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MicroRNA mimics as novel therapeutics for psychiatric disorders: The case of microRNA-135

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Zusammenfassung

Die Pathophysiologie einer schweren depressiven Störung (Major Depression Disorder - MDD) und die Reaktion auf Antidepressiva werden voraussichtlich zumindest teilweise durch epigenetische Mechanismen beeinflusst, die die Transkription und Translation regulieren. Diese molekularen Prozesse sind entscheidend für die Vermittlung von adaptiven oder maladaptiven Veränderungen im Gehirn. Umweltfaktoren spielen eine wesentliche Rolle bei der Entwicklung und Ätiologie von Depressionen, und es wird davon ausgegangen, dass epigenetische Mechanismen den Zusammenhang zwischen genetischer Veranlagung und Umweltfaktoren vermitteln. Zu den gut untersuchten Mechanismen der epigenetischen Regulierung gehören nicht-kodierende RNAs, insbesondere microRNAs (miRNAs). Jüngste Daten haben Belege dafür geliefert, dass Veränderungen in der miRNA-Regulierung oder -Funktion mit verschiedenen psychiatrischen Störungen, einschließlich Major Depression, in Verbindung stehen.

Selektive Serotonin-Wiederaufnahme-Hemmer (Selective Serotonin Reuptake Inhibitor, SSRI), die die Serotonin (5-HT) -Signalübertragung verstärken, sind weit verbreitete Antidepressiva. Daher wurde die Rolle der microRNAs (miRNAs) im serotonergen System eingehend untersucht. Eine umfassende Studie, die von Issler et al. (2014) durchgeführt wurde, verglich die Werte von miR-135 durch Blut und Gehirnproben von Patienten mit Major Depression (MDD) und gesunden Personen und stellte fest, dass die Werte in der depressiven Gruppe niedriger waren. Darüber hinaus wurde miR-135 als starker Regulator innerhalb des serotonergen Systems identifiziert. Sowohl Zellkultur- als auch Tierstudien haben gezeigt, dass miR-135 die Niveaus der Serotonintransporter (SERT) und die Transkriptionen der 5-HT1A-Rezeptoren (5-

HT1AR) direkt kontrolliert. Bei Mäusen führte die absichtliche Erhöhung von miR-135 speziell in serotonergen Neuronen zu ähnlichen Ergebnissen wie die Wirkung von Antidepressiva und Medikamenten gegen Angstzustände. Umgekehrt hatte eine Verringerung von miR-135 den gegenteiligen Effekt, indem es angstähnliche Verhaltensweisen verstärkte und die Reaktion auf antidepressive Medikamente verringerte. Diese Ergebnisse deuten darauf hin, dass miR-135 ein potenzielles therapeutisches Ziel für die Behandlung von Angstzuständen und Depressionen sein könnte.

In der aktuellen Forschungsarbeit untersuchte ich die molekularen und verhaltensbezogenen Effekte, die zuvor in einem transgenen Mausmodell beobachtet wurden, das miR-135 in serotonergen Neuronen des dorsalen Raphe-Kerns (DRN) spezifisch überexprimierte, unter Verwendung einer synthetischen Version von miR-135, die als miRNA-Mimik bekannt ist. Wir verabreichten die miR-135-Mimik direkt in den DRN, in den Liquor intrazerebroventrikulär (ICV) oder über eine intranasale Verabreichung und untersuchten seine Wirkung auf die Funktionalität des serotonergen Systems, die Expressionsniveaus von miR-135-Zielgenen und seine antidepressiven und anxiolytischen Wirkungen in Mausmodellen. Unsere Ergebnisse liefern überzeugende Beweise für das Potenzial der miR-135-Mimik als ein neuartiges Antidepressivum.

Das Design und das Screening der miR-135-Mimik basierte auf der endogenen Antisense-Sequenz von miR-135, die mit verschiedenen Sense-Sequenzen und chemischen Modifikationen gekoppelt wurde. Wir haben gezeigt, dass die Art und der Ort dieser Modifikationen die Wirksamkeit des Wirkstoffs und sein Potenzial zur Aktivierung angeborener Immunreaktionen beeinflussen können. Um die Herausforderung zu meistern, Oligonukleotide in das Gehirn zu bringen, haben wir die

doppelsträngige miRNA-Mimik mit einem niedermolekularen Medikament (Sertralin) kovalent konjugiert. Diese konjugierte Verbindung zeigte nach nicht-invasiver intranasaler Verabreichung bei Mäusen eine selektive Anreicherung in der anvisierten Hirnregion, insbesondere in dem dorsalen Raphe-Kern.

Zusammenfassend unterstreichen die Ergebnisse der pharmakologischen, molekularen und verhaltensbiologischen Experimente, die in dieser Dissertation vorgestellt werden, dass Moleküle auf der Basis von miRNAs und insbesondere miR-135-Mimik als potenzielle Behandlung für MDD dienen können.

Abstract

The pathophysiology of major depressive disorder (MDD) and the response to antidepressants are suggested to be influenced, at least partially, by epigenetic mechanisms that regulate transcription and translation. These molecular processes are crucial in mediating adaptive or maladaptive changes in the brain. Environmental factors play a significant role in the development and etiology of depression, and epigenetic mechanisms are considered to mediate the mechanistic link between genetic predisposition and environmental factors. Among the well-studied mechanisms of epigenetic regulation are non-coding RNAs, particularly microRNAs (miRNAs). Recent data have provided evidence linking changes in miRNA regulation or function to several psychiatric disorders, including MDD.

Selective serotonin reuptake inhibitors (SSRIs), which enhance serotonin (5-HT) signaling, are widely used antidepressant drugs. Consequently, the role of microRNAs (miRNAs) in the serotonergic system has been extensively studied. A comprehensive study conducted by Issler et al. (2014) compared the levels of miR-135 in the blood and brain samples of major depressive disorder (MDD) patients and healthy individuals, demonstrating lower levels in the depressed group. Additionally, miR-135 was identified as a potent regulator within the serotonergic system. Both cell culture and animal studies have demonstrated that miR-135 directly controls the levels of serotonin transporter (SERT) and 5-HT1A receptor (5-HT1AR) transcripts. In mice, intentionally increasing miR-135 specifically in serotonergic neurons resulted in outcomes similar to those of antidepressants and anti-anxiety medications. Conversely, decreasing miR-135 had the opposite effect, increasing anxiety-like behaviors and

diminishing the response to antidepressant drugs. These findings indicate that miR-135 could be a potential therapeutic target for the treatment of anxiety and depression.

In the current research thesis, I investigated the molecular and behavioral effects, previously observed in a transgenic mouse model that specifically overexpressed miR-135 in serotonergic neurons of the dorsal raphe nucleus (DRN), using synthetic version of miR-135, known as a miRNA mimic. We administered the miR-135 mimic directly to the DRN, to the CSF intracerebroventricularly (ICV) or via intranasal administration and examined its effect on the functionality of the serotonergic system, the expression levels of miR-135 target genes, and its antidepressant and anxiolytic-like effects in mouse models. Our results provide strong evidence supporting the potential of miR-135 mimic as a novel antidepressant drug.

The design and screening of the miR-135 mimic was based on the endogenous antisense sequence of miR-135 coupled with different sense sequences and chemical modifications. We demonstrated that the type and location of these modifications can influence the compound's efficacy and its potential to activate innate immune responses. Moreover, to address the challenge of delivering oligonucleotides to the brain, we covalently conjugated the double-stranded miRNA mimic with a small molecule drug (Sertraline). This conjugated compound exhibited selective accumulation in the targeted brain area, specifically the dorsal raphe, following non-invasive intranasal administration in mice.

Taking together, the results obtained in the pharmacological, molecular and behavioral experiments presented in this thesis strongly emphasized that miRNAs-based molecules and more specifically miR-135 mimic can serve as potential treatment for MDD.

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2′-F	2'-Deoxy-2’-fluoro
2’-MOE	2′-O-methoxyethyl
2′-OMe	2′-O-methyl
3’UTR	3 prime Untranslated Region
5-HT	5-hydroxytryptamine / Serotonin
5-HT_{1A}	5-hydroxytryptamine receptor 1A protein
5HTT/SERT	Serotonin Transporter
5HTTLPR	Serotonin-Transporter-Linked Polymorphic Region
AAV	Adeno-Associated Virus
aCSF	Artificial Cerebrospinal Fluid
ACTH	Adrenocorticotropic Hormone
Ad	Adrenaline
AD	Alzheimer Disease
AEG-1	Astrocyte Elevated Gene-1
Ago2	Argonaute RISC Catalytic Component 2
AMPA	Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid
APOE	Apolipoprotein E
ASGPR	Asialoglycoprotein Receptor
ASO	Antisense Oligonucleotide
BDNF	Brain Derived Neurotrophic Factor
Cb	Cerebellum
CDC	Centers for Disease Control and Prevention
circRNAs	circular RNA
CPLX1	Complexin-1
CPLX2	Complexin-2
CPu	Caudate Putamen
CRF	Corticotropin Releasing Factor
CRHR1	Corticotropin Releasing Hormone Receptor 1
CSF	Cerebrospinal Fluid
DA	Dopamine
DGCR8	DiGeorge Syndrome Critical Region 8
DLT	Dark Light Transfer test
DMD	Duchenne Muscular Dystrophy
DRN	Dorsal Raphe Nucleus
ECT	Electroconvulsive Therapy
eEF2	Eukaryotic elongation factor 2
EMA	European Medicines Agency
ESC	Enhanced Stabilization Chemistry
FDA	Food and Drug Administration
FKBP51	FK 506 binding protein 51
FMR1	Fragile X Mental Retardation 1
FST	Forced Swim Test

GalNAc	N-Acetylgalactosamine
GR	Glucocorticoid Receptor
GNA	Glycol Nucleic Acid
GNB3	G-Protein β3 Subunit
GWAS	Genome-Wide Association Studies
GxE	Gene by environment
HDAC	Histone Deacetylase
HPA	Hypothalamic Pituitary Adrenal
HPC	Hippocampus
Htr1a	5-Hdroxytryptamine Receptor 1A (Gene or mRNA)
huPBMC	human PBMC
ICV	Intracerebroventricular
IND	Indatraline
ITH	Intrathecally IT
LNA	Locked Nucleic Acid
lncRNA	long non-coding RNA
LNP	Lipid Nano Paticles
LTD	Long-Term Depression
MAO	Monoamine oxidase inhibitor
mRNA	messenger RNA
MDD	Major Depressive Disorder
Met	Methionine
miRNA	microRNA
MOP	Methoxypropylphosphonate
MP	Methylphosphonate
mPFC	Medial Prefrontal Cortex
MR	Mineralocorticoid Receptor
MSD	Meso Scale Discovery
MT	Melatonine
MTHFR	Methylenetetrahydrofolate reductase
MW	Molecular Weight
NAc	Nucleus Accumbens
NAd	Noradrenaline
ncRNA	non-coding RNA
NMDA	N-Methyl D-Aspartate
OB	Olfactory Bulb
ODN	Oligodeoxyribonucleotide
PBMC	Peripheral Blood Mononuclear Cell
PFA	Paraformaldehyde
PFC	Prefrontal Cortex
PK	Pharmacokinetics
PMO	Phosphorodiamidate Morpholino Oligom
PNA	Peptide Nucleic Acid
pre-miRNA	Precursor microRNA
pri-miRNA	pri-microRNA

PS	Phosphorothioate
PVN	Paraventricular Nucleus
rER	rough Endoplasmic Reticulum
RISC	RNA-Induced Silencing Complex
RNAi	RNA interference
SARI	Serotonin Antagonist reuptake inhibitor
shRNA	short-hairpin RNA
siRNA	small interference RNA
Slc6a3	Solute Carrier Family 6 member 3
Slc6a4	Solute Carrier Family 6 member 4
SMA	Spinal Muscular Atrophy
SMN	Survival Motor Neuron
SND1	Staphylococcal Nuclease Domain-Containing Protein 1
SNP	Single Nucleotide Polymorphism
SNRI	Serotonin and Norepinephrine Reuptake Inhibitor
SSRI	Selective Serotonin Reuptake Inhibitor
STC	Standard Template Chemistry
TLR	Toll-Like Receptor
TPH	Tryptophan Hydroxylase
TRD	Treatment Resistant Depression
UNA	Unlocked Nucleic Acid
UTR	Untranslated Region
Val	Valine
YLD	Years Lived with Disability

1. Introduction

1.1. The etiology and pathophysiology of MDD

Major depressive disorder (MDD) is a chronic, complex, psychiatric illness which is a widespread and disabling health problem that affects people all over the world and it is closely linked to an elevated vulnerability to various other health ailments, including diabetes, cardiovascular disorders, and cancer[1]. Furthermore, individuals suffering from MDD face an alarming 20-fold increased risk of suicide compared to the general population[2] . It was estimated that approximately 250 million cases of MDD occurred in 2017, and over the course of nearly three decades, MDD has consistently maintained its position as one of the top three leading causes of years lived with disability (YLD)[3]. COVID-19 pandemic added to the global growing burden of MDD, recently, the Centers for Disease Control and Prevention (CDC) released data depicting a significant rise in the prevalence of MDD from 7% prior to the pandemic to 27% during the first year of the pandemic and similar alarming increase was presented for the prevalence of MDD with anxiety disorders with prevalence going from 11 to 38%[4,5].

According to the DSM-V, MDD identified by minimum two weeks of the presence of 5 or more of the following symptoms: depressed mood and anhedonia which are the two cardinal symptoms of depression; a change in body weight of more than 5% in a month.; a significant change in sleep patterns; a change in physical activity level; fatigue; feeling worthless or excessive/inappropriate guilt; concentration deficit; and suicidality[6]. It is interesting to note that depression manifestation varies between genders. Women suffering from depression are more likely to report physical ailments such as headaches, gastrointestinal problems and muscle pain and they are also more

likely to demonstrate emotional effects like stress and heightened emotional sensitivity[7]. On the other hand, men with depression are more likely to demonstrate and report anger, acts of aggression, substance abuse and risky behavior[8].

The etiology of MDD is multifaceted, encompassing biological, psychological, and environmental factors. The heritability component in MDD has been estimated to be about 40% based on twin studies[9,10], whereas molecular SNP studies demonstrating 20% heritability. A possible explanation for this 'heritability gap' could stem from a genetic and prenatal environment interaction (e.g., stress in utero)[11]. Numerous Genome-wide association studies (GWAS), linkage studies, and candidate gene approaches have provided substantial evidence supporting the role of genetic factors in depression. However, these studies have only identified a limited number of consistently associated genetic markers, thereby challenging the notion of a singular, comprehensive genetic framework responsible for pure endogenous depression with distinct, independent factors. A possible explanation for the lack of consistency could lay in the fact that none of the GWAS studies could incorporate environmental risk factors[12–14]. Depression is conceptualized as a stress-related disorder with environmental factors playing a role in its etiology, such as early adverse childhood experience, different types of abuse, such as physical, emotional, and sexual abuse, and stressful life events such as: family conflicts, loss events, financial difficulties and circumstances associated with adverse health issues[15]. While various environmental factors have consistently been demonstrated to play a direct role in the development and manifestation of depression[16] , it is improbable that these factors alone could account for the entirety of the disease etiology, given the high heritability of the disorder. Thus, the etiology and the development of MDD cannot be reduced to one

factor and it is rather a result of interplay between genetic predisposition and environmental factors with different relative weights for each factor resulting in different manifestations.

1.1.1 Genetic and environmental factors

As stated earlier, genetic variation accounts for a substantial part of the variance in MDD as demonstrated in twin and single nucleotide polymorphism (SNP) studies (40% and 20% respectively). Analyzing severe cases of MDD, where hospitalization is required or patients suffer a severe recurrent depression, delineate that heritability factor plays a bigger role in those cases with 48-72% variance explained by it[11,17,18]. On top of twin and SNP studies, indirect methods further supported the marked genetic effect in MDD, for example, using enrichment methods, different biological pathways were identified to be suggestively enriched specifically in MDD susceptibility alleles, the most prominent pathway that emerged was "protein phosphatase type 2A regulator activity," a pathway previously linked to serotonergic neurotransmission[19]. The considerable amount of evidence supporting strong heritability of MDD together with the ever - decreasing costs of genotyping, accelerated the growing research aimed to unravel the implied genetic underpinning of depression. Initially, a substantial number of association studies were conducted with the aim of pinpointing specific genetic variations, those studies were involved with identifying particular alleles or genotypes with a higher likelihood of being linked to MDD. A meta-analysis of findings collected from 183 publications revealed that researchers had investigated about 400 genetic polymorphisms within 100 different genes in connection with depression. Among these, only 22 variants met the inclusion criteria of that particular study. The meta-analysis highlighted 5 MDD susceptibility genes: the apolipoprotein E (APOE)

genotype, known to be a genetic susceptibility factor for conditions such as neurodegenerative diseases, and cardiovascular ailments**;** G-Protein β3 Subunit Gene (GNB3) 825T Allele; Methylenetetrahydrofolate reductase (MTHFR) 677T allele, which is linked with reduced DNA methylation; solute carrier family 6 member 3 (SLC6A3) (44bp Ins/Del) – encoding for the dopamine transporter that mediate the active reuptake from the synapse; and solute carrier family 6 member 4 (Slc6a4) (40bp VNTR, serotonin-transporter-linked polymorphic region (5HTTLPR))[20]. Slc6a4 encoding the serotonin transporter (5HTT), which operates as a plasma membrane transporter responsible for terminating serotonin's activity by recycling it into presynaptic neurons through a sodium-dependent process. Notably, 5HTT serves as the primary target for the majority of commonly prescribed antidepressant medications. Both SERT and 5-hydroxytryptamine receptor 1A (Htr1a for the gene, 5-HT_{1A} for the protein) - which functions as an inhibitory G protein-coupled receptor, expressed both as an autoreceptor on serotonin producing cells and postsynaptically throughout the brain at sites receiving 5HT projections, were studied extensively in correlation to MDD, and have shown a predominant involvement in regulating the function of the serotonergic system, influencing the pathophysiology of MDD, and shaping responses to treatment[21,22]. Specifically, 5-HT1A autoreceptors play a role in inhibiting serotonergic neurons and limiting the release of serotonin in nerve terminals. This mechanism has been proposed as one of the factors contributing to the delayed therapeutic onset frequently observed with the use of serotonin-related antidepressants like SSRIs[21]. Decreasing the activity of both SERT and presynaptic 5-HT_{1A} is expected to increase the brain serotonin levels, which are commonly seen with effective treatment and reduction in the depressive symptoms[23–26]. Despite the enormous amount

of work done so far, the replicability of the findings which associated genes and MDD (i.e., GWAS) could not be reliably achieved so far.

Besides genetic factors, environmental factors have a significant role in the etiology of depression. A research investigation that tracked over 2 million offspring as part of the Swedish Extended Adoption Study has shown that the similarity between parents and their offspring in the context of treated MDD is attributable to a combination of genetic factors and rearing experiences, with both factors contributing to the risk of MDD in the offspring in an additive manner[10], this serves as a significant evidence for the important role of environmental stressors. Further investigations revealed a significant association between exposure to chronic or acute stressors and the incidence of depression in women. The authors of the study also note that stressors were 2.5 times more prevalent among individuals with depression compared to controls, and observation within a community found that 80% of individuals with depression had experienced major negative life events in their anamnesis preceding the onset of the condition[27,28]. Additional studies have demonstrated the link between depression and various environmental factors such as inadequate nutrition, prenatal conditions, childhood adversities, stressful events in adulthood, occupational stress, among others[17]. Despite the evidence demonstrated in these publications, it would be hasty to conclude that environmental factors driven depression is common, as while the prevalence of the different stressors is very common, it provokes depression in less than a fifth of the cases[29].

Gene by environment (GxE) interaction studies have examined the influence of several MDD related candidate genes and gene variants on the association between different environmental stressors and MDD. One of the most studied variants is the

polymorphism Slc6a4 promoter region, polymorphic region associated with the serotonin transporter (5-HTTLPR; serotonin-transporter-linked polymorphic region). This polymorphism consists of an insertion of 44 base pairs (L) or their suppression (S). The L and S alleles of 5-HTTLPR have different transcriptional efficiencies, where allele L results in higher levels of SERT protein. A seminal prospective-longitudinal study published in 2003 aimed to comprehend why certain individuals develop depression following stressful experiences while others do not. The findings highlighted that the presence of the short (S) allele in 5-HTTLPR acted as a moderator, influencing the relationship between childhood maltreatment and the occurrence of depressive symptoms in response to a higher number of stressful life events. The epidemiological study unveiled a gene-environment interaction, suggesting that an individual's genetic makeup moderates their response to environmental stressors. Specifically, individuals with one or two copies of the short allele of the 5-HTT promoter polymorphism showed a higher likelihood of experiencing depressive symptoms, being diagnosed with depression, and showing suicidality when faced with stressful life events, in contrast to those who were homozygous for the long allele[30]. This study generated interest in the field and additional studies have suggested similar interaction effects between childhood trauma and 5-HTTLPR[31,32]. A study by Cicchetti et al. found that children with the S/S or S/L genotypes of the 5-HTTLPR variant were more likely to express suicidal risk if they had experienced one or two types of childhood maltreatment. However, children who had experienced three to four types of maltreatment were more likely to express suicidal risk, regardless of their genotype[33]. Meta-analysis studies were done to further examine this interaction, and while some of them provided evidence for the robustness of the interaction between stress and 5-HTTLPR in depression[31,34,35], other did not replicate the original findings[36–38]. Some

experts recommend employing comprehensive phenotype data in GxE investigations, emphasizing the significance of considering specific and frequently overlooked elements that can significantly impact results. One instance is a research experiment that illustrated an interplay between 5HTTLPR and economic hardships, with no comparable effect observed in response to other stressors concerning depression[39]. It is interesting to note that 5-HTTLPR polymorphisms was also found to interact with antidepressant treatments, linking S allele with higher incidence of treatment resistance[40–42] and the L with the best responders[42–44]. Val66Met is a genetic variation found in the human brain-derived neurotrophic factor (BDNF) gene, which involves the replacement of valine (Val) with methionine (Met) at codon 66, was investigated repeatedly in a GxE setup in relation to depression. Two meta-analyses studies established the presence of a significant gene-environment interaction effect in depression, specifically related to the Val66Met polymorphism and exposure to life stress[45,46]. Additional frequently examined monoaminergic genes in this context include MAOA, encoding monoamine-oxidase known to be involved in the monoamine catabolism that has a polymorphism shown to interact with childhood adversity and maternal challenges, impacting the development of depression [11,47,48], but other studies couldn´t replicate those results[47] ,therefore, the extend of the significance of this gene in depression related gene-environment interaction is uncertain. Catechol-O-Methyltransferase (COMT) which is involved in the metabolism of noradrenaline and dopamine, together with SLC6A2 which encodes the noradrenaline transporter, exhibited an interaction effect with different stressors (Ibid). Another monoaminergic gene of special interest –5-hydroxytryptamine receptor 1A (5-HT_{1A}), the gene coding for the serotonin 1A receptor, has a single nucleotide polymorphism (SNP) called C(-1019)G, also known as rs6295. The presence of the G allele instead of C produces the

disruption of the binding site of the transcription factor NUDR, responsible for the repression of 5-HT_{1A} receptor expression[49–51]. The presence of the G allele is related to an increase in depressive symptomatology frequency and lower response to antidepressant treatment[49,52,53]. Important to note that although initial observations have suggested that this polymorphism can selectively control the expression and function of the 5-HT_{1A} autoreceptor without affecting postsynaptic 5-HT_{1A} receptor expression[49], neuroimaging studies showed that both receptors are affected[54]. In a family-based study comprising both parents and offspring who had attempted suicide, a potential gene-environment (GxE) interaction involving the HTR2A gene, which encodes another serotonin receptor and contains a SNP (rs6313), was linked to the cumulative experience of various types of lifetime stressful events[55]. Same study also demonstrated that additional SNP (es7322347) in the same gene, has a significant GxE interaction with early life/adolescence physical assault for females suffering from depression[55]. Researchers have also explored GxE interactions involving HPA axis genes in relation to depression. Ben-Efraim et al. documented a potential GxE interaction involving the CRHR1 rs7209436 SNP and adverse experiences during childhood and adolescence in patients with suicide attempts, as well as a comparable interaction was observed involving the CRHR1 rs16940665 SNP and incidents of assault during adulthood in male patients with suicide attempts. Furthermore, the CRHR1 rs16940665 SNP displayed a GxE interaction with the accumulation of stressful events in male patients and their history of suicide attempts[56]. Another HPA axis related gene, FKBP5, was investigated thoroughly where GxE interaction was found between FKBP5 rs1360780 SNP and was linked to the development of depression when combined with experiences of physical abuse[57], Additionally, GxE interactions involving the FKBP5 rs1360780 SNP and traumatic life events were

associated with the emergence of depression [58] and the GxE interaction between FKBP5 rs9296158 SNP and childhood maltreatment is linked with depression[59]. Although GxE studies have, to some extent, a good replicability record, the results stemming from those studies remain inconclusive and call for a larger sample size, corresponding to the substantial variability in the prevalence of environmental stressors across different populations. While, to date, no particular gene associated with depression risk or gene-environment interaction has been consistently pinpointed, investigations into GxE interactions in depression hold significant value as an exploratory approach in the quest for potential new candidates, and some of these candidates are awaiting replication and confirmation.

