulation efficiency and improved synthesis of LNP siRNA systems in the range of 20-100 nm[96].

LNP-mediated delivery of saRNA follows a methodology similar to that of more experimented siRNA-LNP systems. The first goal is cellular uptake via endocytosis of LNPs into cells and endosomes. The second step is saRNA release into the cytoplasm of the targeted cell through a pH difference mechanism in endosomes to instigate LNP release of the RNA. There is also a sequence-specific association of the saRNA for the upregulation of the targeted gene. These saRNA LNP systems would be used for delivery into the RVLM, would be uptake by neural cells, and would associate with the genetic sequence for GRK2 and GRK5 to stimulate gene expression[97].

**ICV Delivery of saRNA-Loaded LNPs**

LNPs have emerged as a leading delivery system for siRNA-based therapies, with optimized cationic lipids demonstrating effective gene silencing in multiple clinical studies. LNP-siRNA systems have been successfully used to silence genes in the liver following IV administration in animal models, and clinical trials have shown efficacy in treating cardiovascular diseases. Additionally, studies have demonstrated the feasibility of LNP delivery for silencing androgen receptor (AR) gene expression in vivo. However, neuronal delivery of LNPs remains challenging due to the restrictive nature of the BBB[98].

A study investigating LNP-siRNA delivery for neuronal gene silencing reported promising results both in vitro and in vico. In

cell culture, 100% of neurons successfully internalized LNPs with no observable toxicity, leading to a significant reduction in protein expression. In vivo, ICV administration of siRNA-loaded LNPs facilitated widespread diffusion and uptake throughout the brain, resulting in a consistent decrease in mRNA and protein expression without inducing toxic effects [99].

Further studies have confirmed the feasibility of this approach. In one experiment, lucierferase siRNA-loaded-LNPs were injected into rat brains, demonstrating that ICV delivery enables diffusion across extracellular spaces, effectively silencing target genes at reasonable distances from the injection site. These findings highlight the potential of ICV administration for precise and sustained gene modulation in the CNS [99].

Despite these results, ICV delivery of LNPs presents several obstacles. Immune response and neuroinflammation: the lipid composition of LNPs plays a critical role in their interaction with the immune system. Ionizable lipids, essential for endosomal escape and cytosolic release of saRNA, may also interact with toll-like receptors (TLR) on glial cells, provoking an inflammatory response. Excessive neuroinflammation could compromise the therapeutic efficacy and potentially leading to neurotoxicity. Toxicity mitigation strategies include the use of cationic lipids which are necessary for endosomal escape and cytosolic release of the saRNAs, reducing their proportion and incorporating biodegradable lipid components, such as trehalose glycolipids exhibit lower toxicity in major organs like the heart and liver compared to conventional LNP formulations. Also, systematic innate immune activation due to saRNA delivery could lead to unwanted gene overexpression, raising concerns about potential toxic outcomes. Strategies to fine-tune saRNA sequences and optimize nanoparticle surface properties may enhance specificity while minimizing adverse effects [100].

### Alternative Delivery Methods

IV administration presents a non-invasive alternative to ICV but faces challenges in crossing the BBB. To enhance BBB penetration and targeted delivery to regions like the RVLM, advanced nanoparticle modifications are necessary. For example, peptide-functionalized LNPs offer a promising pharmaceutical approach by improving cellular uptake and BBB translocation. Cell-penetrating peptides (CPPs) facilitate targeted delivery of nucleic acids by enhancing cellular internalization. Studies on liposome-siRNA-peptide complexes show that covalent bonding between siRNA-liposomes and targeting peptides increases receptor affinity, improving cellular uptake. Additionally, polyethylene glycol (PEG)-modified liposomes, enhance stability and circulation time by reducing immune clearance. In vivo studies have demonstrated reduced serum degradation of siRNA and improved brain targeting. However, clinical translation remains limited—only 24 clinical trials have employed siRNA therapy to date. The primary obstacles include lack of in vivo validation since most CPP-based delivery studies have been conducted in vitro, the potential toxicity from endogenous interactions may lead to off-target effects, and uncertain BBB penetration (successful cellular uptake does not guarantee adequatee BBB transversal) [101].

Given the challenges associated with liposomal delivery, exosome-based carriers present a promising IV alternative. Exosomes naturally transport genetic material between cells, and engineered versions, such as siRNA-loaded RBG exosomes, have shown potential in targeting neurodegenerative diseases, including Parkinson' and Alzheimer. However, several key challenges must be addressed: immune evasion, while exosomes are biocompatible, immune recognition can still limit efficacy; defective endosomal systems, inefficient endosomal escape may reduce therapeutic potency; optimization of cargo modification, enhancing exosomal loading to reduce pathological proteins while increasing neuroprotective factors remains a critical challenge [102].

While ICV delivery is highly effective in penetrating the BBB, its invasivenesss limits widespread clinical use. IV administration with either peptide-functionalized LNPs or exosome-based systems is a promising alternative, but further research is needed to improve BBB penetration, targeting specificity, and in vivo validation. Further strategies should focus on optimizing nanoparticle modifications to ensure safe, efficient, and clinically viable siRNA delivery to the CNS.

## Methods

### Hypothetical Lab Methods

#### dsRNA Design and Syntheis

Using the University of California at Santa Cruz Genome Database (http://genome.ucsc.edu) 1 kb of promoter sequence was retrieved for the GRK2 and GRK5 genes, and each was scanned for 4 saRNA targets based on agreed-upon saRNA design rules. For the promoter analysis, the sequences before the first exon of gene transcripts were isolated and defined in genome databases, and basic bioinformatics sequence analyses were run on the promoter sequence to identify the target gene sequence for saRNA selection. Repetitive elements were excluded to eliminate off-target effects. High frequency of CPG islands and stretches of DNA with unusually high GC content were identified by the MethPrimeer CPG island prediction program (https://www.urogene.org/methprimer/). In order to avoid hindering the Ago processing of the passenger strand and transcription gene silencing via DNA methylation, the CPG islands were not included in saRNA targets. Candidates best aligned with the rules of functional saRNA designs were selected. Each target saRNA was 19 nucleotides long and had AT-rich 3′ nucleotide overhang. A sense RNA and complementary antisense sequence were created based on the target sequence. Finally,

[U][U] overhangs were added to the 3′-end of both RNA strands. GRK2 saRNA sequences are listed in FIGURE 1. The chemical synthesis and annelation of RNA strands are performed to create a dsRNA duplex [103,104].

**Table 1** GRK2 saRNA Strands: 3 sets of complementary sense and antisense GRK2 saRNA designs formulated following the above procedure

| GRK2 saRNA designs |
|---|
| Sense: CCAGGUUCUGGGUGACCUA-CUAA[U][U]<br><br>Antisense:<br>GGUCCAAGACCCACUGGAU-GAUU[U][U] |
| Sense:<br>AGUGUGGGUCCCAGGUUA-GAC[U][U]<br><br>Antisense:<br>UCACACCCAGGGUCCAAU-CUG[U][U] |
| Sense:<br>UCUGGAGUUGACAGUCCAA-UCUA[U][U]<br><br>Antisense:<br>AGACCUCAACUGUCAGGUU-AGAU[U][U] |
| Sense:<br>GAUCGAGGUCAUGGUCCAA-AAU[U][U]<br><br>Antisense:<br>CUAGCUCCAGUACCAGGUU-UUA[U][U] |

**Lipidoid synthesis**

For the synthesis of C12-200 lipidoid, amine 2[4-2( 2-aminoethyl)amino)-ethylpiperazine-1-YL)ethan-1-amine (200)i reacted with the tail 1,2-epoxyhexadecane (C12) at a molar ratio of 1:5. Glass scintillation vials are used to mix amines and tails without solvent at 90 degrees celsius for a 3 day period. A Teledyne ISCO chromatography system is used for lipidoid purifn [105]. A study found 15 to 50-fold greater protein expression when modifying the delivery vehicle of therapeutic mRNAs, the study found this method of lipdoid synthesis to be most effective.