1.1.2. Deficiency of monoamines

The monoamine system in the human brain is divided into the Indoleamines group that consists of Melatonine (MT) and Serotonin (5-HT) neurotransmitters; the Catecholamines groups which consists of Adrenaline (Ad), Dopamine (DA) and Noradrenaline (NAd) neurotransmitters; and the Imidazoleamines group which consists of Histamine neurotransmitter. 5-HT, DA and NAd were studied extensively in the context of depression. The majority of serotonergic, noradrenergic, and dopaminergic neurons reside within the midbrain and brainstem nuclei. Despite their relatively low numbers, with, for instance, the human dorsal raphe nucleus (DR) containing approximately quarter of a million neurons out of a total of 10^{11} neurons in the entire brain, these three neuronal groups project extensively throughout the brain. Their axons branch extensively, resulting in an exceptionally high axonal density, with over one million serotonergic nerve endings per cubic millimeter reported in the neocortex[60]. This anatomical arrangement implies that the monoaminergic systems participate in

regulating a diverse array of brain functions. And has been demonstrated to influence the functioning of the prefrontal cortex, impacting the processing of working memory, as well as playing a role in regulating behavior and attention. It was also shown to influence acquisition of memories[61–63]. DA was strongly associated with the modulation of the reward and motivation function but also plays a role in working memory and attention[63,64], while 5-HT innervates almost every brain area and the serotonergic neurons form the largest and most complex efferent system in the human brain[63,65]. It is evident that imbalances in the production and transmission of several monoaminergic neurotransmitters are commonly observed in the central nervous system of individuals suffering from MDD[66] and several post-mortem investigations have confirmed the presence of reduced serotonin levels in the brains of individuals suffering from depression[67–69]. Changes in physical functions that are observed in depression like sleep, sex, appetite and circadian rhythm were closely linked to changes in serotonin levels in the Brain[70]. On top of that, post-mortem examinations of brain of depressed patients revealed low levels of serotonin[67–69]. Similarly, DA and NAd abnormalities were linked alone or together with 5HT abnormalities, to a broad spectrum of depressive symptoms[64,71,72]. Practically every compound created with the aim of inhibiting NAd or 5HT reuptake has been demonstrated to be an effective antidepressant in clinical practice[73]. Monoamine oxidase has been identified as a promising target due to the observation showing that its inhibition leading to increased monoamine availability, aligning with the antidepressant effects[74]. In general, monoamines serve as the primary focus for the majority of medications utilized in the treatment of neuropsychiatric disorders. This includes reuptake inhibitors, like antidepressants, precursors such as L-DOPA, and receptor agonists and antagonists. This increasing evidence have contributed to the development of the monoamine

deficiency hypothesis, which is often associated with depression. This theory proposes that a depletion of the monoamine neurotransmitters: serotonin, norepinephrine, and dopamine, is the underlying pathophysiological basis of depression[75,76].

Serotonin is the most thoroughly researched neurotransmitter concerning depression. Tryptophan depletion, leading to a reduction in serotonin synthesis, has been demonstrated to trigger the emergence of depressive symptoms in individuals predisposed to depression. Since tryptophan serves as a precursor to serotonin, these findings establish a direct connection between serotonin and its role in depression[77,78]. An additional observation reinforcing this connection comes from studies revealing imbalances in the expression of the serotonin transporter within the brains of individuals with depression. On top of that, changes in the availability of the 5-HT_{1A}, which regulates serotonin function, were found across the brain of depressed individuals[24,79]. Important to note that this changes in the 5-HT_{1A} expression were found also in cases of panic disorder[80] and epilepsy[81], which could serve as a possible explanation for the comorbidity of these conditions.

On top of the substantial evidence linking 5HT to MDD, converging lines of evidence suggest a significant role for NA in the pathophysiology and treatment of MDD. This neurotransmitter appears to be involved in a few psychological processes that are involved in depression, including vigilance, learning, sleep regulation and in regulating the stress response[82]. The main brain region of NA production is in the locus coeruleus, from which widespread projections innervate key regions known to be involved in psychiatric diseases, among them the cortex and the limbic system and the brain stem. Studies demonstrated that the noradrenergic system also plays a major role in the underlying mechanism of MDD as evidenced by previously mentioned reuptake inhibitors and also by studies investigating depressed patients' brains demonstrating a

heightened activity of the tyrosine hydroxylase, an enzyme responsible for catalyzing the precursors for NAd. Moreover, NAd metabolism was shown to slow down, and lower level of the norepinephrine transporter in the brains of depressed patients[83]. Moreover, postmortem studies demonstrated a reduced density of neurons in the locus coeruleus, and changes in the expression of the adrenergic receptors in individuals who had depression and died by suicide[84]. On top of that, preclinical evidence where genetic manipulation of the NA system was used to increases NA neurotransmission had a protective effect and animals were resilient to depression induced by exposure to stressors, whereas chemical manipulation that reduced the NA increases the susceptibility to a depressive relapse among recovered patients[85,86]. More evidence of NA involvement in depression comes from the use of therapeutic agents which specifically increase NA activity are effective antidepressants like noradrenergic TCAs and the a2-adrenergic antagonist mirtazapine[87], and the selective noradrenaline reuptake inhibitor -NRI- reboxetine[88]. Since there is currently no tool to specifically deplete centra NAd or suitable imaging tools for its study, there is a lack of substantial evidence regarding the involvement of the NAd system in depression.

Besides the dysfunction of the 5HT and NAd pathways, several studies have provided support for the association between alternation in the DA pathways and MDD[89]. Both dopamine reuptake inhibitors and receptor agonists demonstrated an antidepressant effect when studied in MDD cases[90]. Moreover, dopamine metabolites were found consistently to be reduced in CSF and plasma of depressed patients[91]. MDD patients showed lower levels of dopamine neurotransmission in the striatum which coincide with reduced dopamine transporter binding and dopamine reuptake[92]. Other studies have shown significantly lower DA transporter binding and increased DA D2/3 receptor binding in caudate-putamen and amygdala of patients with MDD[93,94].

Diminished dopaminergic projection in the striatum which is characteristic to Parkinson disease was associated with MDD[95]. Additional support to the role of dopamine in MDD stems from studies showing that reduction of dopaminergic transmission to the nucleus accumbens results in anhedonic behavior and deficits in tasks that involve reward processing[74,81], those effects are similar to the clinical evidence where depressed patients demonstrated an attenuated reaction to positive reinforcement and an irregular response to negative reinforcement[96].

Despite the extensive evidence described in this section, it has been demonstrated that the causality of depressive symptoms is not solely attributed to a reduction in the levels of central monoamines. Administering antidepressant agents, be it MAOIs, SSRIs, or SARIs, results in an immediate boost in monoamine availability. However, the observable clinical effects on mood typically emerge after several weeks of treatment[97]. The delayed clinical onset raises the question whether the lagged onset is determined by the delayed onset of clinical effects is a result of the time needed for brain networks to transition from a 'depressive' to a ‘normal’ state or if it's a limitation of the available medications. The former hypothesis is backed by an extensive body of clinical and preclinical observations. The delayed clinical onset following antidepressant treatment is attributed to various pre- and post-synaptic mechanisms occurring in the brain, causing it to readapt. Among those, alterations in the expression levels and sensitivity of the receptors, changes in the expression levels of trophic factors, and so forth. The combined impact of these various mechanisms results in the reconfiguration of brain circuits that play a role in the therapeutic effects of antidepressants. Preclinical studies demonstrated these changes following repeated exposure of animal models to antidepressants drugs[98–102]. A recent systematic umbrella review which attracts a lot of attention, examined 17 systematic reviews and meta-

analysis studies, and showed that there is insufficient compelling evidence to link depression with lower serotonin concentrations or reduced serotonin activity[103].

1.1.3. Hypothalamic-Pituitary-Adrenal axis

As mentioned in the previous section, environmental factors, together with genetic factors, play a substantial role in the progression of MDD[104–107]. Aversive environmental events including physical or emotional trauma were associated with the development of MDD[108]. Unfortunately, despite the extensive research done in this field, not much is known regarding the mechanism by which these early aversive events interact with the genetic and epigenetic factors of the individual, which result in susceptibility to MDD. The link between early traumas and the development of MDD in a later stage, might be partially explained by the changes that occur along the HPA axis[109]. Evidence demonstrated that childhood trauma may lead to heightened sensitivity of the HPA axis and as a result, to an increased response to stressors[110,111]. Some of the symptoms of depression including hopelessness, weight loss, decreased libido, disrupt sleep, and exaggerated response to psychological stressors has been correlated with dysfunction of the HPA axis[85,112–114]. The HPA axis is the main neuroendocrine system that regulates control the reaction to stress. Environmental stress activated the release of monoamines in the brain, including 5-HT from the DRN, which in turn activates the paraventricular nucleus (PVN) of the hypothalamus that then secrets corticosterone releasing factor (CRF) that binds to the pituitary gland. Subsequently, adrenocorticotropic hormone (ACTH) is secreted in the circulation and activates the production and release of glucocorticoids (GC) in the adrenal glands. GC, a prominent stress hormone with far-reaching impacts on the body, functions by interacting with glucocorticoid (GR) and mineralocorticoid (MR) receptors. Both

receptors act as transcriptional regulators and are highly expressed in limbic system, but they exhibit distinct affinities for cortisol, with MR having approximately 10 times higher affinity. MR is implicated in the triggering of the stress response, while GR is part of the negative feedback loop aimed to restore hemostasis. Alterations of this network can result in overactivation of the stress response, leading to cell atrophy, decreased neurogenesis and synaptic plasticity, and changes in monoaminergic signaling. These combined factors may ultimately contribute to the onset of depression[115]. It was demonstrated that individuals with MDD have an impaired sensitivity of the GRs which leads to a reduced negative feedback mechanism that subsequently results in hyper-secretion of CRF and heightened production of GCs hinders the body to reinstate homeostasis[116,117]. CRF is also found within neural circuits where it acts as a neurotransmitter in response to stress, offering potential insights into CRF's involvement in the development of depression[113,118–120]. Preclinical studies demonstrated that the administration of CRF directly into the CNS induced animals to present depression-like symptoms[85]. Elevated CRF concentrations have been found in the CSF of depressed patients[121–123]. Similarly, post-mortem studies demonstrated decreased CRF receptor density and mRNA expression, together with increased CRF concentration in the brains of depressed subjects[124,125]. Interestingly, a further support of the role of CRF in depression came from electroconvulsive therapy (ECT) where patients that reacted positively to the therapy also exhibited normalized concentration of CRF in the CSF and similar results obtained following successful treatment with fluoxetine[126]. One of the key players that regulates the sensitivity of the GR is FK 506 binding protein 51 (FKBP51). When this protein is bound to the GR complex it changes the receptor binding affinity for GC. FKBP51 is encoded by *FKBP5* gene, polymorphism within this gene has been shown to be associated with differences in GR

sensitivity[127,128]. *FKBP5* was shown to be implicated in several stress-related disorder including MDD[127], bipolar disorder[129], and suicidal behavior[130,131].

1.1.4. Neurogenesis and neuroplasticity impairments

Increasing body of evidence has demonstrated alterations of structural and neuronal plasticity in MDD patients. Brain imaging studies have shown atrophy in the cortical and limbic regions of those patients. Moreover, decreased volumes of hippocampus were associated with the duration of the illness and treatment response[132–135]. These findings could serve as a possible underlying mechanism explaining the differences observed between the initial depressive episode, typically considered 'reactive' and precipitated by a significant psychological stressor, and subsequent episodes that tend to be more 'endogenous,' meaning they can be triggered less severe stressors or even occur without any trigger[74,106]. The hypothesis proposes that depression left unaddressed causes hippocampal volume reduction, subsequently leading to heightened stress sensitivity and an elevated risk of recurring depressive episodes[136]. Postmortem studies examining the brain of MDD patients showed a decreased size of the pyramidal neurons in the hippocampus[137] and in the dorsolateral PFC[138] as well as reduced number of astrocytes in the cerebral cortical regions in the prefrontal cortex of depressed patients[138,139]. Various animal models of induced depression using chronic stress (e.g. chronic unpredictable stress, chronic social defeat or repeated restraint stress) which lead to behavioral phenotypes that are known to model clinical symptoms like learned helplessness and anhedonia, were also demonstrated to decrease the elongation and branching of apical dendrites and to reduce in both the quantity and functionality of synapses at neuronal spines in the mPFC, the CA3 and the dentate gyrus[140–144]. Several possible mechanisms have been proposed to explain the brain volume loss in depression like glucocorticoid toxicity, glutamatergic toxicity, decreased neurogenesis and decreased neurotrophic factors[74]. Due to the lack of imaging tools that

can directly measure neurotrophic or neurotoxic processes *in-vivo*, none of the proposed mechanisms was reinforced with solid clinical evidence. Brain derived neurotrophic factor (BDNF), an important neurotrophic factor in the brain, which is essential for the survival and directional guidance of neurons throughout their development process as well as critical to ensure the survival and optimal functioning of neurons in the adult brain[145,146], has attracted considerable interest in depression related studies. Studies utilizing animal models demonstrated correlation between stress-induced depressive like behaviors and decreased levels of BDNF and decreased cell proliferation in the hippocampus[147–151]. Additional support for the role of BDNF in depression arises from the hypothesis that this neurotrophic factor mediates the action of antidepressants. The initial evidence for the feasibility of this hypothesis came from a study in which microinfusing of BDNF to the hippocampus resulted in an antidepressant like effect in the forced swim test (FST)[152]. Additional support came from preclinical studies which showed that several antidepressant treatments, including SSRIs, SNRIs and electroconvulsive shock therapy (ECS) increase the expression of BDNF in the hippocampus[153]. Despite the observations seen in the hippocampus, BDNF exerts different and even opposite effects in other brain regions. For example, it was reported that chronic stress increases the BDNF levels in the Nucleus accumbens (NAc) and moreover, infusion of BDNF in this nucleus induced a depression-like behavior as it was measured in a forced swim test, whereas inhibiting the BDNF function in the NAc had an antidepressant like effect[154]. In summary, the observations from preclinical and clinical studies suggest that the disruption of neuronal and synaptic plasticity resulting from prolonged exposure to stressful events may contribute to the pathophysiology of MDD. On top of that, if the mechanisms that regulate synaptic plasticity are disrupted it might result in the loss or destabilization of synaptic connections responsible for regulating mood and emotions, contributing to the development of MDD[143].

1.1.5. Glutamate and depression

"The glutamate hypothesis of depression" was first introduced in the 1990[155] and since then our comprehension of the functions of glutamate has significantly expanded, recognizing its role as the brain's primary excitatory neurotransmitter and its involvement as a regulator of neurotransmission. A mounting body of evidence underscores the contributions of the glutamatergic system to the pathophysiology and management of mood disorders, both in a broader context and in MDD[156–160]. Alternation of glutamate levels in the CNS and blood of MDD patients was reported[161]. Postmortem analysis demonstrated a significant increase in tissue glutamate levels in the frontal cortex of individuals with history of MDD and bipolar disorders[156] and similar elevation was found in dorsolateral frontal cortex of individuals who have background of bipolar disorder[162]. Other reports demonstrated that treatment with antidepressant agents may decrease the glutamate plasma levels in depressed individuals[161,163,164]. The potential role of the glutamatergic system in MDD finds support in preclinical research showcasing the antidepressant effects of N-methyl D-aspartate (NMDA) receptor antagonists[155,165]. The antidepressant-like activity of NMDA receptor antagonists has been known for almost three decades now with various compounds of both competitive and non-competitive NMDA receptor antagonists, demonstrating a decrease in depressive-like behavior as evidenced by reduced immobility in both the forced swim test and the tail suspension test[155,166]. Over time, these preclinical discoveries led to the approval of esketamine[167168]. The medication received approval for use in adults with treatment-resistant depression (TRD), in co-administration with a recognized antidepressant. It's important to note that as many as one-third of MDD patients are identified with the treatment-resistant variant[165]. Observations from studies monitoring patients with treatment-resistant depression who

received ketamine treatment have documented the drug's capacity to elicit rapid and enduring antidepressant and anti-suicidal effects[169–171]. Besides the enthusiasm of the newly approved drug for such a great unmet need like TRD, its psychotogenic, as well as its sedative effect, remain a serious limitation for the use of esketamine and research efforts directed towards blunting these effects are made.

Ketamine's impact on depression operates through heightened neuroplasticity, even though the compound blocks NMDA receptors, which are known to play an important role in mediating plasticity[172,173]. Ketamine prevents the phosphorylation of eukaryotic elongation factor 2 (eEF2), increases the expression of BDNF, and promotes AMPA receptors shuttling to the synapse and promoting synaptic connectivity and plasticity. Clinical studies showed that ketamine increased prefrontal glutamate–glutamine cycling, indicative to more release of glutamate[174]. Preclinical studies have shown that ketamine administration can lead to a rapid increase in dendritic spine proliferation. This effect is linked to the recovery of functional connectivity seen in in fMRI scans of individuals with depression[174]. Focusing on specific aspects of ketamine's mechanisms of action has the potential to serve as a new class of antidepressants. For example, serotonergic hallucinogens like psilocybin, muscarinic receptor agonists, mGluR2 antagonists among others, all increase glutamate release[172,173].

1.1.6. Epigenetic in depression – focusing on microRNAs

The term epigenetics was first introduced in 1942 by Conard Waddington and was defined as “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being”[175]. Since then, the term was

redefined, and nowadays common definition of epigenetics includes "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence"[176]. Following the completion of the Human Genome Project and the failure to explain human pathologies merely by analyzing the raw sequence of genes, the focus of molecular biology shifted toward regulation of genes in response to intra and extracellular stimuli[177]. The pathophysiology of MDD, as well as the response to antidepressants, is suggested to be controlled, at least in part, by epigenetic regulation of transcription and translation[178–181]. The regulation of gene expression was shown to be a critical molecular mechanism mediating (mal)adaptive changes in the brain[179] in form of neuronal plasticity that includes long lasting modifications in synaptic structure and function[182–184]. Environmental factors significantly contribute to the pathophysiology of depression, and epigenetic mechanisms are proposed to act as a link between environmental and genetic factors. Many clinical and preclinical studies demonstrated disruptions in transcription in various brain regions of MDD patients and of animal models of depression[178,179,185,186]. The extensively examined mechanisms of epigenetic control encompass DNA methylation, chromatin remodeling, and non-coding RNAs.

DNA methylation typically decreases gene expression by chemically attaching a methyl group to cytosine residues within CpG dinucleotides[187]. Both human and animal studies have associated early life stressful events that are known to heightening the susceptibility to psychiatric disorders including depression, with DNA methylation[188]. Studies focusing on particular genes suggested DNA methylation regulation of several genes, such as mineralocorticoid receptor (NR3C1), BDNF and SERT transporter (Slc6a4), to be associated with depression[189–191]. Furthermore, DNA methylation of the BDNF gene was suggested to serve as a biomarker to differentiate between MDD patients and healthy individuals[192]. Decreased methylation levels within NR3C1 gene were found in bpth cases

of childhood trauma and MDD in adults[193] and blood samples revealed a hypomethylation of the same gene in MDD patients compared to healthy controls[194]. Many GWAS studies tried, so far in vain, to identify sequence variations that influence susceptibility to MDD, it is suggested that DNA methylation, as well as other epigenetic regulations, might emerge as better candidates to be employed as biomarkers for depression.

Chromatin remodeling was demonstrated to be involved in different physiological and pathological processes within the brain, such as learning and memory[195] addiction[196] and depression[100,197,198]. Covalent histone modification is the main mechanism that affects chromatin structure and hence modulates gene expression. The main histone modifications are acetylation and deacetylation, and the latter is catalyzed by histone deacetylases (HDACs). These enzymes were demonstrated to be involved in stress response, depression related behavior and antidepressant action[132]. For example, mice that were subjected to chronic stress protocol showed an enhancement of HDAC2 function in the ventral striatum that coincided with depression-like behavior[197]. Same research groups also demonstrated that HDAC4/5 inhibitors administrated into mice hippocampus prevents depression-like behaviors that are induced by chronic stress[142] suggesting that this enzyme might have a role in the action of antidepressants. Many more studies were demonstrating the association between DNA methylation, chromatin remodeling and MDD but those are outside of the scope for this dissertation.

Regulation of gene expression through non-coding RNA (ncRNA) at the post-transcriptional level is another branch of epigenetics which was studied extensively in relation with MDD. ncRNA is a special class of RNA that is transcribed from DNA but not being translated into protein yet exerts a functional role[199]. This class of RNA consists of few members, long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), intronic RNAs, repetitive RNAs, PIWI-interacting RNAs, small nucleolar RNAs, large intergenic

non-coding RNAs, microRNAs (miRNAs), which are the central subject of this dissertation, and possible other types that are yet to be discovered.

miRNAs make up an extensive group of small non-coding RNAs, usually around 22 nucleotides long of a double strand RNA that act as a posttranscriptional regulator of gene expression[200]. It is estimated that miRNAs regulate the transcription of nearly half of all the mammalian genes that encode proteins, which emphasize their important role in developmental and pathological processes. The majority of miRNAs begin their life cycle as pri-miRNA sequences. These sequences are generally transcribed by either RNA polymerase II or III, originating either from specialized genes solely devoted to miRNA production or from gene clusters specifically transcribed for this purpose. Additionally, some are found within the introns or untranslated regions of genes that encode proteins[201–204]. In the canonical pathway, pri-miRNA is processed in the nucleus by a microprocessor complex, consisting of two multiprotein units, into a precursor miRNA (pre-miRNA) - a hairpin-shaped sequence of approximately 70-110 nucleotides. The microprocessor complex contains Drosha, a class 2 ribonuclease 3 enzyme, and the microprocessor complex subunit DiGeorge syndrome critical region 8 (DGCR8)[205]. Drosha snips both the 5' and 3' ends of the pri-miRNA's hairpin structure, while DGCR8 engages with the pri-miRNA to both stabilize it and act as a guide for the exact location of the cut. This process results in a pre-miRNA with a 5'-monophosphate and a 3'-2-nucleotide overhang[206–208]. In a complex involving Ran-GTPase, the pre-miRNA is transferred from the nucleus to the cytoplasm by the transporter known as exportin-5 (XPO5)[201,206,209]. Once reached the cytoplasm, RNase III endonuclease Dicer is cutting the loop of the pre-miRNA which result in to generate a ~22 nucleotide miRNA duplex[207] which is then guided to the RNA-induced silencing complex (RISC)[210] that perform the gene silencing. This machine is composed of different proteins that form “a multiprotein complex” with various functions [210–212]. Once

loaded onto the RISC complex the miRNA will be separated into two strands: the guide stand, which consist of the complementary sequence to the sequence of the target mRNA, and the passenger strand, which will go through degragation[213]. The guide strand is also known as the active strand, leading strand or miR. The passenger strand is also known as miR*[214]. Usually targeting the 3'untranslated region (3'UTR) of its mRNA counterpart, the miRNA-guided RISC complex—often termed miRISC—operates based on nucleotide complementarity. The "seed sequence" of the miRNA, which is a 6-8 nucleotide-long segment located at the 5' end positions 2-7 of the mature miRNA, is typically responsible for this complementary interaction[215,216]. However, non-canonical base pairing has been identified, like in the 5'UTR of the target mRNA[217]. Following the recognition of the target mRNA by miRISC, the mRNA remains stable, but the complex interferes with its translation by inhibiting the translation elongation[218,219]. Recent studies in the field demonstrated that translational repression always occurred before mRNA degradation[220–223], suggesting that gene silencing by the miRNA machinery causing the inhibition of the protein translation which in turn result in the mRNA target degradation and it is secondary consequence[224]. Interesting to add that studies have shown that whereas the process in which the RISC complex is loaded with the guide strand of the miRNA and the targeted mRNA is performed on the rough endoplasmic reticulum (rER) membrane, together with the process of translational repression, the degradation of the target mRNA (deadenylation and decay) was demonstrated to occur in the endosome/multivesicular bodies (MVBs)[225,226]. Since the first discovery of the miRNA, thousands of miRNAs have been identified and the need to organize them was immediate and nomenclature system has been developed[227]. In summary, the lowercase 'mir' is used to denote pre-miRNA, while the uppercase 'miR' signifies the mature form. If miRNAs have similar mature sequences, they are differentiated by lowercase letters (e.g., miR 135a vs. miR 135b). For identical mature

sequences coming from different precursor sequences or genomic locations, an additional number is used (e.g., miR 135a1 vs. miR 135a2). The species origin should precede the miRNA annotation, as in hsa-miR 135 for a human miRNA. The strand of the mature miRNA is named based on its origin: if it comes from the 5′-end of the pre-miRNA, it's called a 5p strand (miRNA-5p), and if it comes from the 3′-end, it's called a 3p strand (miRNA-3p)[228].

An overwhelming amount of evidence supporting the role of microRNAs in different biological processes has accumulated[215]. It was shown that a single miRNA can bind to numerous mRNAs, as well as to long noncoding RNAs, resulting in significant cumulative effects. Developmental studies showed that miRNAs can completely block their target gene(s) expression, it was demonstrated that in these cases the miRNA and the target genes commonly have mutually exclusive expression patterns[215]. Alternatively, in adult tissues, it is often observed that miRNAs and their target genes are often expressed together and act as 'fine-tuners' of their expression levels[215,229]. A vast majority of miRNA have been detected in mammalian nervous system and on top of their involvement in processes on neuronal development, it was demonstrated that they 'fine-tune' target gene expression and affect the function and structure of neuronal networks[230,231]. Like other fields of biology, the studies of miRNAs in the field of neuroscience were primarily focused at first on their involvement in the developmental aspects[232] and a considerable amount of work was done in relation to neurodegenerative disorders[233–235].

1.1.7. The Role of miR-135 and Other Key miRNAs in Depression and Anxiety

Data published in the last decade backs the link between various mental health conditions, such as MDD, and alterations in miRNA activity or regulation. These shifts

have been detected in everything from post-mortem brain samples to cerebrospinal fluid and even peripheral blood in individuals with MDD[228,236,237]. Similarly, various animal models that mimic MDD have illustrated the role of miRNA pathways in the fundamental biological processes underlying depressive behaviors[238]. Drugs that boost serotonin levels, such as SSRIs, are widely employed as antidepressants in medical settings. Consequently, a substantial amount of research has explored the impact of miRNAs on the serotonin system. In a comprehensive study by Issler et al. in 2014, it was found that levels of miR-135 were reduced in both blood and brain samples of MDD patients compared to those without the disorder. The study identified miR-135 as a potent regulator of the serotonin system, revealing that it directly regulates transcripts for SERT and 5-HT1AR, among others. Overexpressing miR-135 specifically in serotonin neurons led to effects similar to antidepressants and anxiety-reducing drugs in mice. On the flip side, reducing miR-135 levels resulted in increased anxiety-like behaviors and diminished responsiveness to antidepressants. Moreover, administering the fluoxetine, either acutely or over an extended period, led to a rise in miR-135 levels in the mice's raphe nuclei[239]. In a different study it was demonstrated that adolescent rats exposed to stress displayed anxious behaviors as adults, a situation that was linked with decreased miR-135 and elevated 5-HT_{1A}R expression in the medial prefrontal cortex. Administering SSRIs mitigated these anxiety-like actions while normalizing the expression of both miR-135 and 5-HT_{1A}R[240]. Moreover, *in-silico* analysis and *in-vivo* experiments suggested that miR-135 directly regulates the mineralocorticoid receptor transcript[241]. SERT was shown to be a target of miR-16[242]. Administering miR-16 directly into the mice's DRN counteracted the behavioral issues caused by ongoing stress. This hints that the therapeutic properties of SSRIs could be partly due to their impact on miRNA expression levels[242]. Another investigation

discovered that MDD patients who were not on medication had lower levels of miR-16 in their cerebrospinal fluid compared to a control group. The levels of this miRNA were inversely related to the severity of MDD, and directly related to the levels of serotonin in the CSF[243,244]. When rats were given an ICV dose of an antagomir targeting miR-16, they exhibited behaviors akin to depression. These rats had decreased miR-16 levels in the CSF, elevated CSF serotonin levels, and a boost in SERT protein levels in the raphe compared to the control group[243].

As discussed above, MDD is a disorder associated with alterations in synaptic function and those alterations have been found to be closely associated with abnormal expression of miRNAs[245]. An increase in anxiety-like behavior was observed following the pharmacological blocking of miR-135 in the amygdala, which was attributed partly to a direct regulation of complexin-1 (CPLX1) and complexin-2 (CPLX2) - two key regulators of synaptic vesicle fusion that regulate both pre- and post-synaptic exocytosis and are known to regulate AMPA receptor and related to spine morphogenesis, by miR-135[246]. The regulation of complexin-1 and -2 by miR135 was demonstrated to be required for prolong spine remodeling following induction of NMDAR-long term depression (LTD) which causes long-lasting synaptic depression and spine loss[247]. The involvement of miR-135 in synaptic formation was also suggested by the demonstration of reduction of this miR in the hippocampal neurons of mice models for Alzheimer disease. Inhibition of miR-135 was sufficient to mimic the effects and result in synaptic dysfunction and memory deficits. These effects partially occurred via excess Rock2 and adducin 1 signaling pathways[248]. Several studies showed that increased hippocampal levels of miR-124 inhibit depression-like behavior induced by chronic stress, whereas reduced expression levels of the same miRNA enhance behavioral susceptibility to stress. These behavioral observations coincided with changes in the dendritic structure

of neurons in the dentate gyrus, more specifically, chronic stress led to a decrease in spine density, an effect that was counteracted by the overexpression of miR-124[132]. Yoshino and colleagues investigated the connection between miRNAs and their synaptic function in the context of MDD. They did this by analyzing tissue samples from the dorsolateral prefrontal cortex of individuals with MDD and control subjects. Of the 351 miRNAs found in the synaptic tissue, eight showed different expression levels between the MDD and control groups. Subsequent gene ontology study indicated that these specific miRNAs play roles in both synaptic plasticity and neurogenesis[249]. Decreased levels of miR-1202 have been observed in individuals with depression. This miRNA is specific to primates and is abundant in brain tissue. It regulates the gene that encodes for metabotropic glutamate receptor-4, the expression of which was found to be elevated in samples from patients with MDD[250]. Additional study demonstrated that the miRs that were shown repeatedly to be involved in depression (miR-135, miR-16 and miR-1202) appeared at reduced levels in the blood serum of people with depression, indicating their possible utility as biomarkers for MDD[251]. miR-144-5 was shown to target the protein C and β-catenin pathways associated with MDD[252], and this miRNA was also demonstrated to play a role in the efficacy of mood-stabilizing medications as well as in stress response[253]. Examining the blood from patients with MDD led to the identification of other miRNAs with reduced expression compared to controls. Notably, these include miR-30, miR-34, and miR-221. These miRNAs are responsible for regulating genes involved in pathways related to MDD including corticotrophin releasing factor receptor (CRFR1) and the glutamate transporters[254]. Post-mortem analysis of brain tissue from individuals who had MDD showed elevated levels of miR-124-3p in the prefrontal cortex (PFC) compared to controls. This miRNA targets transcripts of glutamate receptors 3 and 4, as well as the glucocorticoid receptor

(NR3C1)—genes that have been demonstrated to play roles in stress response and neuroplasticity[255]. Expression analysis of miRNAs in rats' brain after prolonged corticosterone treatment showed differences in the expressions of 26 miRNAs within the PFC. Analysis to identify potential targets for the most significantly changed miRNAs after corticosterone exposure - namely miR-124 and miR-218 - pointed to key genes previously linked to MDD and stress-related conditions. These included glucocorticoid receptors (NR3C1 and NR3C2) as well as BDNF, among several others[256].

Numerous miRNAs have been identified as regulators of BDNF, a gene that was shown to have a critical role in MDD pathophysiology[257]. Insightful findings were obtained from studying the expression levels of BDNF and the miRNAs that target it, both in MDD patients and in mouse models exhibiting depression-like behaviors. For a more comprehensive analysis, please refer to our review[238]. A compelling case involves miR-182 and miR-132, two miRNAs whose levels were elevated in the serum of MDD patients, coinciding with a decrease in BDNF levels. Intriguingly, a robust negative relationship was found between serum BDNF levels and scores on the Self-Rating Depression Scale, while a positive relationship existed between the scale's scores and the serum levels of miR-132[258]. In a different study, rats expossed to chronic stress demonstrated an increased hippocampal level of miR-124a and miR-10b which coincided with lower BDNF levels[259,260]. Overexpressing miR-124a in the hippocampus intensified stress-triggered behaviors resembling depression. On the flip side, silencing miR-124a activity in the hippocampus through a lentiviral inhibitor restored the antidepressant effects observed in animals with elevated BDNF levels[259]. These studies suggest that miRNAs have the potential to be targeted for the creation of novel therapeutic treatments by overexpressing and/or administrating miRNA mimics

to increase specific miRNA expression. To induce miRNA loss of function here are three general methods: a genetic knockout, antisense oligonucleotide inhibitors and sponges.

1.2. Oligonucleotides therapy

Targeting proteins was the focus of drug development over the past century with the main compounds utilized for the task were small molecules and monoclonal antibodies. To date, protein targeting is still the main approach in drug discovery. Despite many years of protein targeting, it is still complicated to recognize the spatial conformation of the target protein, a paramount process in developing a compound capable of activating or inhibiting a protein function. In addition, not all classes of proteins (e.g., transcription factors, structural proteins etc.) can be approached using a protein targeting modality. Another method for adjusting protein activity involves altering its levels of expression. This can be done by targeting its mRNA, a molecule that has its intrinsic property to complementary Watson–Crick base-pairing which makes it a very attractive target. Oligonucleotides are short (~20mers) sections of single- or double- strand DNA or RNA that function through full Watson-Crick base pairing with specific DNA or RNA sequence, hence theoretically speaking, oligonucleotides can target any gene and modulate its splicing, translation, or abundance[261,262] and consequently, modulate the expression of any protein. On top of that, oligonucleotides can modulate ncRNAs, including miRNAs, which are gaining recognition as prospective therapeutic targets. It is also important to note that since oligonucleotides requires a high base-pair complementary with its target (in the case of ASOs and siRNAs), its target specificity should be proved higher than a small molecule drug.

The concept of using oligonucleotides originated from the research conducted by Clercq and colleagues, who studied interferon induction by synthetic polynucleotides[263], but the potential of oligo therapy was really first proposed only in 1978 by Zamecnik and Stephenson, where they utilized "antisense oligodeoxyribonucleotides(ODNs) to bind and inhibit the replication of Rous sarcoma virus (RSV) RNA"[264]. To advance this technology for medical use there were and still are, several issues to be solved, mainly the stability, delivery and specificity. Chemical optimization of the oligonucleotides was the focus of many studies exploring various modifications to both the phosphodiester bonds and the sugar groups. The objective has been to enhance the stability of these oligonucleotides in plasma by making them more resistant to nucleases, increasing their affinity for serum proteins, and boosting the specificity for their intended target sequences[265]. The delivery challenge was improved by different formulations and conjugations with specific chemical groups, which enabled tissue specific delivery[265].

Oligonucleotides therapy comprises of antisense oligonucleotide (ASO), small interference RNA (siRNA), miRNA, anti-miRNA (antagomir) and short-hairpin RNA (shRNA). Since the first oligonucleotide-based therapy to be approved -Fomivirsen, an ASO based drug treating cytomegalovirus retinitis in immunocompromised patients, by the FDA (1998) and by EMA (1999), 14 additional oligo therapeutics have been approved (as of March 2022) in the US or the EU, including 5 RNAi therapies (siRNA-based drugs)[266]. As of the end of 2021 there are about 80 oligonucleotide compounds in 130 clinical trials (phase 2 or higher), approved or tested for more than 100 different indications[267].

1.2.1. Antisense oligos

The most represented class of oligonucleotides among the approved/in advanced clinical phase stage compounds, is the antisense oligo (ASO), with about 2/3 of the compounds. Based on their mechanisms of action, ASOs can be categorized into two main groups: those that inhibit gene expression and those that modulate splicing. Expression inhibitors acting via the activation of RNAse H (see Figure.1), an enzyme that recognizes and binds to double strand of RNA and DNA and degrades the RNA strand. Acting via RNase H mechanism requires the ASO to possess a chemical structure similar to DNA. Splicing modulators are engineered to target and bind to the "intron-exon junction" of the pre-mRNA and at these locations, they create a structural obstacle to hinder splicing processes[268]. The ASOs in this class could have an RNA-like chemical structure or similarly to the expression inhibitors, a DNA-like chemical structure, in the later, chemical alternation of nucleotides were used to prevent RNAse-H recognition of the duplex consist of the ASO and the pre-mRNA. It took many years of chemical optimization until the first ASO compound – Fomivirsen, was approved in 1998. Ionis Pharmaceuticals (formally known as Isis Pharmaceuticals) licensed this ASO from the NIH and spent many years developing it. The lessons learned through its development were shared in peer reviewed papers by Ionis and had a great deal in progressing the oligo therapy field. Fomivirsen contains phosphorothioate (PS) in which a sulfur atom takes the place of a non-bridging oxygen in the phosphate backbone of the oligonucleotide. This alteration enhances the linkage's resistance to degradation by nucleases[269,270]. PS is considered as the first-generation ASO modification, and still most ASOs, especially the expression-inhibiting ones, contain this modification as it is quite efficient in increasing the stability of ASOs in plasma. The second ASO to be approved in 2013, Mipomersen/Kynamro, contained on top of PS modifications, 2′-O-

methoxyethyl (2′-MOE)-modified ribose (see Figure.2) on both the 5' and 3' ends of the molecule, and this (i.e. PS + 2'-MOE) is considered to be the second generation ASO modification. The central deoxyribonucleotides, forming a gap region of 10 nucleotides, remain unmodified[270]. 2′-MOE increases the oligo's resistance to breakdown by nucleases, together with improving its affinity to the target RNA affinity and minimizing its non-specific protein binding toxicity. The use of 2′-MOE is limited to the edges of the molecule as its presence inhibits the RNAse-H activity, hence in the "Gapmer" design where short DNA antisense oligonucleotide structures is combined with RNA-like sections flanking both ends of the sequence, the use of 2′-MOE is limited to the RNA sections, and it is not interfering with the gene-expression inhibition[270]. The second generation ASO modification (PS+2'-MOE), was extensively used thereafter and can be found in several compounds approved (e.g., Tegsedi, Volanesorsen) and in about 30 ASOs in advanced developing phases[267]. Important to note that about a third of those compounds are aimed to target the CNS, and it is doable as 2'-MOE was shown to be highly stable in the CSF after intrathecal administration[271]. Two other chemical modifications that can be found in compounds in advanced stages of development, both are sugar modifications: (S)-constrained ethyl (cET) and locked nucleic acid (LNA)[270]. LNA and cET have a methylene bridge linking the 2'-oxygen and the 4'-carbon of the ribose which predominantly favor the RNA ribose sugar conformation over the DNA one[272], and results in increased affinity and specificity to the target and reduced recognition by nucleases. Like the 2'-MOE, those modifications can interfere with the RNAse-H activity hence are not used on the DNA section in the gapmer.

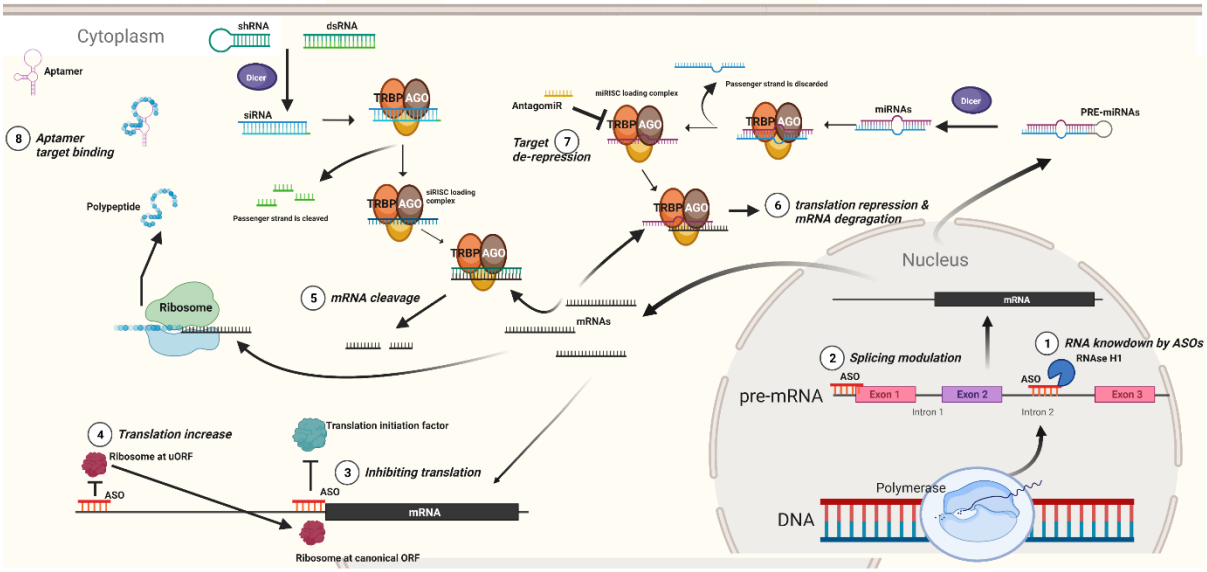


Fig. 1. Schematic representation of oligonucleotide's mechanisms of action. 1. ASO can bind anywhere in the pre-mRNA (messenger RNA), then RNAse-H is recruited to cleave the target RNAs, an action resulting in RNA knockdown. 2. ASO causes an exon to be preferentially included or excluded resulting in splice modulation. 3. ASO sterically blocks the translation machinery, lowering protein but not RNA levels. 4. ASO blocks upstream open reading frames (uORFs) increasing translation efficiency. 5. Endogenous or exogenous shRNAs are processed by Dicer to form siRNAs that bind to RNA-induced silencing complex (RISC). The siRNA-RISC complex binds to complementary mRNA sequences, resulting in the enzymatic cleavage of the target mRNA. The cleaved mRNA is rendered nonfunctional. 6. microRNA binds to RISC, and the miRNA-RISC complex binds to a seed match in the mRNA sequences, resulting in translation repression or mRNA degradation. 7. Antagomir sterically blocks microRNA from binding to its target mRNA, which prevents the degradation of the target mRNA via RISC. 8. Aptamers are single-stranded oligonucleotide molecules that bind to protein targets by folding into a three-dimensional conformation, like antibodies. TRBP – TAR. RNA-binding protein, Ago – Argonaute. Figure and legend adapted from Bortolozzi et al., 2021[238]

A limited number of ASO splicing modulator compounds have also received approval for treatment. Spinraza© stands out as the best-known and first drug in this category to be approved for treating spinal muscular atrophy[273] which is a rare genetic disorder. The mutation in the Survival Motor Neuron 1 (SMN1) gene is the underlying cause of the disease. The drug aims to elevate the levels of fully functional SMN protein by targeting a specific sequence within the SMN2 gene's intron. Though nearly identical to its paralog, the SMN1 gene, SMN2 typically produces only 10-20% functional SMN due to alternative splicing[274]. Spinraza compound has PS bonds and 2'-MOE

throughout all its sequence and the administration is done via the intrathecal route twice a month[275]. Other splicing modulator ASOs were approved for the treatment of Duchenne muscular dystrophy, which is a very prevalent type of inherited myopathy[276], the pathophysiology involves mutations in a gene that encodes the dystrophin protein. This gene, named DMD, is considered the longest human gene. Different kinds of mutations along the gene sequence may disrupt the reading frame and lead to the loss of the protein it produces[277]. These ASOs (Eteplirsen, Golodirsen, Casimersen, and Viltolarsen), targeting axon53 in the DMD gene, received approval due to their efficacy in enhancing dystrophin protein production. Those ASOs feature a chemical adjustment that relies on phosphorodiamidate morpholino oligomer (PMO). With a DNA base connected to a methylene-morpholine ring structure through phosphorodiamidate groups, PMO has a specific composition (see Figure 2). PMO are suitable for MOAs that don't require RNAse-H as upon binding to its target RNA, PMO obstructs other molecules from accessing the RNA due to steric hindrance, without triggering RNA degradation. While PMOs boast considerable stability and a strong safety profile, their limited ability to bind with proteins hampers their pharmacokinetics and reduces their effectiveness. Efforts are being made to overcome these limitations by developing peptide conjugated PMO oligomers (PPMOs) which should increase cellular uptake of PMOs[278].

1.2.2. Small interfering RNAs

Small interfering RNA (siRNA) is the second most developed class of oligo therapeutics with 5 approved drugs (as of March 2022)[266] and 30 compounds in advanced development stages (as of November 2021)[267]. siRNA is a double-strand RNA molecule that by utilizing the miRNA machinery (i.e., the RISC complex) and

Watson-Crick base pairing, it can either degrade or inhibit the translation of its target RNA, through a mechanism commonly known as RNA interference (RNAi)[279]. The discovery of RNA interference (RNAi) was initially made by Fire and Mello, who were awarded the Nobel Prize for their research into how external RNA could inhibit gene function in C. elegans[280]. 20 years after the discovery of the RNAi in 1998[281], the first RNAi drug (patisiran/Onpattro) was approved[282]. The development of siRNA has many challenges that limit its extensive clinical application. Like the case of ASOs, the main issues were and still are, delivery, stability, and specificity[279]. Although siRNA and miRNA share comparable physicochemical characteristics as short RNA duplexes, they induce gene silencing through unique mechanisms of action. Consequently, they vary in both their sequence configurations and their uses in medical treatments. On the other hand, they share many of the hurdles: poor *in-vivo* stability, delivery challenge and off-target effects, and so the employed solutions are common between siRNA and miRNA mimics. To avoid redundancy, problems and approaches that are common to both siRNA and miRNA DRUG development will be discussed in this section and the compound will be referred to as 'RNAi modalities' or 'dsRNA'.

A carefully designed siRNA sequence is essential for achieving a compound which is potent and specific to its targeted mRNA. siRNA sequence will consist of ~20nt in a double stand (duplex) design with two nucleotides overhands at the 3' end, which are important for its recognition by the RISC complex[283]. Longer sdRNA that first will be processed by dicer, have shown the potential of enhanced potency, but on the downside, has a greater potential of activating an immune response via IFN pathways[284]. It was shown that the efficiency of siRNA to successfully silence a gene depends greatly on the mRNA region it targets. A combination of in-silico and *in-vitro* studies are performed to identify and validate successful gene targeting[285,286]. When

using RNA duplexes aimed for RNAi therapy, whether it is siRNA or miRNA, it should be taking into consideration that both strands could potentially be loaded into the complex and an incorrected loading orientation will result in discarding of the intendent guide strand while the remaining strand base-pairs to unintended mRNAs, resulting in off-target effects. To increase the chances of a correct strand selection by the RISC the design should follow two key principles: (i) the asymmetry rule and (ii) 5' nucleotide preference. Those principles are applied in both siRNA and miRNA design. The asymmetry rule suggests that the selection of the guide strand loaded by AGO to the RISC complex is favored by a less thermodynamic 5' end, which means that the strand with higher A/U content at this end has a better chance to be selected as the guide strand. The second principle suggested that there is a preference for an Uracil (U), or less favorably adenine (A), at the 5' of the intended guide strand [285]. The overall G/C content of the siRNA seems to influence its activity where the tendency is that high (over 50%) G/C content lowers the efficacy[287,288]. Moreover, it was shown that a consecutive nine or more nucleotides of G/C may reduce the efficiency of siRNA[289]. siRNA can induce off-target effects in a miRNA like fashion, where the 5' terminus of the siRNA guide strand matches the 3' untranslated region of an unintended mRNA. To avoid this miRNA-like-effect the seed region which is located at nucleotides number 2–7 from the 5′end, should not have a base-paring with the 3′UTR of mRNA. Typically, judicious application of bioinformatics tools can help evade this, by ensuring that seed sequences of miRNAs (which can be identified using miRNA databases) are not included. Another kind of off-target effect, which is not reliant on sequence, happens when the RNAi machinery becomes saturated due to the presence of synthetic siRNAs or by miRNA that compete with endogenous miRNAs, hence the regulations of genes by those endogenous miRNAs is disrupted which could result in unforeseen off-target

effect[290,291]. Both off-target effects are dose dependent hence the goal is to use the lowest concentration possible when administrating RNAi modalities. It is also recommended to avoid certain sequence motifs that are known to activate the immune system through IFN pathways[292,293].

The molecular weight (MW) of siRNA and miRNA (~ 15 kilodalton) which is about twice of ASO, and their negative charge, present a major hurdle in their ability to cross the cell membrane. Concerning their stability, both the phosphodiester bonds and the 2'-OH group on the ribose contribute to the susceptibility of RNA to hydrolysis. Double-stranded RNA (dsRNA) shows greater resistance to enzymatic degradation compared to its single-stranded counterpart. Once siRNA is introduced into the bloodstream, it is rapidly broken down by endonucleases and exonucleases. This rapid degradation prevents the intact molecules from accumulating in the target tissue. The specificity issue arises from the fact that the RNAi machinery can tolerate some mismatches, this could result in unintended silencing of genes that only have minor nucleotide differences. Furthermore, the sense strand of siRNA or miRNA, when loaded into the RISC complex, may also cause the inadvertent knockdown of unrelated genes. On top of that, unmodified and unformulated dsRNA may activate Toll-like receptor 3 (TLR3) which will result in undesired effects on the blood and lymphatic systems[294]. To mitigate the unwanted side effects and address the pharmaceutical challenges associated with RNAi approaches, while maximizing their potency, various chemical modifications were tested as well as different delivery pathways. A few of the lessons learned from ASO development were applied (e.g., PS, 2’-OMe) and the progress made in siRNA development was used for the development of miRNA mimics as therapeutic agents (discussed here and below). In the development phase of siRNAs, both entirely unmodified and minimally modified siRNAs were employed for localized

drug delivery, such as to the eyes, and were demonstrated to mediate gene silencing *in-vivo*. However, these approaches displayed limited efficacy and were subject to off-target effects. Notably, they triggered the activation of Toll-like receptor 3 (TLR3), leading to the secretion of interleukin-12 and interferon-γ[295]. To improve specificity, and reduce the activation of innate immunity by siRNA, one common strategy involves altering the 2'-OH hydroxyl group on ribose. Well-known substitutions for this purpose include 2′-O-methyl (2′-OMe)[296] and 2′-methoxyethyl (2′-MOE)[297] groups. Likewise, modifying specific nucleotides in the sequence with alternatives like locked nucleic acid (LNA)[298], unlocked nucleic acid (UNA)[299] or glycol nucleic acid (GNA)[300], can achieve comparable enhancements. For a more comprehensive list of these modifications, please refer to Figure 2. The implementation of these chemical modifications is evident in clinical settings. For example, Onpattro/Patisiran[301] is an anti-transthyretin (TTR) RNAi medication that incorporates both 2′-O-methyl (2′-OMe) and 2′-deoxy-2′-fluoro (2′-F) chemical modifications into its oligonucleotide sequence (see Figures 2 and 3). Another significant example is Inclisiran, an approved siRNA compound for treating hypercholesterolemia. This compound utilizes a combination of phosphorothioate (PS) linkage along with 2′-O-methyl (2′-OMe), 2′-deoxy-2′-fluoro (2′-F), and 2′-deoxy modifications[302,303]. Overall, it was demonstrated that different modifications of the siRNA can determine the levels of its efficacy, specificity, and stability as well the levels of its toxicity and immunogenicity.

As previously noted, phosphorothioate (PS) linkage has been employed in the modification of antisense oligonucleotides (ASOs). This change is accomplished by substituting a sulfur atom for one nonbridging oxygen in the phosphodiester bond (see Figure 2). The PS linkage enhances the oligonucleotides' resistance to nucleases and facilitates their binding with plasma proteins,[304] in particular, albumin[305]. This can lead

to an extended circulation time in the bloodstream. Additionally, protein binding is generally advantageous for the cellular uptake of oligonucleotides. Some data have shown that PS can potentially cause a reduction of the binding affinity with target sequence[306] and sequences that contain a high level of phosphorothioate linkage have been associated with toxicities[307,308]. It was demonstrated that the exact positions as well as the total number of PS linkages will affect the oligo efficacy and toxicity profiles. Alnylam, one of the leading pharma companies in the field of oligo therapies, has incorporated a pair of PS linkages at the edges of the 3' and 5' end of the oligo (see Figure.3). On top of the location, the stereochemistry of the phosphorothioate can affect the stability of the linkage[309]. Besides phosphorothioate (PS), other chemical modifications have been employed to replace the phosphodiester group, thus altering the properties of the oligonucleotide, including phosphorodithioate, peptide nucleic acid, among others (see Figure.2). Incorporation of methylphosphonate and methoxypropylphosphonate was demonstrated to reduce ASO protein binding, whereas PS2 modification showed its capacity to increase the affinity between the siRNA and RISC. The 5′-phosphate is essential for properly loading the oligonucleotide into the RISC complex. Nonetheless, studies have shown that various analogs can replace the phosphonate at the 5′-end and still retain similar conformational and electronic characteristics to natural phosphates, in addition to being more resistant to dephosphorylases. Currently, the most effective and metabolically stable method is the use of 5′-(E)-VP, which swaps out bridge oxygen and carbon with E-vinyl phosphonate elements at the 5′-end[307,308].