**Formulation of LNPs**

To form the LNPs, C12-200 lipidoid, cholesterol, and mPEG2000-DMG must be mixed at a molar ratio of 50:38:35 in 90% (v/v) ethanol and 10% (v/v) 10 mM sodium citrate. A saRNA solution is prepared via the dilution of saRNA in a 10mM solution of sodium citrate buffer to produce a 10:1 final weight ratio of lipidoid. Microfluidic device SHM is used to mix equal volume amounts of lipid and saRNA solutions. Then any particles were dissolved in phosphate buffer saline. Following that, for 90 minutes in 3500 g/mol molecular weight cutoff cassettes LNPs were dialyzed against the phosphate buffer saline [106]. A study followed this formulation of LNPs for siRNA drugs, and the result was a consensus 95% protein silencing in vivo. The study recommends using their chemical design criteria for degradable lipidoids to reliably predict in vivo siRNA delivery efficacy without cell culture assays and animal testing prior [105].

**In Vivo GRK SaRNA delivery**

Administration of LNPs: injection 0.5mg/kg GRK saRNA. ICV injection of the drug into the RVLM would allow for saRNAs to hyperactivate GRK2 and GRK5 expression [96]. Studies with IV delivery of LNP-mRNA systems saw greater protein expression at 0.5mg/kg dosing [105,106]. Expression of the saRNA could be analyzed 48 hours post-injection [107]. The implementation of synthetic serum markers expressible in the brain but transportable in the blood for less invasive analysis of gene activation in the RVLM through a blood test could be utilized to assess the functionality of the saRNA-induced GRK overexpression. A study can monitor gene expression in the brain because of the development of synthetic serum markers [108].

**Search Methods**

To evaluate the potential of saRNA for upregulating transcription of GRK2 and GRK5 in neural cells for hypertension management, this paper e was conducted following these criteria. Studies were identified through Pubmed using keywords AT1R, $\beta$-arrestin signaling, GRKs, RNAa, gene therapy, saRNAs, and hypertension. Criteria for inclusion required experimental studies on gene activation in neural cells, GRK activation, and hypertension-related outcomes and literature reviews on AT1R signaling pathways. Search strategies included the use of boolean operators such as “and” and “or,” which were used to find studies relating to $\beta$-arrestin AT1R signaling and GRK overexpression. For precise search strategies, this review used MeSH(medical subject headings) terms, a feature of Pubmed that is used for indexing articles, using MeSH terms like Hypertension/gene therapy paired with more specific keywords like saRNA and GRKs for a precise query. The filtered search option was implemented to limit results to clinical trials, literature reviews, and meta-analyses. All of these components are compiled in the advanced search.

## Discussion

The promotion of AT1R signaling via the $\beta$-arrestin pathway was previously thought to only desensitize GPCRs, but $\beta$-arrestin signaling mediates receptor internalization and G-protein independent signaling. Thus, it offers new hypertension therapeutic opportunities targeting neuronal AT1R located in regions of cardiovascular regulation [109].