The ribose modification at the 2' position has been used extensively to protect siRNAs and ASOs from nucleases attack which requires 2'-OH group for the hydrolysis of RNA. 2'-OMe, a naturally occulting ribosugar, is the most frequently used

modification, thus far as it was shown to increase stability by blocking the nucleophilic 2'-OH group. On top of that, 2'-OMe was demonstrated to increase the affinity of the oligo to its target RNA and reduce immunogenicity. Following the recognition of 2'-OMe, a few analogs have been identified like 2'-O-methoxyethyl (2'-O-MOE), that showed a better affinity profile to RNA than 2'-OMe. 2′-deoxy-2′-fluoro (2′-F), is an additional analogue which is widely used, was also showed to have good affinity profile to the RNA. Other analogous are illustrated in Figure.2

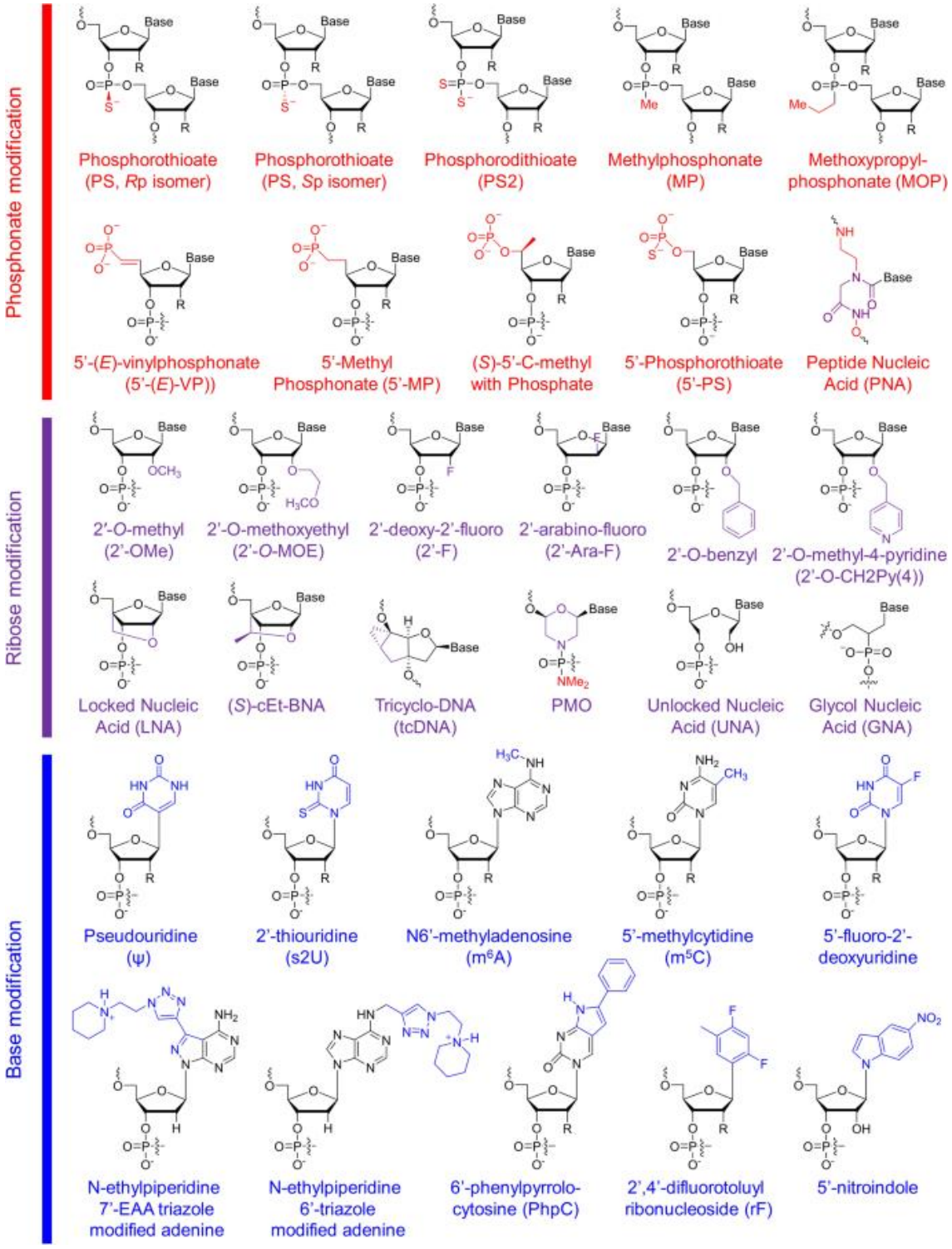


Fig. 2. Structures of chemical modifications and analogs used for siRNA and ASO decoration. According to the modification site in the nucleotide acid, these structures can be divided into three classes: phosphonate modification, ribose modification and base modification, which are marked in red, purple and blue, respectively. R = H or OH, for RNA or DNA, respectively. (S)-cEt-BNA (S)-constrained ethyl bicyclic nucleic acid, PMO phosphorodiamidate morpholino oligomer. Figure and legend extracted from Hu et al., 2020[312].

With the advancement in understanding the impacts of diverse chemical alterations on the pharmacology of oligonucleotide modalities, several patterns of chemical modifications have been established. Alnylam has engineered one such pattern, termed Standard Template Chemistry (STC). In this pattern the sense strand is 21nt long and the antisense stand is 23nt. The exact positions of the PS linkages, 2'-F moieties, and 2'-OMe modifications are distinctly specified (see Figure.3). It is suggested that three consecutive 2'-F moieties placed in the central part of the sense strand facilitates its removal from the RISC complex which is prerequisite of RISC activation. The STC pattern was employed in the development of an siRNA compound named Revusiran, which advanced to Phase 3 clinical trials. This compound exhibited promising stability and affinity profiles, maintaining the sequence's capability to be correctly loaded to the RISC and execute its inhibitory activity through the RNAi machinery[310]. However, the drug did not progress to clinical use due to a higher incidence of death in the test group. Although a comprehensive investigation by the company indicated no correlation between the deaths and the drug, it could not fully alleviate the suspicions surrounding the STC modification pattern. The next-generation pattern, known as Enhanced Stabilization Chemistry (ESC), incorporated fewer 2'-F analogues and additional PS linkages, and was utilized in the approved drug, Givlaari[311]. Further advancements in modification patterns are illustrated in Figure.3 and are reviewed here[312].

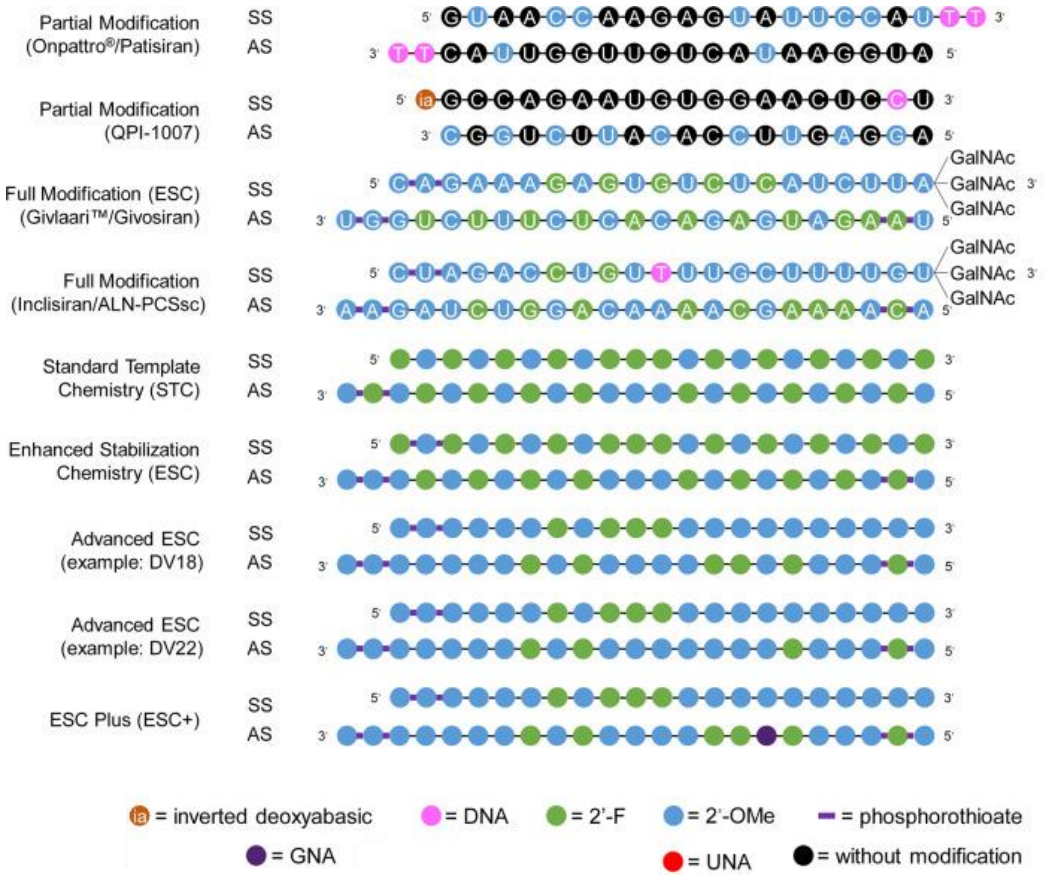


Fig. 3. Representative designs for the chemical modification of siRNA. The sequences and modification details for ONPATTRO®, QPI-1007, GIVLAARI™ and inclisiran are included. The representative siRNA modification patterns developed by Alnylam (STC, ESC, advanced ESC and ESC+) are shown. 2′-OMe 2′-methoxy, 2′-F 2′-fluoro, GNA glycol nucleic acid, UNA unlocked nucleic acid, SS sense strand, AS antisense strand. Figure and legend adapted from Hu et al., 2020[312].

RNAi modalities (siRNA and miRNA) must reach to the cytoplasm for them to achieve gene silencing. A series of hurdles had to be tackled for RNAi therapy to be effective, and while incorporating chemical modifications helped in addressing some issues (e.g., nuclease degradation, short-lived circulation, immune recognition) other challenges remained to be addressed: (i) accumulation of the compound in desired tissue as unspecific binding in blood circulation together with glomerular filtration prevents accumulation of RNAi modalities in the tissue of target; (ii) effective transmembrane trafficking as oligonucleotides are negatively charged and have

relatively high molecular weight, they cannot pass the cell membranes by passive diffusion, as most small molecule drugs do. (iii) endosomal and lysosomal escape into the cytoplasm. To address these issues two main strategies were used: 1. The creation of formulation carriers primarily involves utilizing lipids and/or other nanomaterials to encapsulate the nucleic acid. This encapsulation serves to shield them from RNA degradation and to mask their negative charge. Lipid Nano Particles (LNP) are the principal technology employed in this strategy. 2. The second strategy to tackle delivery challenges involves the deployment of conjugated biomolecules, such as lipids, peptides, or sugars, that aid in enabling receptor-mediated endocytosis. 3. Another strategy leverages virally transduced artificial expression, but this approach is beyond the scope of this dissertation.

Lipid-based nanoparticles (LNPs) were originally used to facilitate the delivery of DNA-based compounds. However, their ability to protect RNA-based oligonucleotides against nucleases and renal clearance, coupled with increased cellular uptake, has marked them as a promising technology for siRNA drug development. Research involving siRNA encapsulated in LNP revealed that when these compounds were systemically administered, they primarily accumulated in the liver, spleen, and kidneys[313]. Based on their varying charge properties under neutral conditions, LNPs can be categorized into different classes: ionizable, cationic, and neutral. Their charge will determine the release of their load. For instance, Ionizable LNPs become protonated once entering endosomes and lysosomes which have a low PH environment relative to the circulation. Their main interaction with apolipoprotein E3 (ApoE3), enables their transportation into hepatocytes. In contrast, cationic LNPs consistently exhibit a positive charge, maintaining this characteristic both in circulation and within acidic environments. This class of LNPs also accumulate mainly in hepatocytes but in

ApoE3 independent fashion. Nonspecific binding with plasma proteins makes this class more prone to cause immunogenicity hence most development efforts are focused on developing ionizable lipids for hepatocyte targeted delivery[314,315].

Biomolecule conjugation was also shown to be an effective approach for siRNA delivery, with N-acetylgalactosamine (GalNAc) developed by Alnyam, being the flagship in the oligo field, leading the delivery through conjugation. In this delivery platform an siRNA is conjugated via a proprietary linker structure to GalNAc, which serves as a ligand for the asialoglycoprotein receptor (ASGPR). This endocytic receptor is distinctly and abundantly expressed on the surface membrane of hepatocytes[316]. Following the binding of the GalNAc to the membrane receptor, a clathrin-mediated endocytosis transports the ligand together with the siRNA into the cytoplasm. While the precise mechanism allowing escape across the endosomal membrane is still undetermined, sufficient quantities of siRNAs manage to access the cytoplasm to engage with their target RNA. Studies revealed that conjugation of more than one ligand to each siRNA displays affinity that is orders of magnitude higher compared to a single or double ligand. The ligands are conjugated at the 3' or 5' ends of the sense strand[300,317]. GalNAc conjugates present great pharmaceutical properties both in high target tissue accumulation, high therapeutic index and limited adverse effects, hence allowing subcutaneous administration. On top of that, the chemical modification aimed to enhance stabilization (e.g., ESC) supporting the potential for a quarterly to biannual dosing regimen for these RNAi therapeutics. This excellent performance profile and despite the commercialization of patistran (LNP-based siRNA), Alnylam stopped all their liposome-based programs and focused their development efforts on GalNAc. In addition to Alnylam, GalNAc-oligo conjugate platforms are developed by additional biotech companies (e.g., Arrowhead, Dicerna, Ionis, Wave Life science, Regulus

among others) and Pharma (e.g., Sanofi, and Novartis). The first approved drug utilized GalNAc, which was the second RNAi drug to be approved, was developed for the treatment of acute hepatic porphyria, a rare genetic disease associated with mutation in the genes involved in heme synthesis[318]. Four out of five approved siRNA compounds are utilizing GalNAc conjugates, all of them targeting hepatocytes and among the 30 siRNAs that are in advanced developing stages, 19 are GalNAc conjugates. Also 12 ASOs in advanced stages are using GalNAc, which means that almost third of oligonucleotides in advanced developing stages are using GalNAc, and 75% of the oligos that target the liver use this platform[267].

Other RNAi delivery platform are currently in advanced stages of development, for detailed review please see here[312], including viral vectors encoding shRNAs or miRNAs. Commonly utilized viruses for this objective include lentiviruses, adenoviruses, and adeno-associated viruses[319].

On top of treating rare diseases, siRNA therapy achieved a major milestone and was approved for the treatment of a common disease, e.g., dyslipidemia, Inclisiran. The recent advances in RNAi therapy which enable reduced effective dose (from milligrams to micrograms) and prolonged half-life (from minutes to months) which enables twice-yearly administration, will impact the entire industry. Great efforts are being made to develop the extrahepatic delivery platform.

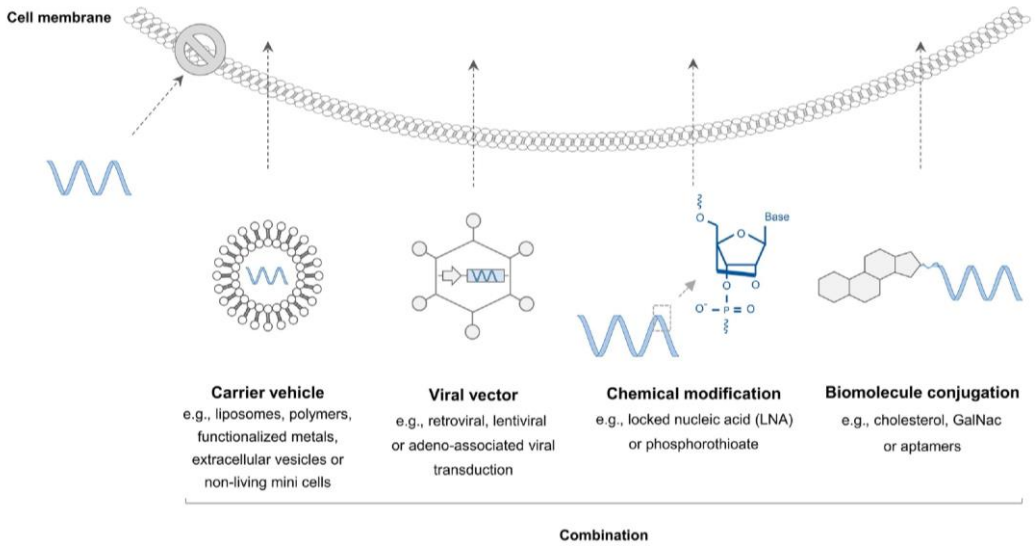


Fig. 4. Nucleic acid–based miRNA therapeutics show a low cell membrane permeability. Efficient intracellular delivery can be achieved by nano-sized carrier vehicles, viral transduction, introduction of chemical modifications, or conjugation to biomolecules that further a receptor-mediated uptake. Abbreviations: GalNAc, N-acetylgalactosamine; miRNA, microRNA. Figure and legend adapted from Diener et al., 2022[384].

1.2.3. miRNA replacement therapy

miRNA-based therapy comprises of two approaches: miRNA replacement and miRNA inhibition (outside of the scope for this dissertation). The latter approach is similar to antisense therapy, with a single strand RNA acting as miRNA antagonist, referred to as anti-miRs or antagonists. The replacement approach, also known as miRNA mimics, shares many similarities with siRNA. Both modulates inhibit genes expression in a post-transcriptional manner via the RNAi machinery, but whereas siRNA is designed to target one gene only, the miRNA-based therapy attractiveness lies in the fact that a single miRNA can regulate a wide spectrum of genes, and especially when this spectrum is related to an entire cellular or indication related pathway. This characteristic makes miRNA mimics suitable for the treatment of complex multigenic disease such as cancer, neurodegenerative disorders and potentially be applied also for the treatment of affective disorders. However, this quality of

miRNAs is a double-edged sword as they induce numerous cellular effects and averting off-target effects is challenging. The miRNA can produce this overall effect by exerting a relatively moderate impact on each of the targeted genes, as demonstrated in the case of miR-34-5p that plays a key regulator of T cell networks[320,321]. In this chapter I will discuss the differences between siRNA and miRNA therapies. The similarities between the two RNAi modalities (e.g., chemical modifications, delivery) were discussed in the '*siRNA*' section above. After processing by Dicer the miRNA duplex is associated with the RISC complex forming what is referred to as miRISC. Within this complex, the miRNA duplex undergoes unwinding, and the passenger strand is discarded. In the case of siRNA, the cleavage of the passenger strand is catalyzed by the AGO2 protein of the RISC complex. The antisense miRNA, still attached to the RISC, forming miRISC, is guiding this complex to its mRNAs to which it can binds through different levels of complementarity resulting in inhibition of the target gene, if via translational repression, degradation, or cleavage (for more details please see miRNA chapter above). Conversely, in the scenario of siRNA, complete complementarity between it and its target mRNA must be established to trigger the activation of AGO2, which subsequently results in the cleavage of the mRNA's phosphodiester backbone[322]. The aim of utilizing miRNA mimics in miRNA replacement therapy is to replicate the inherent biological function of the endogenous miRNA. Consequently, the design of the sequence is more direct compared to that of siRNA, closely resembling, if not being entirely identical to, the endogenous sequence. It was shown that using a miRNA in a duplex form rather than a single strand increases its potency up to 1000 folds[323,324], probably since the double stand formation facilitates the loading of the miRNA mimic into the RISC complex. Designing mature miRNA mimics of about 22nt with a duplex structure has become the standard of miRNA therapeutic development. Even though

the sequence of the miRNA mimics is highly identical to the endogenous one, the development of miRNA faces similar challenges as siRNA, for instance activation of innate immune system via toll like receptors (TLRs) as well as poor stability and lack of accumulation in target cells. To overcome those hurdles and the limited sequence variation, chemical modifications were used extensively as well as different delivery approaches (see siRNA chapter).

MRX34, was the first miRNA mimic to enter a clinical trial[325]. MRX34 was designed as a miRNA replacement therapy, aimed to replenish the levels of miR-34 that were demonstrated to be downregulated in many kinds of tumors and *in-vivo* studies marked this miRNA as a potential tumor suppressor. The miR-34 mimic was delivered systemically using a liposomal delivery approach, designed to release the miRNA explicitly in the acidic environment of tumor tissues. The clinical trials had to be terminated due to severe immune related side effects which caused the death of four patient[326,327]. *In-vivo* studies demonstrated that the miR-34 mimic was accumulating in bone marrow and spleen[328,329] on top of tumorous tissues, which serves as a possible explanation for the severe adverse effects seen in the clinic. The case of MRX34 emphasized the importance of a prior risk assessment of *in-vivo* targeting by miRNA mimics. Now, the most advanced miRNA mimic program is Remlarsen© of Miragen Therapeutics which designed to mimic the activity of miR-29. miR-29 was shown to be downregulated in pathological fibrotic conditions[330] and by mimicking its activity, Remlarsen is aimed to ameliorate the disease condition. Remlarsen has a DNA chemical structure with LNA sugar modifications. Other branch of miRNA replacement therapy is based on Artificially designed miRNA constructs (referred to as amiRNAs) that are a combination of siRNA sequence and scaffolds of primary miRNA which enables high target specificity while their endogenous miRNA-based structure ensures cellular

processing. AmiRNA-based drug combining an siRNA against Huntingtin mRNA together with pri-miR-451 scaffold is currently in clinical testing for Huntington disease[331–333].

2. Materials and experimental procedures

2.1. Animals and housing

Adult C57BL/6 male mice 9-11 weeks old were used. Mice were housed in a temperature-controlled room (22 ± 1 °C). Food and water were available ad libitum. All experimental protocols were performed on male mice and were approved by the Institutional Animal Care and Use Committee.

2.2. Microdissection and preparation of RNA

As previously described[334], the brain was extracted and positioned within a 1 mm metal matrix (cat# 51380; Stoelting Co. Wood Dale, IL). Standard razor blades (GEM, 62-0165) were positioned at known locations to slice the brain into 1 mm or 2 mm sections. These were then swiftly frozen on dry ice. Blunted syringes of varying gauges were employed to obtain the precise brain regions required. The samples were preserved at -80 ºC. The RNA was isolated using the miRNeasy kit (Qiagen, Hilden, Germany) and was subsequently reverse transcribed to cDNA utilizing the High-Capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA). The resultant cDNA was analyzed through quantitative real-time PCR (RT-PCR).

2.3. Cloning of target transcripts 3'UTRs into psiCHEK-2 luciferase expression plasmid

As previously described[239] , the 3'UTRs sequences of Slc6a4 and Htr1a were amplified from mouse genomic DNA using PCR. The PCR fragments of 3'UTRs were then ligated into the pGEM®-T easy vector (Promega), following the manufacturer's instructions, and subsequently sub-cloned into a singular NotI site at the 3' end of luciferase in the psiCHECK-2 reporter plasmid (Promega). The orientation of cloning was confirmed through diagnostic cuts and sequencing.

2.4. Transfections and luciferase assays

As previously described[239] , HEK293T cells were cultured on poly-L-lysine in a 48-well plate until they reached 70-85% confluence and were then transfected with polyethyleneimine using the following plasmids: psiCHECK-2 plasmid containing either the wild type or mutated 3'UTR, and either the overexpressing vector for a specific miRNA or empty overexpression plasmids. The miRs overexpression plasmids were sourced from the miR-Vec library. Twenty-four hours post-transfection, the cells were lysed, and the luciferase reporter activities were measured as described previously[335]. The Renilla luciferase values were normalized to control firefly luciferase levels, which were transcribed from the same vector but were not influenced by the 3'UTR. These values were then evaluated and averaged across six repetitions for each condition.

Table 1: Oligonucleotide primers used for cloning

Gene	Orientation	Primer Sequence
Slc6a4 3'UTR	sense	ATCCGCATGAATGCTGTGTA
Slc6a4 3'UTR	antisense	GTGGGTGGTGGAAGAGACAC
Htr1a 3'UTR	sense	AGTTCTGCCGCTGATGATG
Htr1a 3'UTR	antisense	GCACAAATGGAGAGTCTGATTAAA

Table 2: Oligonucleotide primers used for microRNA real time PCR

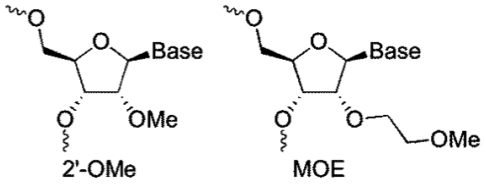
Gene	Primer sequence
miR-135a	TATGGCTTTTTATTCCTATGTGA
miR-135b	TATGGCTTTTCATTCCTATGTGA
miR-375	TTTGTTCGTTCGGCTCGCGTGA
U6	GATGACACGCAAATTCGTGAA
miR-124	TAAGGCACGCGGTGAATGCC
miR-16	TAGCAGCACGTAAATATTGGCG

Table 3: Oligonucleotide primers used for mRNA real time PCR

Gene	Sense primer	Antisense primer
Slc6a4	GGGTTTGGATAGTACGTTCGCA	CATACGCCCCTCCTGATGTC
Htr1a	GTGCACCATCAGCAAGGACC	GCGCCGAAAGTGGAGTAGAT
Hprt1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
Gapd	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
YWHAZ	ACTTTTGGTACATTGTGGCTTCAA	CCGCCAGGACAAACCAGTAT

2.5. Synthesis of miR-135 single strand oligonucleotide

Oligonucleotide sequences were assembled using a standard procedure using standard 2´-deoxy, 2´-O-Me or 2´-MOE phosphoramidite blocks, e.g.:



The synthesis was carried out while employing a typical experimental procedure of solid-phase synthesis on a CPG (Controlled Pore Glass) support. In short, a typical oligonucleotide synthesis preceded through a series of cycles composed of four steps (deprotection, coupling, capping and oxidation) that were repeated until the 5’ most nucleotide was attached (as depicted above).

2.6. Sertraline-conjugated miR-135 synthesis

The guide and passenger RNA strands were synthesized on an automated synthesizer using the phosphoramidite chemistry. The sequences were elongated using rA^{Bz}; rC^{Ac}; rG^{iBu}; rU and $^{2'OMe}A^{Bz}$; $^{2'OMe}C^{Ac}$; $^{2'OMe}U$ phosphoramidite monomers. After the chain elongation, the RNA strands were cleaved from their solid support and deprotected using an ammonium hydroxide and methylamine mixture. The 2’OH positions were deprotected using a solution containing fluorine ions. Then, the RNA strands were purified by anion exchange HPLC.

The Sertraline modified passenger RNA strands were synthesized on an automated synthesizer using the phosphoramidite chemistry. The sequences were

elongated using rA^{Bz}; rC^{Ac}; rG^{iBu}; rU and $_{2'OMe}A^{Bz}$; $_{2'OMe}C^{Ac}$; $_{2'OMe}$U phosphoramidite monomers. Before the deprotection step, the C10 N-hydroxysuccimide ester linker was grafted to the 5' end of the passenger RNA sequence still attached to the solid support. The condensation of the Sertraline-C_6Acyl-NH_2 Ligand with the C10 N-hydroxysuccimide ester linker was carried out in dimethylformamide with 3 % of *N,N*-Diisopropylethylamine for 48 hours at room temperature. After the conjugation, the Sertraline modified passenger RNA strands were cleaved from their solid support and deprotected using an ammonium hydroxide and methylamine mixture. The 2'OH positions were deprotected using a solution containing fluorine ions. Then, the conjugated RNA strands were purified by reverse phase HPLC.

The guide and passenger RNA strands were quantified by UV spectrometry using the extinction coefficient calculated based on the nearest neighbor method. The duplexes were combining equimolar amount of guide and passenger strands. The annealing was done in water. The duplexes were not further purified.

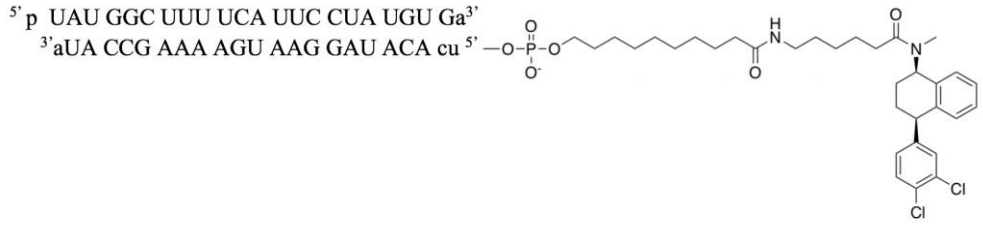
Oligonucleotide primers, RNA strands and sertraline-conjugated RNA strands and RNA duplexes were prepared by Axolabs, GmbH, following the specifications and designs discussed herein.

Structures:

Naked miR-135 Duplex 1 (Duplex 11 per Table 5, and Duplex 1 per Table 7, below)

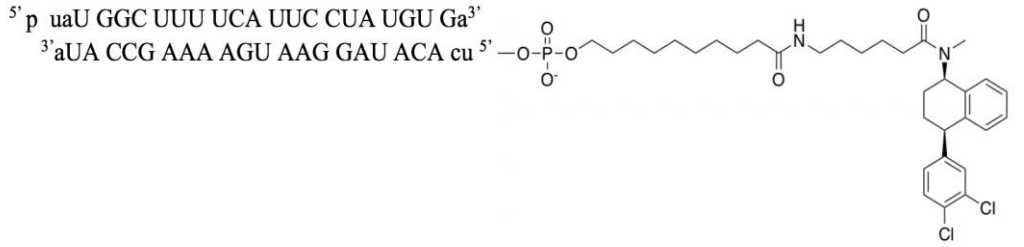
5' p UAU GGC UUU UCA UUC CUA UGU Ga3'
3'aUA CCG AAA AGU AAG GAU ACA cu 5'

Sertraline conjugated miR-135 Duplex 1 (miCure-135-1 per Table 7, below)



Sertraline-AcylC6-NHCO-C9-5'miR-135sequence3'-OH

Sertraline conjugated control (miCure-135-8 per Table 7, below)

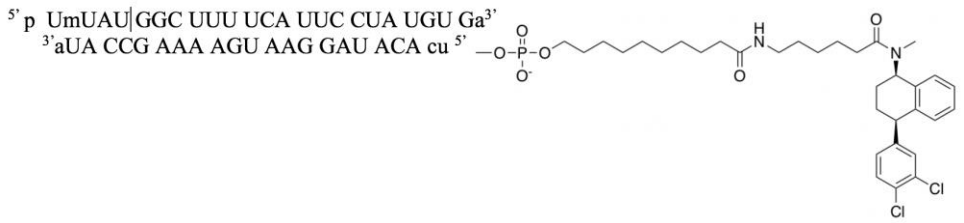


Naked miR-135 Duplex 2

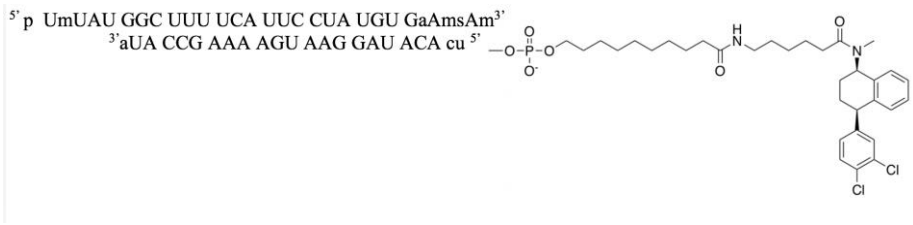
Guide strand - 5'-P/UmUAUGGCUUUUCAUUCCUAUGUGa

Passenger strand - 5'- ucACAUAGGAAUGAAAAGCCAUa

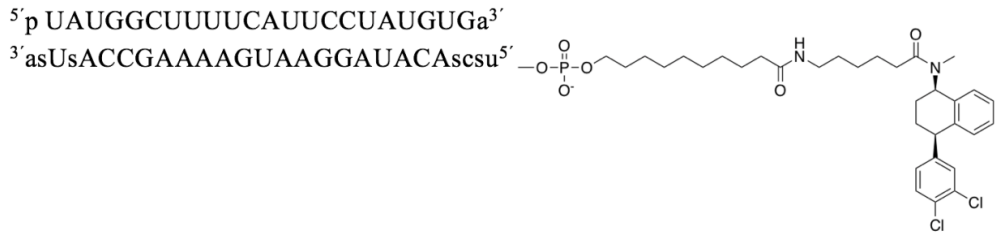
Sertraline conjugated miR-135 Duplex 2 (miCure-135-2 per Table26, below)



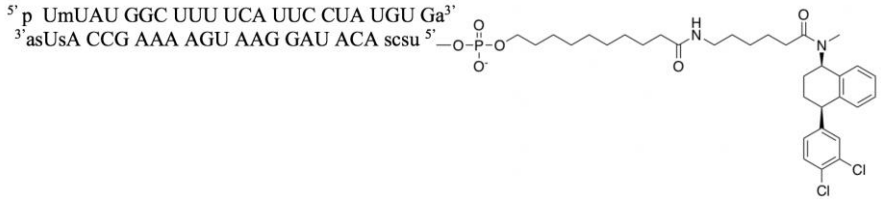
Sertraline conjugated miR-135 Duplex 3 (miCure-135-3 per Table 7, below)



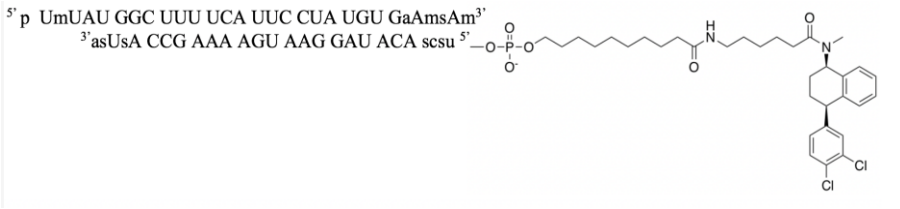
Sertraline conjugated miR-135 Duplex 9 (miCure-135-9 per Table 7, below)



Sertraline conjugated miR-135 Duplex 10 (miCure-135-10, per Table 7, below)



Sertraline conjugated miR-135 Duplex 11 (miCure-135-11 per Table 7, below)



For all duplexes:

High case letters (e.g. N, A, U, C, G): RNA

Lower-case letters (e.g. a, u, c, g): 2'-O-Me modification

Um: 2'-O-MOE-5'-Me Uracil modification

Am: 2'-O-MOE Adenine modification

Lower-case 's': phosphorothioate. No indication means a normal phosphodiester bond.

P: phosphate

Underscore: 2'-fluoro, i.e. 2'-F

(C10): Carboxymodifier C10

(Glen Research: 10-1935)

2.7. Intranasal administration

Mice were mildly anesthetized using 2% isoflurane inhalation and positioned supinely. A 5 µl drop of either conjugated control or conjugated miR-135 was alternately administered to each nostril daily. In total, 10 µl of solution was delivered.

2.8. Intracerebral injection

As previously described [239] , under general anesthesia, mice were positioned on a stereotaxic apparatus, and the compound was administered to coordinates, as defined by the Franklin and Paxinos atlas, targeting the DR: ML 1 mm; AP - 4.5 mm; DV -4.2 mm, with a 20° tilt. The injections were carried out at a rate of 0.2 µl per minute.

2.9. Intracerebroventricular chronic administration

As previously described[336] , the right lateral ventricle was perforated using a brain infusion kit 3 (ALZET, DURECT Corporation, Cupertino, CA; coordinates from the bregma: posterior, − 0.7 mm; lateral, − 1.5 mm; ventral, − 2.0 mm). The infusion cannula was then linked to a mini-osmotic pump (ALZET; pump model 1007D), which was subsequently implanted subcutaneously on the animal's back, just behind the scapula. Prior to implantation, osmotic pumps were primed for approximately 8 hours and were loaded with the test compound/control (0.5 nmol/day). Post-implantation, the pumps continuously delivered the compound or the negative control for 7 days, infusing at a steady rate of 0.5 µl/h.

2.10. Intracerebral microdialysis

Based on previously described protocols[337,338], microdialysis apparatus was used to measure the extracellular 5-HT concentration *in-vivo*In summary, a concentric dialysis probe, made of cuprophan and 1 mm in length, was implanted in the mPFC (AP, 2.2; ML, -0.2; DV, -3.4) of mice under anesthesia. Experiments were conducted 24–72 hours post-surgery. 1 mM citalopram (an SSRI; provided by Lundbeck A/S, Valby, Copenhagen) was introduced to artificial cerebrospinal fluid, which was then propelled at a rate of 6 µl min-1 (using WPI model sp220i), and samples were gathered every 6 minutes. While collecting the 8th fraction tail suspension test was performed.

The concentration of serotonin was measured using high-performance liquid chromatography-amperometric detection (Hewlett-Packard 1049; þ0.6V, Palo Alto, CA, USA) with detection limits of 1.5 fmol. Baseline levels of 5-HT were determined by calculating the average of the four samples taken before drug administration. The correct placement of the probe was confirmed through the use of cresyl-violet staining which was administrated through the probe prior to the decapitation of the animal.

2.11. Behavioral assessments

All behavioral assessments were performed during the dark phase following habituation to the test room for 2 hours before each test. The experimenter running the tests was blind to the mice groups. The behavioral tests below were described previously[334]

Dark-Light Transfer (DLT) test: The DLT test apparatus consists of a box divided into a black dark compartment (14 x 27 x 26 cm) and a connected white 1200 lux illuminated light compartment (30 x 27 x 26 cm). Using a video tracking system, the time spent in the light compartment, as well as the distance traveled in light area, and the number of light-dark transitions were quantified.

Tail suspension test: Mice were hanged from their tail in a height of 30 cm above the surface. For 6 minutes the mice were video recorded and the time they spend immobile was measured.

2.12. 8-OH-DPAT-induced hypothermia

Based on previously described protocols[338] The mice were subcutaneously implanted with programmable subcutaneous microchip transponders (IPTT-300 Extended Accuracy Calibration; Bio Medic Data Systems, Seaford, DE) following the manufacturer's instructions. In short, this procedure involved a quick insertion of a large-

bore needle delivery device containing the microchip, and depression of the plunger on the device that expelled the microchip from the delivery device. Temperature measurements from the microchip transponders were obtained using a compatible reader (catalog no. WRS6007, model IPTT-300, Bio Medic Data Systems). Two baseline temperature measurements were taken. After 10 minutes, the mice were administered i.p. with 8-OH-DPAT in a dose of 1 mg per kg and body temperature was recorded at 15, 30, 60 and 120 minutes following the administration. Data are presented as a change from the average baseline measurement.

2.13. Receptor autoradiography

Based on previously described protocol [337,338]

Tissue preparation for in situ hybridization and receptor autoradiography

14-mm slices of mice brain were cut using a microtome-cryostat (HM500 OM, Microm, Walldorf, Germany), thaw-mounted onto 3-amino- propyltriethoxysilane-coated slides (Sigma-Aldrich, Madrid, Spain) and kept at -20 °C until use.

Receptor autoradiography

The autoradiographic binding assays for 5-HT_{1A} and 5-HT_{1B} receptors and serotonin transporter (SERT) were performed using the following radioligands: (a) [^{3}H]-8-OH-DPAT (233 Cimmol^{-1}), (b) [^{125}I]-cyanopindolol (2200 Cimmol^{-1}) and (c) [^{3}H]- citalopram (70 Cimmol^{-1}), respectively (Amersham- GE Healthcare, Barcelona, Spain and Perkin-Elmer, Madrid, Spain). 8-OH-DPAT, isoproterenol, pargyline and 5-HT were from Sigma-Aldrich, and fluoxetine was from Tocris. Experimental conditions are summarized in Table 4, below.

Table 4: Summary of the conditions for labeling serotonin receptors and transporter

Protein	Ligand	Ligand (nM)	Buffer	Pre-incubation (RT, min)	Incubation buffer	Incubation protocol (RT, min)	Washing protocol (I.c.b., min)	Exposure time	Blank
5-HT $_{1a}$R	[^{3}H] 8-OH-DPAT	1.0	A	30	A + 10 μM pargyline	60	2 x 5	60 days	10 μM serotonin
5-HT $_{1B}$R	[^{125}I]Cyanopindolol	0.1	B	10	B + 100 μM 8-OH-DPAT+30nM isoprotrenol	120	2 x 15	1 day	10 μM serotonin
SERT	[^{3}H] citalopram	1.5	C	15	C	60	2 x 10	45 days	1 μM fluoxetine

2.14. Peripheral blood mononuclear cells (PBMCs) study

The study was performed by *Axolabs(www.axolabs.com).* In short, human PBMCs were isolation from buffy coats of healthy donors (obtained from the Blood Bank Suhl, Germany, Institute for Transfusion Medicine). Buffy coat, at a volume of about 28-32 ml, about 19 hours after blood donation, and delivered sterile in a sealed infusion bag were used. Isolation of human PBMCs (huPBMCs) was carried out using Ficoll gradient centrifugation.

huPMBCs were treated with the different mimetics (as described in the *'Results'* section below) by transfection or direct incubation (at three concentrations for 24 hours). Transfection was carried out using Lipofectamine2000. Supernatants were analyzed for the presence of cytokines using multiplex assays (U-Plex®) run on the meso scale discovery (MSD) platform. This entire procedure was carried out by Axolabs (www.axolabs.com).

2.15. Corticosterone – Chronic Restraint Stress Model

For the Chronic Restraint stress protocol, mice were isolated and handled 3 days before the start of the experiment. Along 28 days, once a day, mice were gently introduced in holed 50 mL Falcon tubes, fixed in a plastic tray, and left for 2 hours in a dark, quiet room. Control mice were not isolated and only handled daily for 1 minute each.

Corticosterone (Cortico, Sigma-Aldrich, Madrid, Spain) was dissolved in commercial mineral water and HCL was used to reach a pH of 7.0–7.4. Isolated mice were presented with Cortico solution for the 28 days of Chronic Restraint stress protocol, at 30 $\mu g\ ml^{-1}$ for 15 days, followed by 15 $\mu g\ ml^{-1}$ for 3 days and 7.5 $\mu g\ ml^{-1}$ for 10 days. Cortico solutions were no more than 3 days old and maintained in opaque bottles to protect them from light. Control mice were presented only with mineral water.

2.16. Immunohistochemistry/ Immunofluorescence

Protocol was previously described here[339]

Mice were anaesthetized and perfused with 4% paraformaldehyde (PFA) in sodium-phosphate buffer (pH 7.4). Brains were extracted, post-fixed 24 hours at 4 °C in PFA, and placed in gradient sucrose solution 10–30% for 3 days at 4 °C. After cryopreservation, serial 30 μm-thick sections were cut to obtain prefrontal cortex (PFC), caudate putamen (CPu), hippocampus (HPC), and dorsal raphe nuclei (DRN). Brain sections were washed and incubated in a 1x PBS/Triton 0.2 % solution containing normal serum from secondary antibody host. Primary antibodies for NeuN (anti-NeuN 1:1000; ref: MAB377, Millipore), Iba1 (anti-Iba1 1:1000; ref: 019-197741, Wako), and TPH (anti-TPH1 1:2500; ref: AB1541, Millipore) were used. Briefly, primary

antibodies were incubated overnight at 4 °C, followed by incubation with the corresponding biotinylated anti-mouse IgG1 (1:200; ref: A-10519, Life Technologies) for anti-NeuN, biotinylated anti-rabbit (1:200; ref: BA-1000, Vector Laboratories) for anti-Iba1, and biotinylated anti-sheep (1:200; ref BA-6000, Vector Laboratories) for anti-TPH1. The color reaction was performed by incubation with diaminobenzidine tetrahydrochloride (DAB) (ref: 18865.02, Quimigen) solution. Sections were mounted and embedded in Entellan (Electron Microscopy Sciences). The number of NeuN-positive cells, Iba1-positive cells and TPH-positive cells in the DRN were assessed in sections corresponding to different antero-posterior levels −4.24 mm to −4.84 mm from the bregma using ImageJ software (v1.51s, NIH, Bethesda, MD, USA). All labeled cells with their nucleus within the counting frame were counted in three consecutive DRN sections.

Primary antibodies Alexa488 (anti-Alexa488 1:1000; ref.: A11094, Invitrogen) and TPH (anti-TPH 1:1541; ref.: ab1541, Abcam) were used. Briefly, primary antibodies were incubated overnight at 4 °C, rinsed and treated with secondary antibodies A555-anti-sheep (1:500; ref.: A-21436, Life Technologies) and Alexa488-anti-rabbit (1:500; ref.: A-21206, Life Technologies) for 120 minutes. Nuclei were stained with Hoechst (1:10.000, ref.: H3570, Life Technologies). Intracellular localization of Alexa488-conjugated-miR-135 in TPH^+ neurons was observed and imaged using an inverted Nikon Eclipse Ti2-E microscope (Nikon Instruments) attached to the spinning disk unit Andor Dragonfly (Oxford Instruments). For all experiments an oil-immersion objective (Plan Apochromat Lambda blue 60×, numerical aperture (NA) 1.4, oil) was used. Samples were excited with 405 nm, 488 nm and 561 nm laser diodes. The beam was coupled into a multimode fiber going through the Andor Borealis unit reshaping the beam from a Gaussian profile to a

homogenous flat top. From there it was passed through the 40 µm pinhole disk. Tissue sections were imaged on a high resolution scientific complementary metal oxide semiconductor (sCMOS) camera (Zyla 4.2, 2.0 Andor, Oxford Instruments Company). Fusion software (Andor, Oxford Instruments) was used for acquisition and ImageJ/Fiji (1.51s, open source software) was used for image processing.

2.17. Western Blot

Based on previously described protocol [337,338]

Tissue samples from various brain regions including OB, PFC, CPu, HPC, DRN, and Cb were carefully dissected and subsequently homogenized in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycolate, 5 mM EDTA, 0.1% SDS, 50 mM Tris, pH 8.0), enriched with protease and phosphatase inhibitors. The protein concentration within these samples was then quantified utilizing the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). A portion of the protein lysate, ranging between 10–15 µg, was subjected to separation through 4–15% SDS-PAGE and subsequently electro-transferred to nitrocellulose membranes. Following the transfer, these membranes were probed with primary antibodies specifically targeting SERT and 5HT1AR, referenced by Abcam, and anti-β-actin from Sigma-Aldrich served as the loading control. Post-probing, the membranes underwent a secondary incubation with corresponding HRP-conjugated anti-goat for SERT and HRP-conjugated anti-rabbit IgG for 5HT1AR, leading to the eventual detection phase carried out through chemiluminescence, employing the SuperSignal™ Chemiluminescence ECL substrate kit (Thermo Fisher Scientific). The resultant images were captured utilizing the ChemiDoc™ Imaging System by Bio-Rad, and subsequent analysis was conducted using ImageLab™ software (Bio-Rad).

2.18. Statistical analysis

Data are expressed as means ± s.e.m. Data were analyzed with Student's t-test, one- or two-way analysis of variance, as appropriate, followed by post-hoc test (Newman–Keuls). The level of significance was set at $P < 0.05$ (two tailed).

3. Results

[This work has been entirely funded by miCure Therapeutics LTD, which also holds ownership of the presented results.
The findings presented in section 3.1 'Design, synthesis, and validation of miR-135 mimetic oligos' were derived from experiments conducted collaboratively by miCure and the Weizmann Institute of Science. These experiments were completed prior to my enrollment at LMU.]

3.1. Design, synthesis, and validation of miR-135 mimetic oligos

miR-135 mimetics (oligos) were designed to mimic the antidepressant effects demonstrated using overexpression of endogenous miR-135 in transgenic mouse model and viral manipulation, in the mouse 5HT neurons or DRN, respectively. The oligos (miR-135 mimetics) were designed based on the endogenous miR-135 with a few modifications aimed to improve stability and cell penetration. The altered oligonucleotides were synthesized by an oligonucleotide manufacturing company called BioSpring (www.biospring.de) using conventional chemistry and each oligo was used as a duplex to improve stability. Seventeen different mimics (see Table 5) were designed, synthesized and screened *in-vitro*, using luciferase assay, for their effects on the validates Htr1a and Slc6a4 target transcripts. For the luciferase assay screening the human cell line HEK293T and the transfection reagent, lipofectamin 2000 (Thermo Fisher Scientific) were used.

Duplex 11 was found to be the most potent miR-135 mimetic oligo, affecting significantly both Htr1a and Slc6a4 levels (using 3'UTR luciferase constructs; Figure.5A&B).

The design of duplex 11 mimetic of miR-135 was as follows:

Guide strand - 5Ph/UAUGGCUUUUCAUUCCUAUGUGa

Passenger strand – ucACAUAGGAAUGAAAAGCCAUa5

Lower-case letters (e.g. a, u, c, g): 2´-O-Me modification

Ph= phosphate, 5 indicates that this is the 5-prime end of the sequence

The mimetic that was identified to consistently reduce the target transcripts (Htr1a and Slc6a4) across preparations and was further used for the *in-vivo* experiments.

To examine the effect of miR-135 mimetics on the serotonergic system *in-vivo,* the miR-135 mimetic (duplex 11) was delivered directly into the dorsal raphe nucleus (DRN) of wild type mice using stereotactic surgery. The naked (i.e., unconjugated) mimetic was administrated acutely (100 µg) and the physiological effect of silencing the 5-HT1A auto-receptor, which manifests as a response of hypothermia, was explored by administering the selective 5-HT1AR agonist, 8-OH-DPAT. In mice, the effect of hypothermia is exclusively mediated by pre-synaptic 5-HT_{1A}R. miR-135 mimetic (duplex 11)-treated mice did not show 8-OH-DPAT-induce hypothermia, while both control groups: mice treated with artificial cerebrospinal fluid (aCSF) or mice treated with the control miR (i.e., naked control), displayed the expected hypothermic response (Figure.5C-F). No difference in baseline temperature was found between the groups (Figure.5G). The kinetics of this effect was demonstrated at 4 different time points (24, 48, 72 and 96 hrs.) following the acute administration (Figure.5C-F)

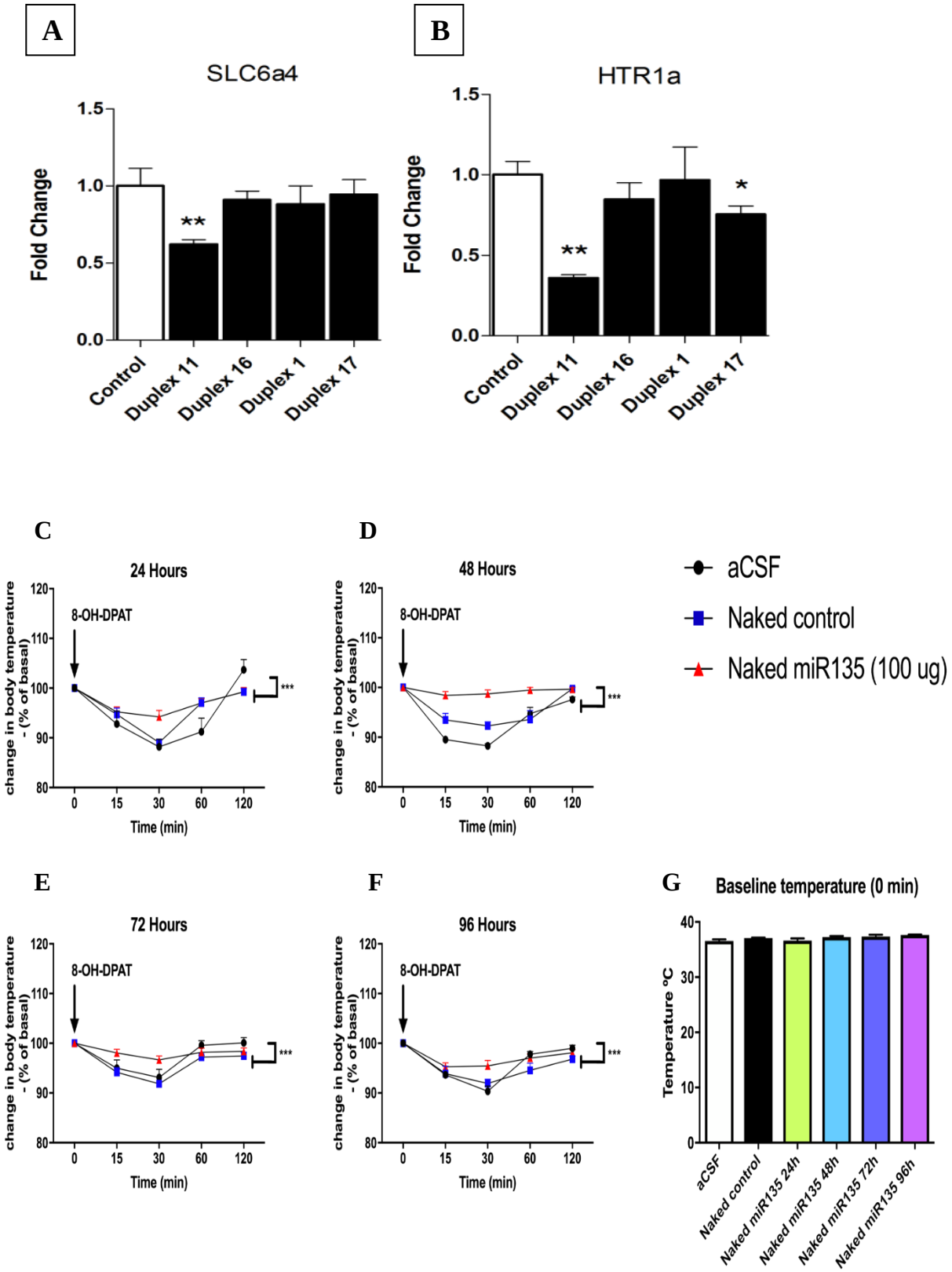


Fig. 5. Validation of miR-135 oligos in-vitro and its effects on the serotonergic function *in-vivo*. Luciferase reporter assay results demonstrating that Duplex 11 targets Slc6a4 3′UTR (A) and Htr1a 3'UTR (B). Systemic 8-OH-DPAT administration (1 mg/kg body weight (BW), i.p.) did not evoke hypothermia in naked (i.e., unconjugated) miR-135-treated (100 µg) mice, 24 (Figure 5C), 48 (D), 72 (E) and 96 (F) hours following treatment. Two-way analysis of variance showed a significant effect ***P < 0.001 versus control groups (n = 5-10). No difference was found in the basal temperature between the groups (G).