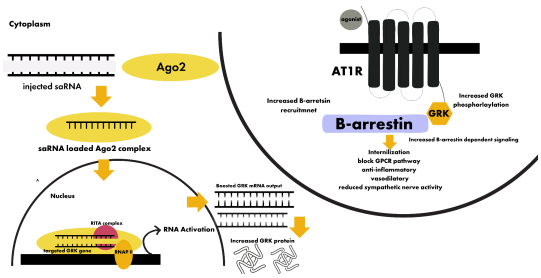


**Fig. 1** saRNA-Mediated $\beta$-arrestin Dependent Signaling:

AT1R-$\beta$-arrestin mechanisms induce antihypertensive effects, including a decrease in vascular resistance, lowered blood pressure, reduced saline intake, and minimized sympathetic outflow. GRKs are the regulators of AT1R selectivity of G protein or $\beta$-arrestin pathways. GRK2 and GRK5 are commonly associated with the AT1R receptor, and following their phosphorylation, $\beta$-arrestins are recruited to the receptor to initiate $\beta$-arrestin-dependent mechanisms. This report suggests the use of RNAa with saRNAs to upregulate the transcription of GRK2 and GRK5 in the RVLM neuronal cells due to its regulatory role in the cardiovascular region and abundance of AT1R receptors. Similar to Zibestarain, a siRNA therapeutic for hypertension whose gene silencing effects last six months, it is possible for saRNA gene activating effects to last patients six months as well following the proposed ICV injection[34,110].

A Simplified diagram of saRNA-induced GRK activation leading to increased GRK-mediated $\beta$-arrestin dependent signaling, resulting in antihypertensive effects.

Injected saRNA into the RVLM interacts with Ago2, forming the saRNA-loaded Ago2 complex, which enters the cytoplasm to assemble the RITA complex. This complex facilitates RNA activation and GRK translation. The translated GRK proteins then phosphorlate AT1R, inducing $\beta$-arrestin signaling, which leads to antihypertensive effects—inclduing receptor interlization, inhibition of the hypertensive GPCR pathway, vasolidation,and reduced sympathetic nerve activity.

Ensuring targeted and efficient delivery of saRNAs to RVLM cells is difficult, which may limit the effectiveness of the therapy. Optimization of LNPs is needed for saRNAs with complex secondary structures, which makes it more challenging to encapsulate and preserve the saRNA. Naked-saRNA delivery has numerous challenges, including membrane impermeability, degradation, molecular phagocyte system entrapment, endosomal escape, and off-target effects. However, LNPs have shown minimal toxicity and off-target effects for saRNa delivery[111].

MTL-CEBPA is the first saRNA drug in clinical development. The RNA therapeutic regulates hepatic and meloid functions via the upregulation of CCAAT/enhancer-binding protein alpha transcription factor, which is a tumor suppressor and regulator of hepatocyte function. CEBPa is a proof of concept for saRNA drugs that enhance the expression of targeted genes to improve treatment outcomes. In the clinical trials for MTL-CEBPA, a patient experienced umor size reduction (partial response) that lasted for more than two years—this means the therapeutic effect of the saRNA drug exists. This opens the possibility that with proper chemical modification of the saRNA the therapeutic effect could have long-lasting effects. However, not enough clinical implementation of saRNA therapy has been done to actually determine the duration of the treatment. The cost effectiveness depend on the duration of the effect and whether repeat treatments are required. If one treatment lasts over 5 years, the saRnA therapy could become competitive with chronic medication costs. But with the ICV model and 2-year limitation, this new form of therapy will be significantly higher[112].

## Conclusion

RNA therapeutics are gaining prominence in the treatment of cardiovascular diseases, especially hypertension, where several studies with antisense RNA siRNA-based drugs have been used to effectively manage high blood pressure in numerous clinical studies[113,114].

RNA therapy for hypertension could renovate the current standard of hypertension care to improve patient treatment outcomes and economically benefit the U.S. healthcare system. If RNA therapy is implemented as first-line therapy, a saRNA injection will result in a long-lasting and sequence-specific induction of a targeted gene. Future clinical studies and research must be continued, but if saRNA therapy proves effective, patients will experience blood pressure control for a possible 2 years or more, a hypertension treatment approach more convenient than several daily doses of blood pressure medication that cause severe issues with patient adherence. This research highlights the potential of saRNA-based gene therapy as an innovative and targeted approach to treating hypertension by modulating key signaling pathways within neural cells.

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