Table 5: miR-135 mimics (oligos) tested

Duplex id		oligo id	Oligo sequence	Comments
DUP-001	Guide	OLG-135-001	5'Ph/UAUGGCUUUUUAUUCCUAUGUGa	minimal mod
	Passenger	OLG-135-002	uaUAGGGAUUGGAGCCGUGGCg	minimal mod
DUP-002	Guide	OLG-135-001	5'Ph/UAUGGCUUUUUAUUCCUAUGUGa	minimal mod
	Passenger	OLG-135-003	ucAUAUAGGGAUUGGAGCCGUg	minimal mod and cut overhangs
DUP-003	Guide	OLG-135-001	5'Ph/UAUGGCUUUUUAUUCCUAUGUGa	minimal mod
	Passenger	OLG-135-004	ucACAUAGGAAUAAAAAGCCAUa	minimal mod and complete match
DUP-004	Guide	OLG-135-005	5'Ph/UAUGGCUUUuuAUUCCUAUGUGa	bulge protection
	Passenger	OLG-135-002	uaUAGGGAUUGGAGCCGUGGCg	minimal mod
DUP-005	Guide	OLG-135-001	5'Ph/UAUGGCUUUUUAUUCCUAUGUGa	minimal mod
	Passenger	OLG-135-006	uaUAGGGAUuGGAGCCGUGGCg	bulge protection
DUP-006	Guide	OLG-135-005	5'Ph/UAUGGCUUUuuAUUCCUAUGUGa	bulge protection
	Passenger	OLG-135-006	uaUAGGGAUuGGAGCCGUGGCg	bulge protection
DUP-007	Guide	OLG-135-007	5'Ph/uaUgGCUUUUUAUUCCUAUGUGa	miR seq seed block
	Passenger	OLG-135-002	uaUAGGGAUUGGAGCCGUGGCg	minimal mod
DUP-008	Guide	OLG-135-008	5'Ph/uaAuUUAAGCUUCUUUGUUCUGg	scamble seq seed block

	Passenger	OLG-135-009	cCAGAACAAAGAAGCUUAAAUUa	scramble pass
DUP-009	Guide	OLG-135-010	5'Ph/UAUGGCUUUUCAUUCCUAUGUGa	minimal mod
	Passenger	OLG-135-011	auGUAGGGCUAAAAGCCAUGGg	minimal mod
DUP-010	Guide	OLG-135-010	5'Ph/UAUGGCUUUUCAUUCCUAUGUGa	minimal mod
	Passenger	OLG-135-012	ucAUGUAGGGCUAAAAGCCAUG	minimal mod and cut overhangs
DUP-011	Guide	OLG-135-010	5'Ph/UAUGGCUUUUCAUUCCUAUGUGa	minimal mod
	Passenger	OLG-135-013	ucACAUAGGAAUGAAAAGCCAUa	minimal mod and complete match
DUP-012	Guide	OLG-135-014	5'Ph/UAUGGCUUUUcauUCCUAUGUGa	bulge protection
	Passenger	OLG-135-011	auGUAGGGCUAAAAGCCAUGGg	minimal mod
DUP-013	Guide	OLG-135-010	5'Ph/UAUGGCUUUUCAUUCCUAUGUGa	minimal mod
	Passenger	OLG-135-015	auGUAGGGcuAAAAGCCAUGGg	bulge protection
DUP-014	Guide	OLG-135-014	5'Ph/UAUGGCUUUUcauUCCUAUGUGa	bulge protection
	Passenger	OLG-135-015	auGUAGGGcuAAAAGCCAUGGg	bulge protection
DUP-015	Guide	OLG-135-016	5'Ph/uaUgGCUUUUCAUUCCUAUGUGa	miR seq seed block
	Passenger	OLG-135-011	auGUAGGGCUAAAAGCCAUGGg	minimal mod
DUP-016	Guide	OLG-135-016	5'Ph/uaUgGCUUUUCAUUCCUAUGUGa	miR seq seed block
	Passenger	OLG-135-013	ucACAUAGGAAUGAAAAGCCAUa	minimal mod and complete match
DUP-017	Guide	OLG-135-007	5'Ph/uaUgGCUUUUUAUUCCUAUGUGa	miR seq seed block
	Passenger	OLG-135-004	ucACAUAGGAAUAAAAAGCCAUa	minimal mod

3.2. Effects of conjugated miR-135 mimetic on serotonergic function

Following *in-vitro* and *in-vivo* validation of miR-135 mimetic efficiency, a previously reported approach was used[340]for non-invasive delivery of oligonucleotide to the brain using sertraline-conjugated intranasal (nose-to-brain) administration. Ferrés-Coy et al., (2016, ibid) previously reported that intranasal administration of a sertraline-conjugated siRNA silenced SERT expression and function in mice.

Modified miR-135 mimetic and control oligo were conjugated to a non-functional sertraline (purchased from Nedken Solutions. S.L., Barcelona, Spain) and were administrated acutely into the dorsal raphe nucleus (DRN) of naïve mice. Mice treated with the sertraline-conjugated miR-135 mimetic Duplex 11 (termed miCure-135-1) at a dose of 100 µg, did not show hypothermia response following 8-OH-DPAT administration. This effect lasted up to 7 days following the acute administration (Figure.6A-D).

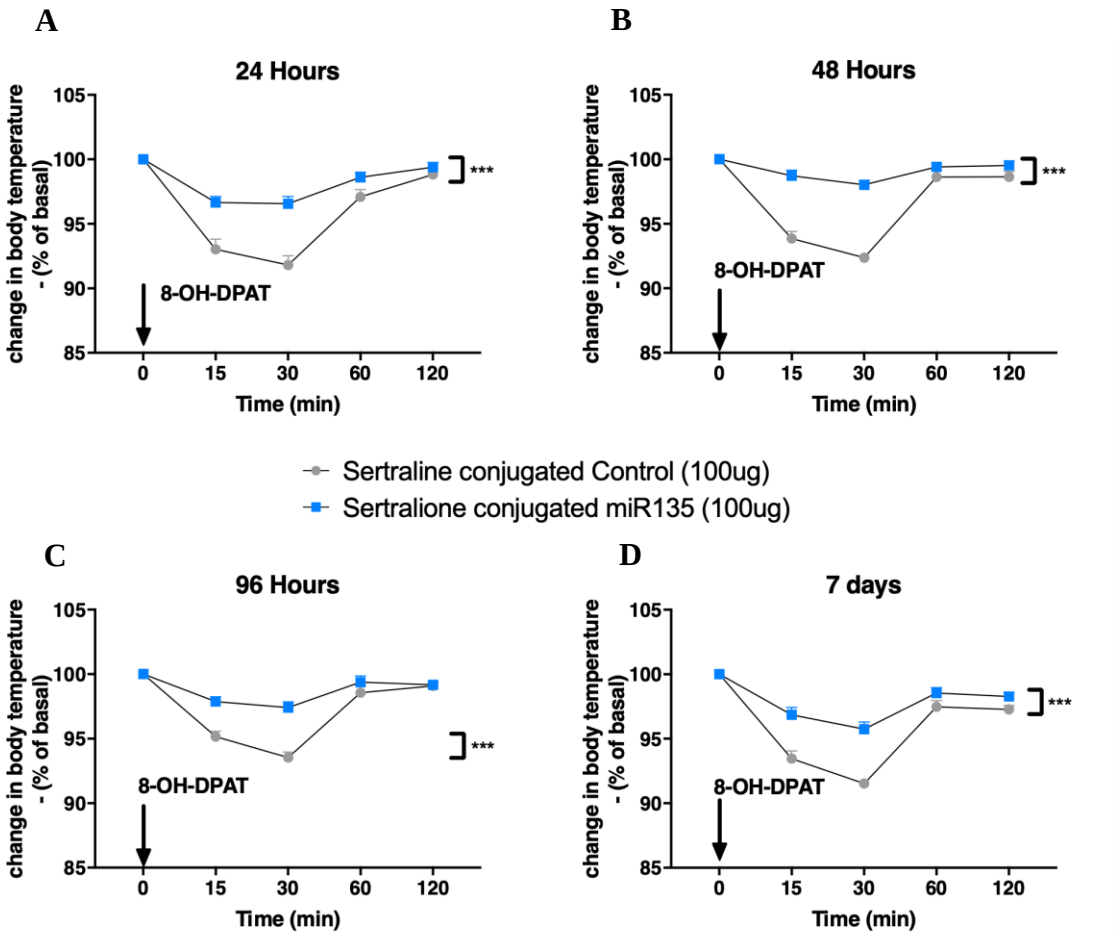


Fig. 6. Sertraline-conjugated miR-135 mimic effects on serotonergic function following direct dorsal raphe nucleus. Intraperitoneal administration of 8-OH-DPAT (1 mg/kg BW) did not induce hypothermia in sertraline-conjugated miR-135-treated (100 µg) mice, 24 hours (A), 48 hours (B), 96 hours (C) and 7 days (D) following treatment. Two-way analysis of variance showed a significant effect ***P < 0.001 versus control groups (n = 10).

3.3. Sertraline-conjugated miR-135 evokes anti-depressant-like responses

To further explore the efficacy of sertraline-conjugated miR-135 in downregulating the expression of genes in the serotonergic system that were previously demonstrated to be directly regulated by miR-135, a lower dose of miCure-135-1 (30 µg) was acutely administrated into DRN of adult mice. First, as was done with the higher dose (100 µg), the physiological consequences of 5-HT$_{1A}$-auto receptor (5-HT$_{1A}$) silencing were examined using the hypothermia response induced by 8-OH-DPAT. The results indicated that a single

administration of miCure-135-1 in a dose of 30 µg was sufficient to abolish the hypothermia induced by selective 5-HT_{1A} agonist, up to 7 days following the treatment and without altering the baseline temperature (Figure.7A-E).

Next, a microdialysis probe was fixated into the medial prefrontal cortex (mPFC) of mice which enabled the collection of CSF in real time and measure subsequently the 5-HT levels using liquid chromatography. Mice were single caged and 15 fractions of 6 minutes each were collected. The 8^{th} fraction was collected during a tail suspension test (a test used for assessing depression-like behavior in mice). The results revealed that mice treated with miCure-135-1 showed a significant reduction in immobility time compared to control group (Figure.7G) which indicates the anti-depressive like effect of sertraline-conjugated miR-135. Remarkably, coincides with the behavioral results, mPFC 5-HT levels of the treated mice were significantly higher (Figure.7F), suggesting a better coping mechanism of sertraline-conjugated miR-135 treated mice. Next, the present inventor evaluated whether sertraline-conjugated miR-135 could silence the SERT gene. Histological examination at 1-4 days post-administration revealed that SERT binding densities were significantly reduced in the dorsal raphe 24 hours and 96 hours post injection (75 % of control; Figure.7H).

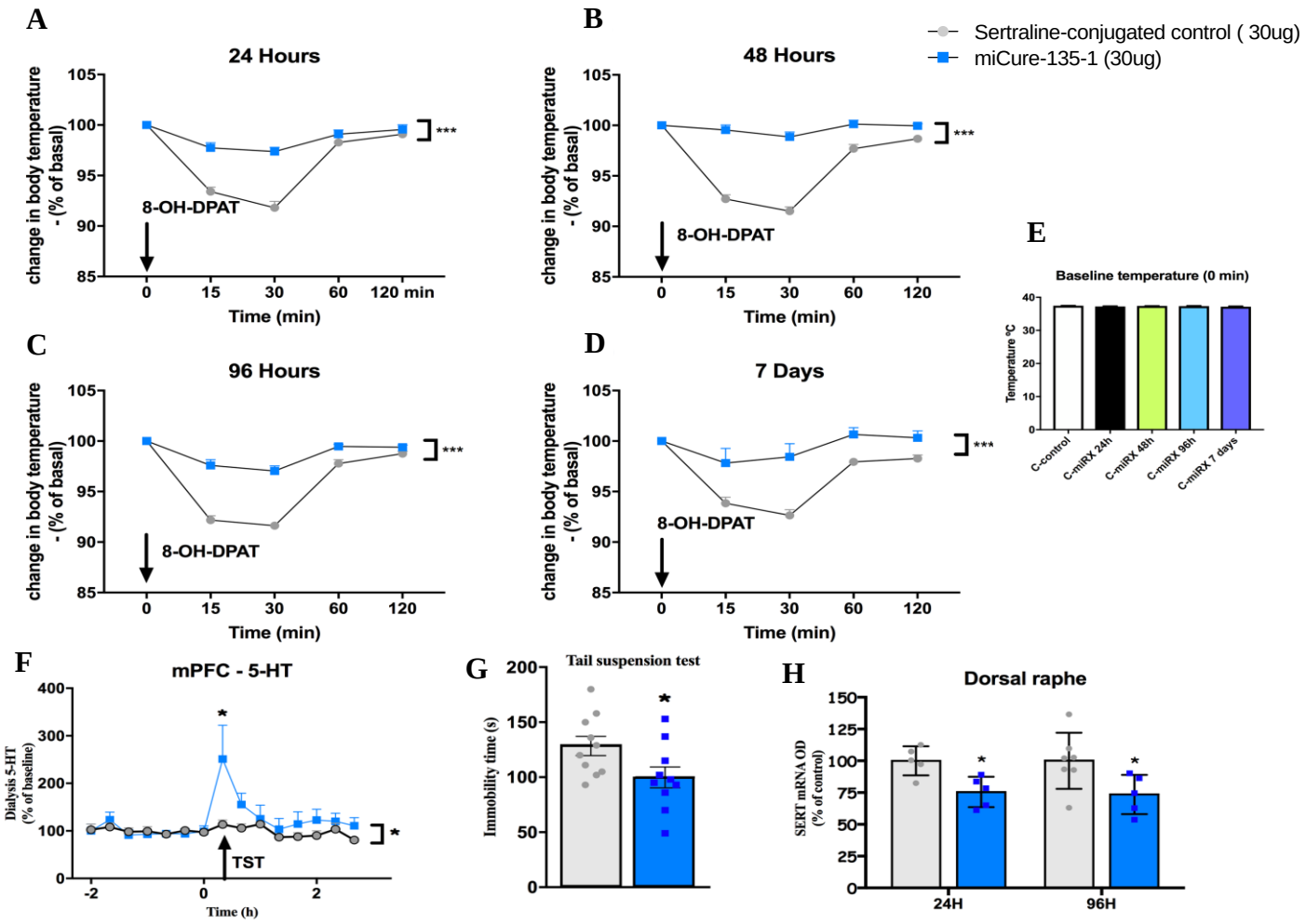


Fig. 7. Acute administration of sertraline-conjugated miR-135 (miCure-135-1) at a lower dose (30 µg) silences 5HT1a and SERT and evokes anti-depressant-like responses. Intraperitoneal administration of 8-OH-DPAT (1 mg/kg BW) did not induce hypothermia in sertraline conjugated miR-135-treated (30 µg) mice one day (A), two days (B), four days (C) and seven days (D) following treatment. Two-way analysis of variance showed a significant effect ***P < 0.001 versus control groups (n = 10). No difference was found in the basal temperature between the groups (E) (n = 10-40). Increased extracellular serotonin was uncovered in medial prefrontal cortex (mPFC) of miCure-135-1 treated mice during the tail suspension test. Significant effect *P < 0.05 versus controls (F). Single intracerebral miCure-135-1 administration (30 µg) induced a reduction in immobility time in the tail suspension test (n = 8-9) (G). Autoradiography of [3H]-citalopram binding showed a reduction of SERT density in the dorsal raphe of treated mice compared to control (n = 5-7) *p < 0.05 (H).

3.4. Effects of intranasal administration of miR-135 mimetic

The potential of miCure-135-1 to serve as an anti-depressant following a non-invasive delivery method was examined using intranasal delivery where anesthetized mice received 5 µl of miCure-135-1 in each nostril and a total of 166 µg. 8-OH-DPAT induced hypothermia

was examined 5 days following treatment and revealed that the miCure-135-1 treated mice group showed scientifically lower hypothermic response (Figure.8A), a physiological consequence of Htr1a silencing. On top of that, an anti-depressive like effect was shown by decreased immobility time at the tail suspension test of miCure-135-1-treated mice compared to controls (Figure.8B). Further testing of the compound via intranasal administration didn't evoke a robust effect not on Htr1a levels nor on behavioral phenotype, even when testing a higher dose (Figure.8C-F)

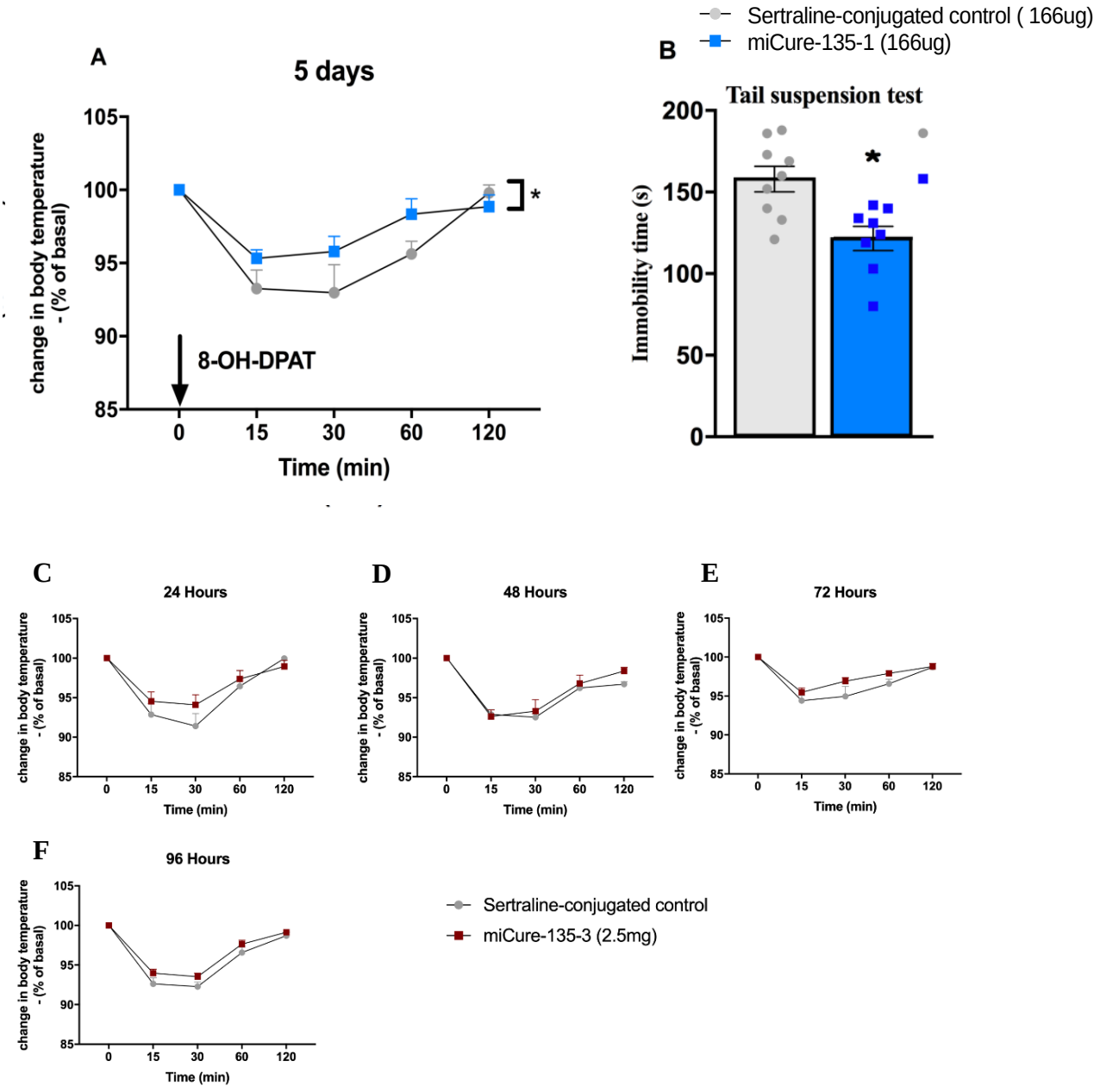


Fig. 8. Acute intranasal administration of miCure-135-1 (166 µg) silences Htr1a and evokes anti-depressant-like responses. Of note, Intraperitoneal administration of 8-OH-DPAT (1 mg/kg BW) did not evoke hypothermia following intranasal delivery of sertraline conjugated miR-135 treated (166 µg) mice 5 days following treatment. Two-way analysis of variance showed a significant effect for groups (n = 5) *P < 0.05 versus control (A). Single intranasal miCure-135-1 administration (166 µg) induced a decreased immobility time in the tail suspension test 4 days following treatment (n = 8-9) *P < 0.05 versus control (B). Intraperitoneal administration of 8-OH-DPAT (1 mg/kg BW) induced hypothermia following intranasal delivery of sertraline conjugated control and sertraline conjugated miR-135 (2.5 mg) (C-F)

3.5. Design, synthesis and validation of advanced chemically modified miR-135 mimetics

Additional miR-135 mimetics (oligos) were designed based on the endogenous miR-135 as described above (see Table.5, above), but comprised advanced chemical modifications aimed to improve stability and reduce innate immune toxicity without leading to reduced potency.

Sixteen different mimics were designed and synthesized using conventional chemistry (as described in the *'Materials and Experimental Procedures'* section above), and each oligo was used as a duplex to improve stability. The sixteen different miR-135 mimetics were screened *in-vitro*, using luciferase assay, for their effects on the validates Htr1a and Slc6a4 target transcripts. For the luciferase assay screening the human cell line HEK293T and the transfection reagent, lipofectamin 2000 (Thermo Fisher Scientific) were used.

miCure-135-1, miCure-135-2, miCure-135-3, miCure-135-9, miCure-135-10 and miCure-135-11 were found to be the most potent miR-135 mimetic oligos, affecting significantly both Htr1a and Slc6a4 levels (using 3'UTR luciferase constructs; Figure.9A-B). The mimetics that were identified to consistently reduce the target transcripts (Htr1a and Slc6a4) across preparations were further used in additional experiments.

Table 6: Chemical medications on miR-135 mimic

Olg-no.	Description	Strand	Previous Name	5' Mod	Sequence (5'-3')
1	miR-135 mimetics Positive Control	Guide	OLG-135-010	P	UAUGGCUUUUCAUUCCUAUGUGa (SEQ ID NO: 10)
2	+ T (5')	Guide		P	UmUAUGGCUUUUCAUUCCUAUGUGa (SEQ ID NO: 41)
3	+ AsA (3')	Guide		P	UmUAUGGCUUUUCAUUCCUAUGUGaAmsAm (SEQ ID NO: 42)
4	alternate ribose modifications	Guide		P	UmUaUgGcUuUuCaUuCcUaUgUgaAmsAm (SEQ ID NO: 43)
5	PS backbone modifications	Guide		P	UmsUsaUsgGscUsuUsuCsaUsusCscsUsasUsgsUsgsasAmsAm (SEQ ID NO: 44)
6	removed ribose mod from seed region (SERT and 5-HT1a)	Guide		P	UmsUsAUsGGsCUsUUsuCsAUsusCscsUsasUsgsUsgsasAmsAm (SEQ ID NO: 45)
7	removed ribose and PS modification from seed region	Guide		P	UmsUAUGGCUUUsuCAUusCscsUsasUsgsUsgsasAmsAm (SEQ ID NO: 46)
8	modified control	Guide	OLG-135-016	P	uaUgGCUUUUCAUUCCUAUGUGa (SEQ ID NO: 16)
9	Original	Passenger	OLG-135-013		ucACAUAGGAAUGAAAAGCCAUa (SEQ ID NO: 13)
10	minor PS at backbone	Passenger			uscsACAUAGGAAUGAAAAGCCAsUsa (SEQ ID NO: 47)

Table 6 depicts the additional miR-135 modifications carried out. The modifications are as follows:

High case letters (e.g., A, U, C, G): RNA

Lower-case letters (e.g., a, u, c, g): 2'-O-Me modification

Um: (2´-O-MOE-5´-Me) U modification

Am: (2'-O-MOE) A modification

Lower-case 's': phosphorothioate. No indication means a normal phosphodiester bond.

P: phosphate

Green: 2'-fluoro, i.e., 2'-F

Table 7: Additional miR-135 mimics (oligos) tested

Sertraline-Conjugated Duplex No.	Guide	Passenger	Remarks
miCure-135-1	Olg-1	Olg-9	Original Positive (Previously designated Duplex 11, per Table 5, above)
miCure-135-2	Olg-2	Olg-9	
miCure-135-3	Olg-3	Olg-9	
miCure-135-4	Olg-4	Olg-9	
miCure-135-5	Olg-5	Olg-9	
miCure-135-6	Olg-6	Olg-9	
miCure-135-7	Olg-7	Olg-9	
miCure-135-8	Olg-8	Olg-9	Original Negative (Previously designated Duplex 16, per Table 5, above)
miCure-135-9	Olg-1	Olg-10	Original guide
miCure-135-10	Olg-2	Olg-10	
miCure-135-11	Olg-3	Olg-10	
miCure-135-12	Olg-4	Olg-10	
miCure-135-13	Olg-5	Olg-10	
miCure-135-14	Olg-6	Olg-10	
miCure-135-15	Olg-7	Olg-10	
miCure-135-16	Olg-8	Olg-10	Original negative guide

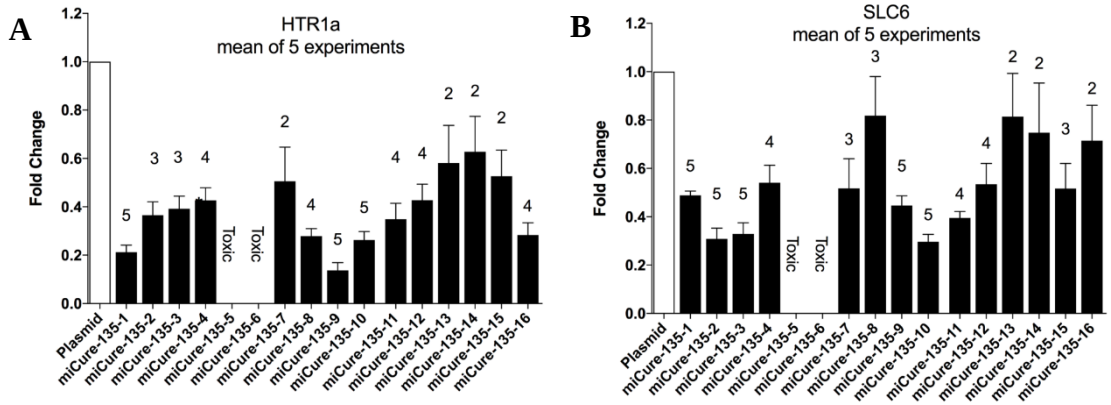


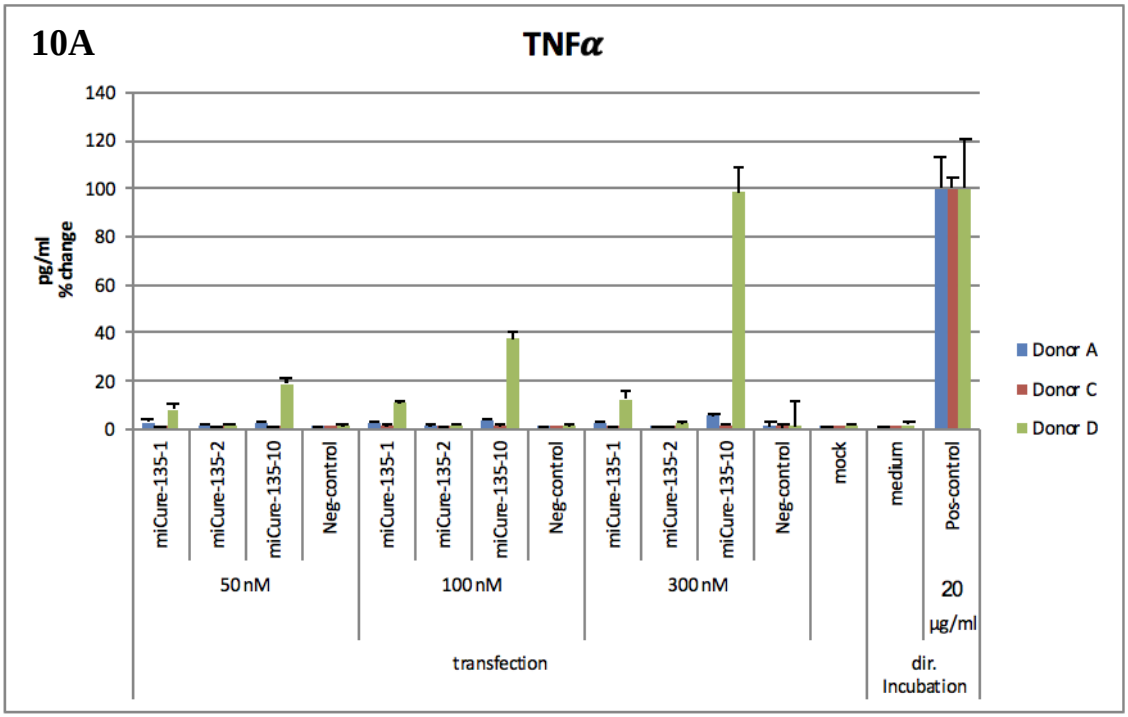
Fig. 9. Validation of advanced chemically modified miR-135 mimetic oligos *in-vitro*. Luciferase reporter assay demonstrating that miCure-135-1, miCure-135-2, miCure-135-3, miCure-135-9, miCure-135-10 and miCure-135-11 significantly target Slc6a4 3′UTR (B) and Htr1a 3'UTR (A). The numbers above the bars indicate the number of significant statistical differences found (out of 5 experiments).

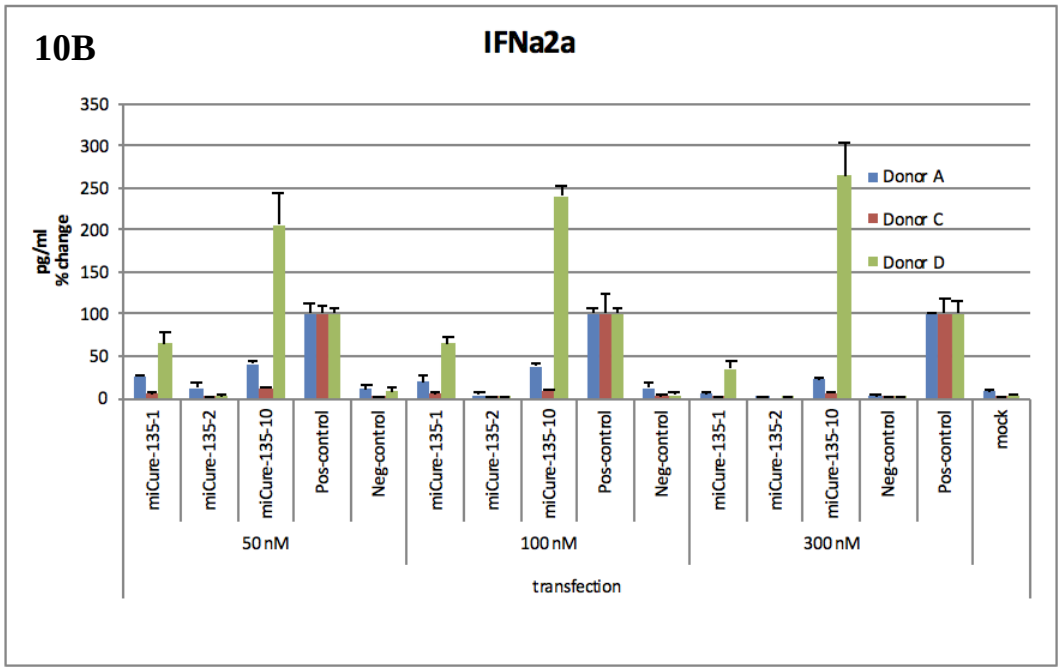
3.6. Advanced chemical modifications changed the pattern of cytokine secretion

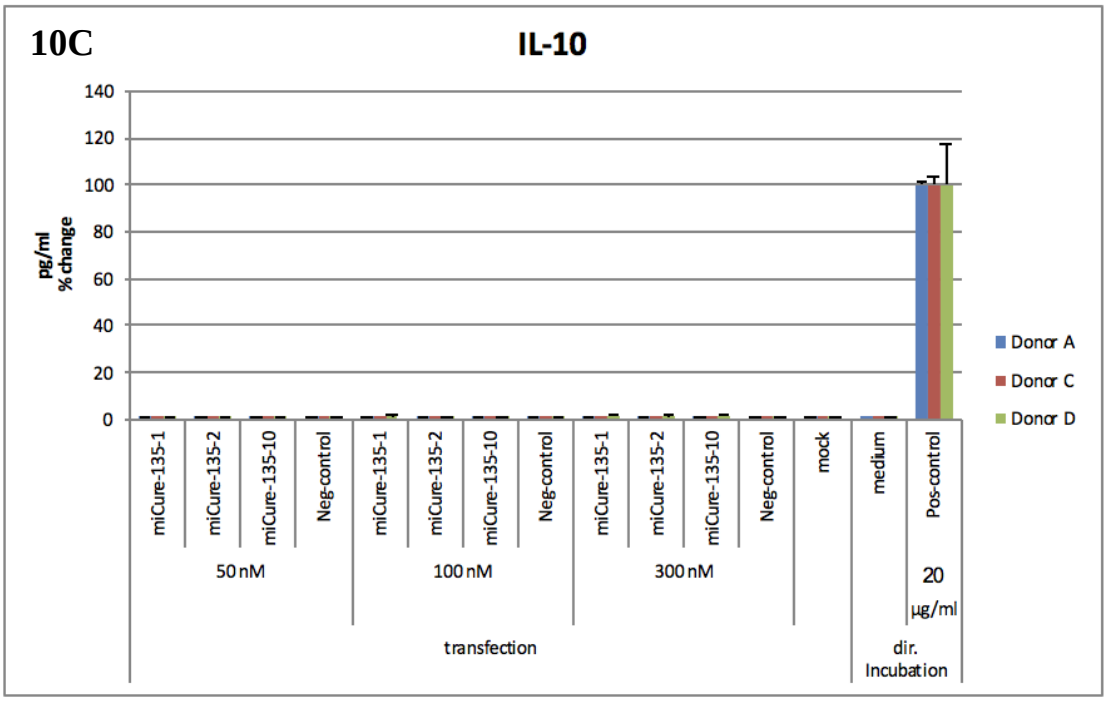
Oligonucleotides that the body does not recognize as self, typically induce an immune response through the release of pro-inflammatory cytokines. Chemical modifications of oligonucleotides are aimed at increasing potency and have the potential to reduce innate immune activation. In order to test the pattern of immune activation induced by the new modifications, human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation starting from fresh buffy coats of healthy volunteers (obtained from a blood donation center, as described in the *'Materials and Experimental Procedures'* section above). PMBCs were treated with different mimetics for 24 hours and the supernatant was analyzed for the presence of cytokines using multiplex assays run on the Meso Scale Discovery (MSD).

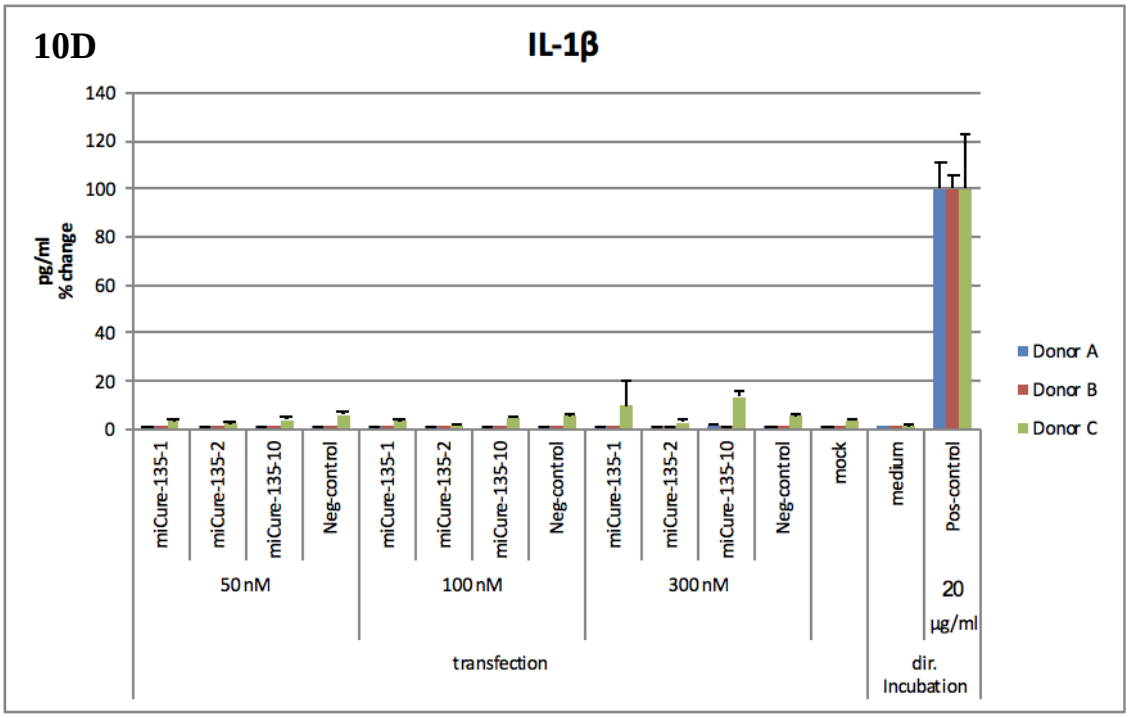
The results of this assay illustrated that 2 of the new duplexes, miCure-135-2 and miCure-135-3 did not induce secretion of the tested cytokines (Figure.10A-E and Figure.11A-F, respectively), miR-135-1 showed a modest activation of TNF-alpha (Figure.10A) and IFN-alpha-2a (Figure.10B), miR-135-9 induced high secretion of IFN-alpha-2a (Figure.11A) and low secretion TNF-alpha (Figure .11B), miR-135-11 induced

high secretion of IFN-alpha-2a (Figure.11A), and low secretion of TNF-alpha (Figure.11B) and IFN-gamma (Figure.11C). MiCure-135-10 results revealed a very high secretion and TNF-alpha and IFN-alpha-2a (Figure.10A and Figure.10B, respectively). The duplexes that were identified to induce the lowest level of secretion (i.e. miCure-135-2 and miCure-135-3) were further tested *in-vivo*. It is possible that the relatively high cytokine secretion induced by miCure-135-9, miCure-135-10 and miCure-135-11 relate to the shared passenger strand that consists of two phosphorothioate bonds replacing the normal phosphodiesters at the 5' end and one at the 3' end, this is tested.




10B
IFNa2a
pg/ml
% change
350
300
250
200
150
100
50
0
Donor A
Donor C
Donor D
miCure-135-1
miCure-135-2
miCure-135-10
Pos-control
Neg-control
mock
50 nM
100 nM
300 nM
transfection


10C
IL-10
pg/ml
% change
140
120
100
80
60
40
20
0
Donor A
Donor C
Donor D
miCure-135-1
miCure-135-2
miCure-135-10
Neg-control
mock
medium
Pos-control
50 nM
100 nM
300 nM
20
µg/ml
transfection
dir.
Incubation



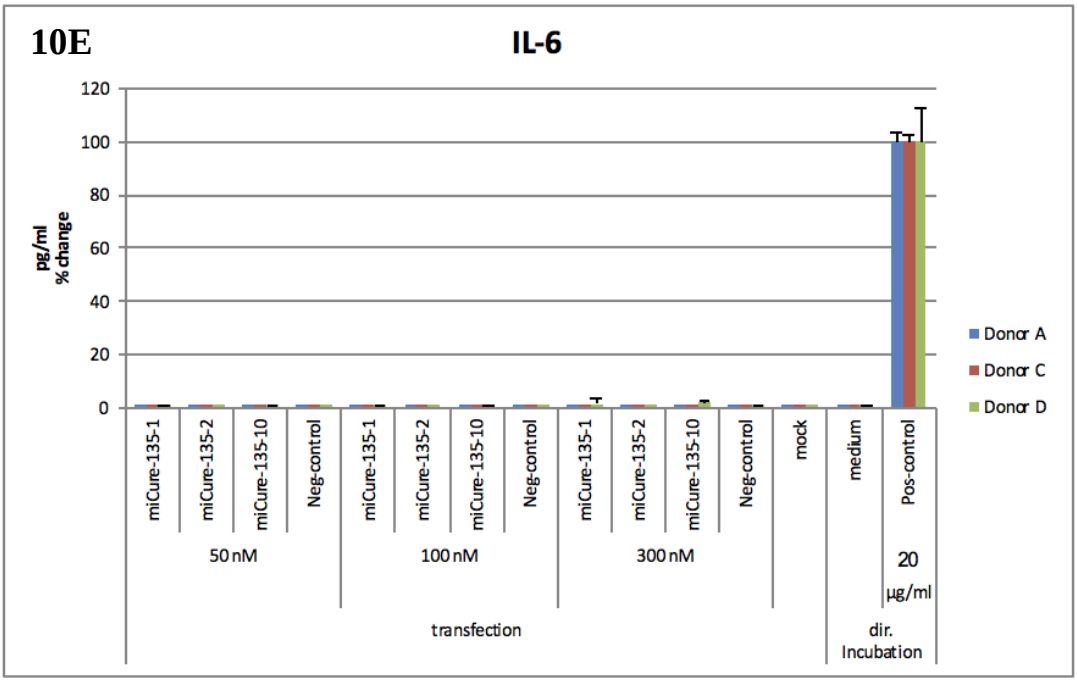
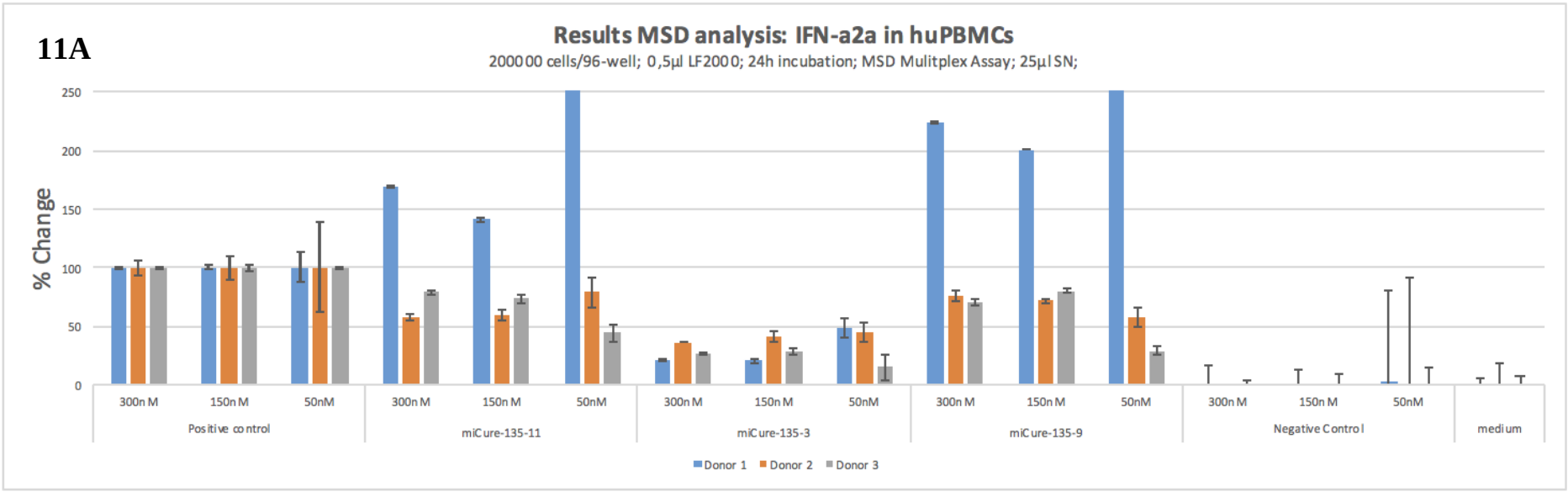
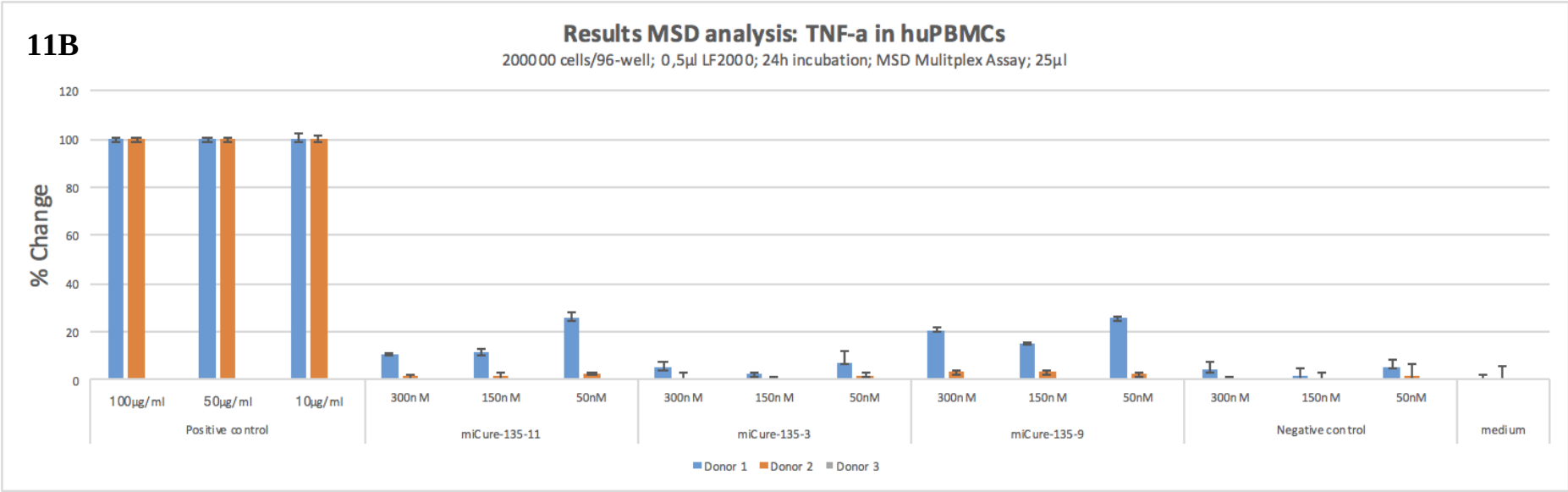


Fig. 10. The immune response of human peripheral blood mononuclear cell following treatment with miR-135 conjugated duplexes in-vitro. Of note, miCure-135-10 induced high secretion of TNF-alpha cytokine especially at the concentration of 300 nM, while miCure-135-1 moderately activated this cytokine and miCure-135-2 induced almost no secretion (A). The same pattern of secretion was demonstrated for IFN-alpha-2a cytokine (B), where miCure-135-10 induced a high level of secretion, miCure-135-1 induced moderate levels of secretion and miCure-135-2 no secretion. None of the tested duplexes induced secretion of IL-10 (C), IL-1beta (D) nor IL-6 (E). In Figure. A, C-E: dir. incubation refers to direct incubation.

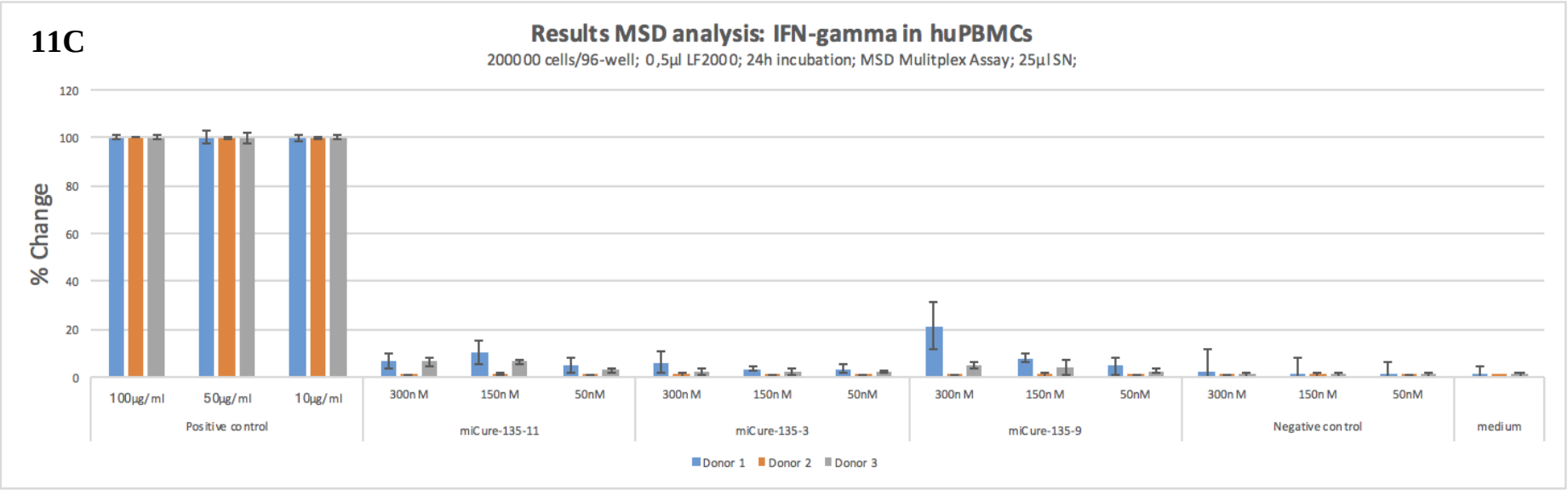
11A


Results MSD analysis: IFN-a2a in huPBMCs
200000 cells/96-well; 0,5µl LF2000; 24h incubation; MSD Mulitplex Assay; 25µl SN;
% Change
250
200
150
100
50
0
300n M
150n M
50nM
Positive control
miCure-135-11
miCure-135-3
miCure-135-9
Negative Control
medium
Donor 1
Donor 2
Donor 3

11B


Results MSD analysis: TNF-a in huPBMCs
200000 cells/96-well; 0,5µl LF2000; 24h incubation; MSD Mulitplex Assay; 25µl
% Change
120
100
80
60
40
20
0
100µg/ml
50µg/ml
10µg/ml
Positive control
300n M
150n M
50nM
miCure-135-11
miCure-135-3
miCure-135-9
Negative control
medium
Donor 1
Donor 2
Donor 3

11C


Results MSD analysis: IFN-gamma in huPBMCs
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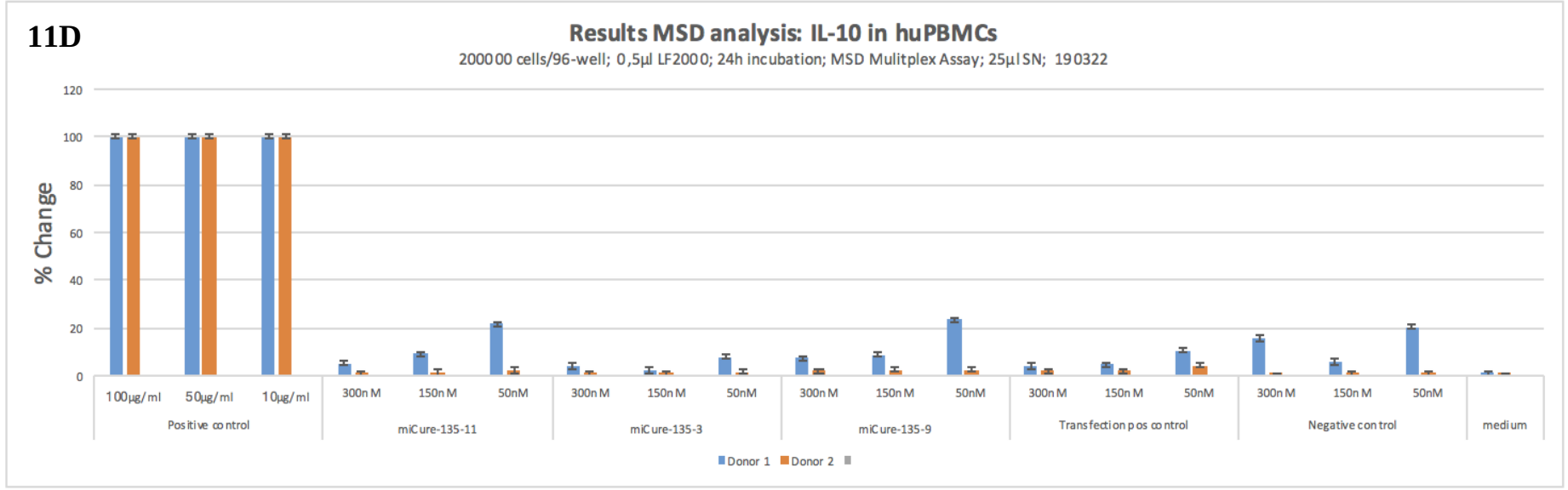
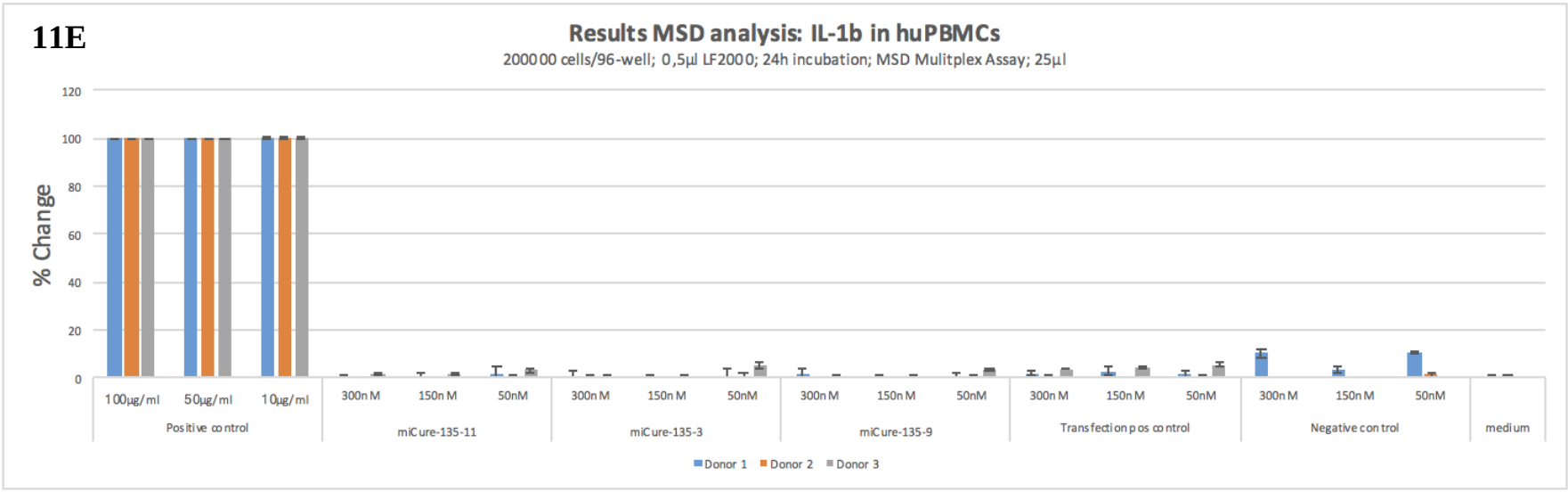
% Change
120
100
80
60
40
20
0
100µg/ml
50µg/ml
10µg/ml
Positive control
300n M
150n M
50nM
miCure-135-11
miCure-135-3
miCure-135-9
Negative control
medium
Donor 1
Donor 2
Donor 3

11D


Results MSD analysis: IL-10 in huPBMCs
200000 cells/96-well; 0,5µl LF2000; 24h incubation; MSD Mulitplex Assay; 25µl SN; 190322
% Change
120
100
80
60
40
20
0
100µg/ml
50µg/ml
10µg/ml
Positive control
300n M
150n M
50nM
miCure-135-11
miCure-135-3
miCure-135-9
Transfection pos control
Negative control
medium
Donor 1
Donor 2



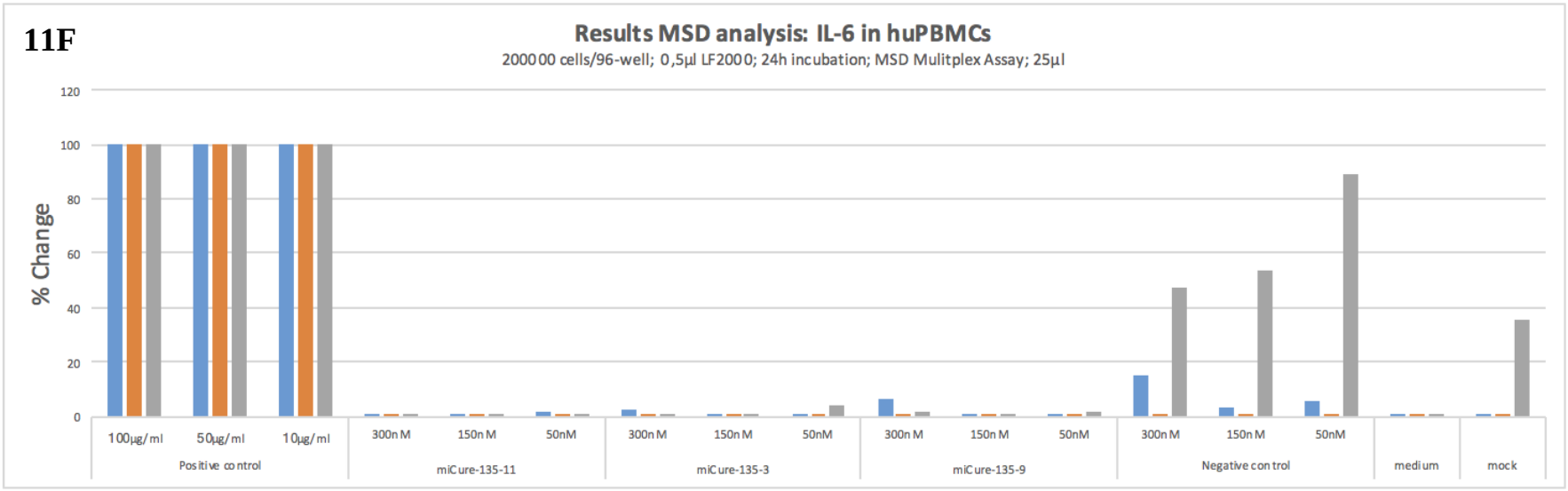
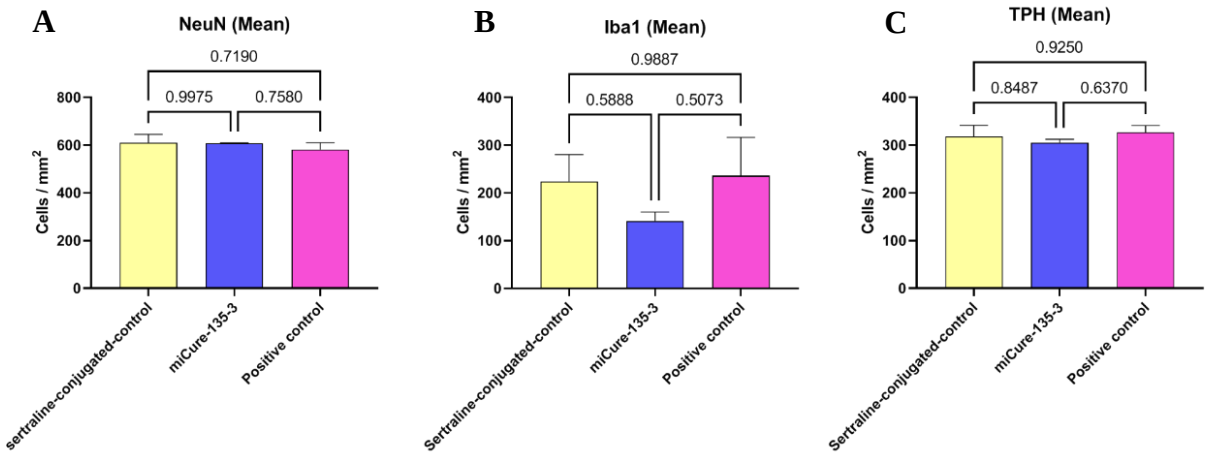


Fig. 11. The immune response of human peripheral blood mononuclear cells (PBMCs) following treatment with an additional set of 3 miR-135 conjugated duplexes in-vitro. Of note, miR-135-11 and miCure-135-9 induced high secretion of IFN-alpha-2a while miCure-135-3 induced almost no secretion thereof (A). A low level of TNF-alpha secretion was induced by miCure-135-11 and miCure-135-9 where no secretion was induced by miCure-135-3 (B). miCure-135-9 at a 300 nM concentration showed a low level of secretion of IFN-gamma, whereas miCure-135-3 and miCure-135-9 did not show secretion thereof (C). Neither of the 3 tested duplexes lead to a noticeable production of cytokine IL-10 (D), IL-1b (E), nor IL-6 (F).

3.7. miR-135 mimetic has no effect on cellular viability in the brain

To further examine the safety of miR-135 mimetics, mice received a single direct injection directly into the dorsal raphe nucleus of a conjugate-control, miCure-135-3 or a positive control that was reported previously to have no effect on cellular viability (Conjugated-sertraline-siRNA against SERT as taught previously[341]). Sections of the midbrain raphe nuclei were stained with neuronal (NeuN-positive), serotonergic neurons (TPH-positive) or microglial (Iba-1-positive) markers. Immunohistochemistry analysis demonstrated that miCure-135-3 did not induce neuronal degeneration (Figure.12A), serotonergic neuron degeneration (Figure.12C) nor immune responses (Figure.12B). These can also be seen in the representative images of midbrain raphe nuclei stained with NeuN, Iba1 and TPH under the different treatments (Figure.12D). This data supports the specificity and safety of miCure-135-3.



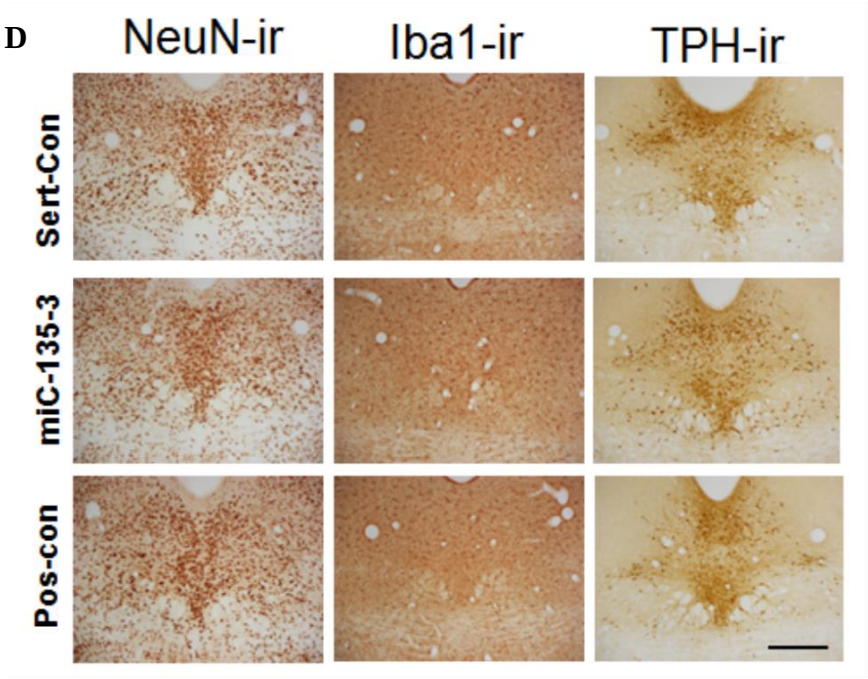


Fig. 12. Immunohistochemical assessment of cellular viability in the raphe nuclei after acute administration of miCure-135-3 (100 µg). Conjugate-control, miCure-135-3 or a positive control that was reported previously to have no effect on cellular viability were acutely delivered directly into the dorsal raphe nucleus. Sections (30-µm-thick) through the raphe nuclei were stained with (i)neuronal NeuN (A), (ii)microglial Iba1 (B), or (iii) serotonergic TPH (C) markers. Representative images of midbrain raphe nuclei stained with NeuN, Iba1 and TPH under the different treatments are represented in 'D'. Of note, no differences were found between all experimental groups for any of the markers. Representative pictures are depicted in 'D'. Scale bar: 100 µm.

3.8. Acute DRN of sertraline-conjugated miR-135 mimic affects serotonergic function

To further examine the effect of miR-135 mimetics on the serotonergic system *in-vivo*, miR-135 mimic conjugated to a non-functional sertraline was delivered directly into the DRN using stereotactic surgery.

The synthetic miRNA was administrated acutely (30 µg) and the physiological effect of silencing the 5-HT1A auto-receptor, which manifests as a response of hypothermia, was explored by administering the selective 5-HT1AR agonist, 8-OH-DPAT. In mice, the effect of hypothermia is exclusively mediated by pre-synaptic 5-HT_{1A}R. As illustrated in Figure.13A-C, sertraline-conjugated miR-135-treated mice did not show hypothermia, while

both control groups, i.e., mice treated with artificial cerebrospinal fluid (aCSF) and mice treated with the control miR, displayed the expected hypothermic response. No difference in baseline temperature was observed between the groups (Figure.13E). The kinetics of this effect were demonstrated at three different time points following the acute administration.

Next, the miCure-135-3 and sertraline conjugated control were administrated acutely (100 µg) into the mice DRN, and the functional consequences of serotonin transporter (SERT) silencing were examined. To that end, a microdialysis probe was fixated into the medial prefrontal cortex (mPFC) of mice which enabled the collection of CSF in real time and the subsequent measure of 5-HT levels using liquid chromatography. Mice were single caged and 12 fractions of 20 minutes each were collected. Starting from the 7th fraction a local selective serotonin reuptake inhibitor (Citalopram 10 µM) was infused by reverse-dialysis and resulted in an increase of extracellular 5-hydroxytryptamine (5-HT). The increase of the 5-HT levels in the miCure-135-3-treated mice was significantly lower compared with the levels in the control group (Figure.13F) suggesting that miCure-135-3 lead to a decrease in the available serotonin transporter. The same experimental group, i.e., mice that were injected once to the DRN with 100 µg of miCure-135-3 or conjugated control were tested in the tail suspension test. The results revealed that mice treated with miCure-135-3 showed a significant reduction in immobility time compered to control group (Figure.13G) which indicates the anti-depressive like effect of sertraline-conjugated miR-135 mimetics (e.g., miCure-135-3). The intracerebroventricular (ICV) delivery method was aimed to model intrathecal administration as in both cases the drug is administrated directly into the CSF. In this experiment, a subcutaneous osmotic mini-pump was connected to a cannula that was placed in the mice brain ventricle. miCure-135-3 was delivered constantly to the 2nd brain ventricle in a rate of 200 µg/day for 7 days. A lower reduction in body temperature was observed in the miCure-135-2 treated group compared to control (Figure 13D).

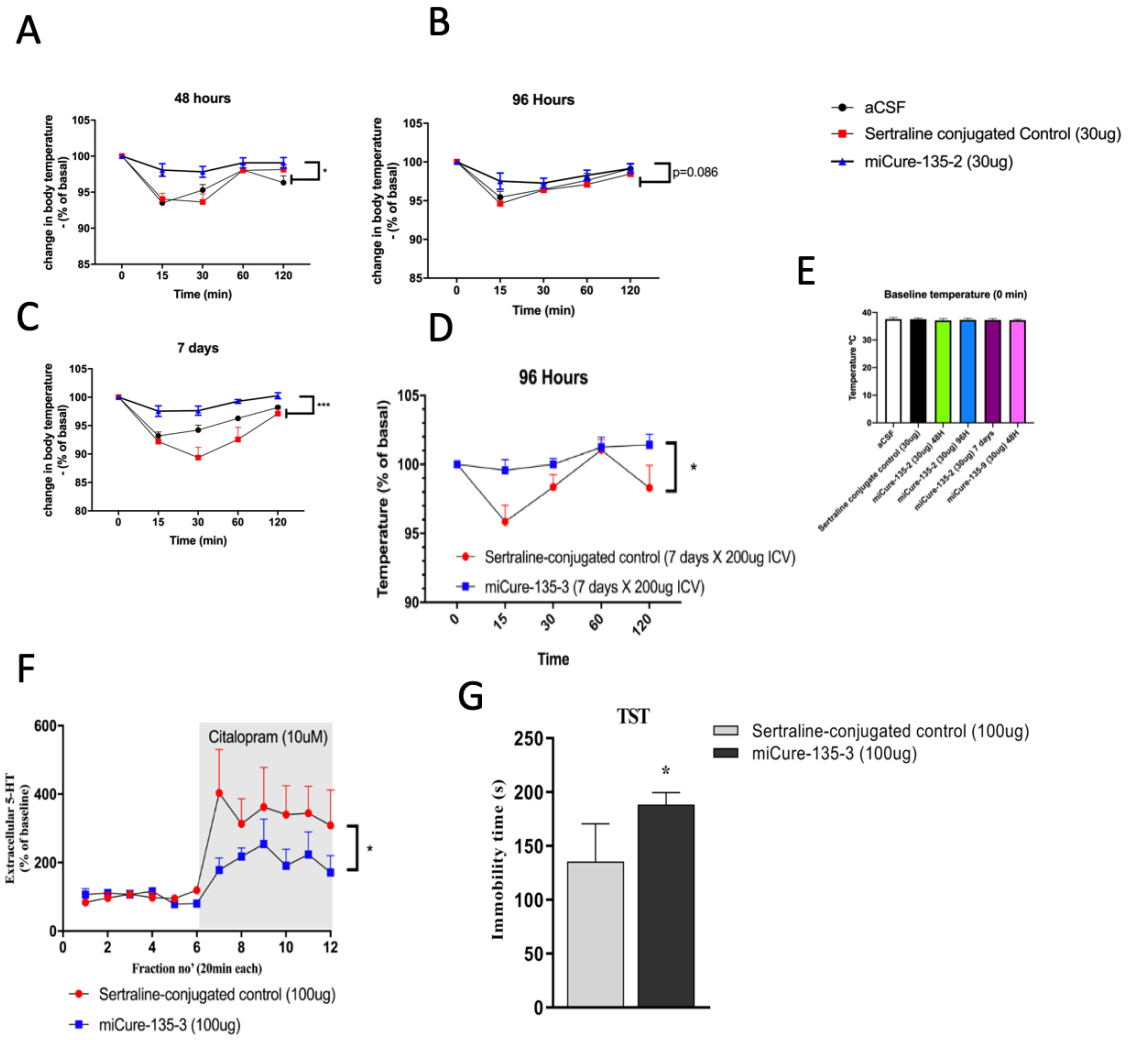


Fig. 13. The effect of the sertraline conjugated miR-135 mimics on serotonergic function and their anti-depressant-like effect. Following intraperitoneal administration of 8-OH-DPAT (1 mg/kg BW) did not induce hypothermia in treated (30 µg) mice two days (A), four days (B) and seven days (C) days following treatment. The same effect was illustrated following 7 days of ICV administration of miCure-135-3 (200 µg/day) 96 hours following treatment (D). Two-way analysis of variance showed a significant effect Two-way analysis of variance showed a significant effect * P<0.05, ***P < 0.001 versus control groups (n = 4-5). No difference was found in the basal temperature between the groups (E) (n=4-19). Local Citalopram (10 µM) infusion by reverse-dialysis lead to an increase of extracellular 5-HT in the PFC of sertraline conjugated control (100 µg) treated mice as compared to miCure-135-3 (100 µg) treated mice (F). Two-way analysis of variance showed a significant effect *P < 0.05 versus control groups (n = 6). Single intracerebral miCure-135-3 administration (100 µg) induced a reduction in immobility time in the tail suspension test (n = 6) *p < 0.05 (G).

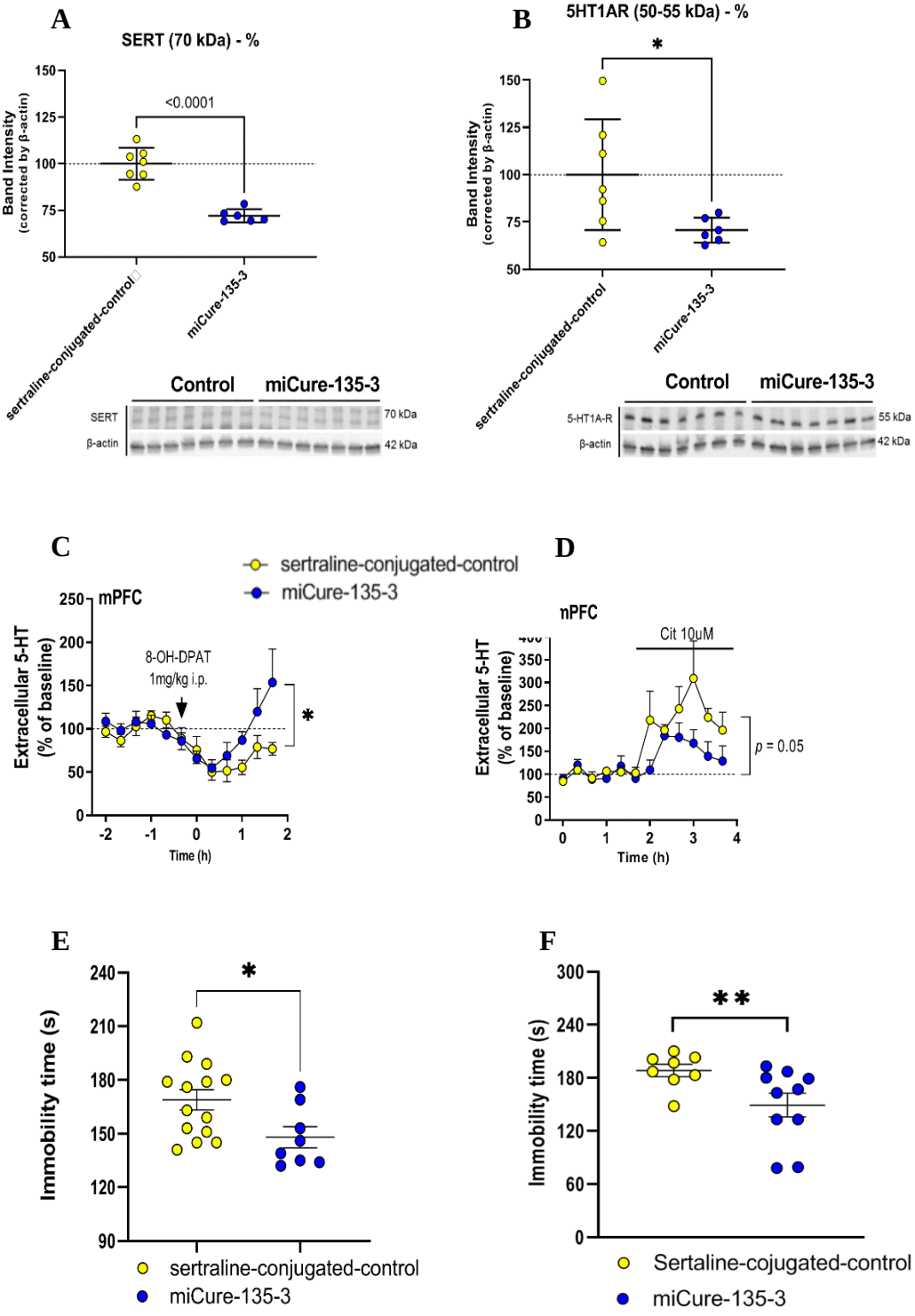
3.9. Intranasal administration of miCure-135-3 affects the serotonergic function

To further explore the effects of sertraline-conjugated miR-135 mimetics that were administrated in a non-invasive fashion, on the serotonergic functions, miCure-135-3 was delivered to wildtype mice using an intranasal route of administration. In order to evaluate whether sertraline-conjugated miR-135 could reduce the protein levels of SERT and the 5-HT_{1A}, mice were treated acutely with miCure-135-3 and immunoblotting analysis of samples taken 3 days post-administration revealed that SERT and Htr1a protein levels were significantly reduced in the dorsal raphe 72 hours post injection (75 % of control for both proteins; Figure.14A-B).

Next, to examine the effect of the sertraline-conjugated miR-135 mimetics on the levels of serotonin transporter (SERT), a microdialysis probe was fixated into the medial prefrontal cortex (mPFC) of mice which enabled the collection of CSF in real time and subsequent measure of the 5-HT levels using liquid chromatography. 48 hours post administration, mice were single caged and 12 fractions of 20 minutes each were collected. Starting from the 7^{th} fraction a local selective serotonin reuptake inhibitor (Citalopram 10 μM) was infused by reverse-dialysis and resulted in increase of extracellular 5-hydroxytryptamine (5-HT). The increase of the 5-HT levels in the miCure-135-3-treated groups was significantly lower compared with the levels in the control group (Figure.14D) suggesting that the intranasal administration of sertraline-conjugated miR-135 mimetics (e.g., miCure-135-3) decreases the serotonin transporter in the dorsal raphe nucleus.

Using the same probes that were fixated into the mPFC, the same group of mice underwent an additional microdialysis experiment aimed to examine the effect of acute intranasal administration of miCure-135-3 on the levels of Htr1a. 72 hours post administration, CSF was collected in real time in order to measure the 5-HT levels using liquid chromatography. Mice were single caged and 12 fractions of 20 minutes each were

collected. On the 6th fraction an i.p. injection of a selective 5-HT$_{1A}$ agonist (8-OH-DPAT 1mg kg^{-1},i.p.) resulted in a decrease of extracellular 5-hydroxytryptamine (5-HT). The decrease of the 5-HT levels in the miCure-135-3-treated groups was significantly shorter compared with the levels in the control group (Figure.14C) suggesting that the intranasal administration of sertraline-conjugated miR-135 mimetics (e.g., miCure-135-3) decreases the 5-HT$_{1A}$ in the dorsal raphe nucleus. The same experimental group, i.e. mice that were administrated once intranasally with 2500 µg of miCure-135-3 or with a conjugated control were tested in the tail suspension test. The results revealed that mice treated with miCure-135-3 showed a significant reduction in immobility time compered to control group (Figure.14E) which indicates the anti-depressive like effect of sertraline-conjugated miR-135 mimetics (e.g., miCure-135-3). The potential of sertraline-conjugated miR-135 mimetics to serve as anti-depressant agents was demonstrated using a depression-like mice model. Mice were treated with corticosterone in their drinking water and were subjected to restrained stress for 2 hours every day for 28 days. Mice subjected to the paradigm displayed increased depression-like behavior. Following this protocol mice were treated with one dose of miCure-135-3 (2500 µg). An anti-depressive like effect was shown 3 days following the treatment by decreased immobility time at the tail suspension test of miCure-135-3-treated mice compared to controls (Figure.14F).


A
SERT (70 kDa) - %
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Band Intensity (corrected by β-actin)
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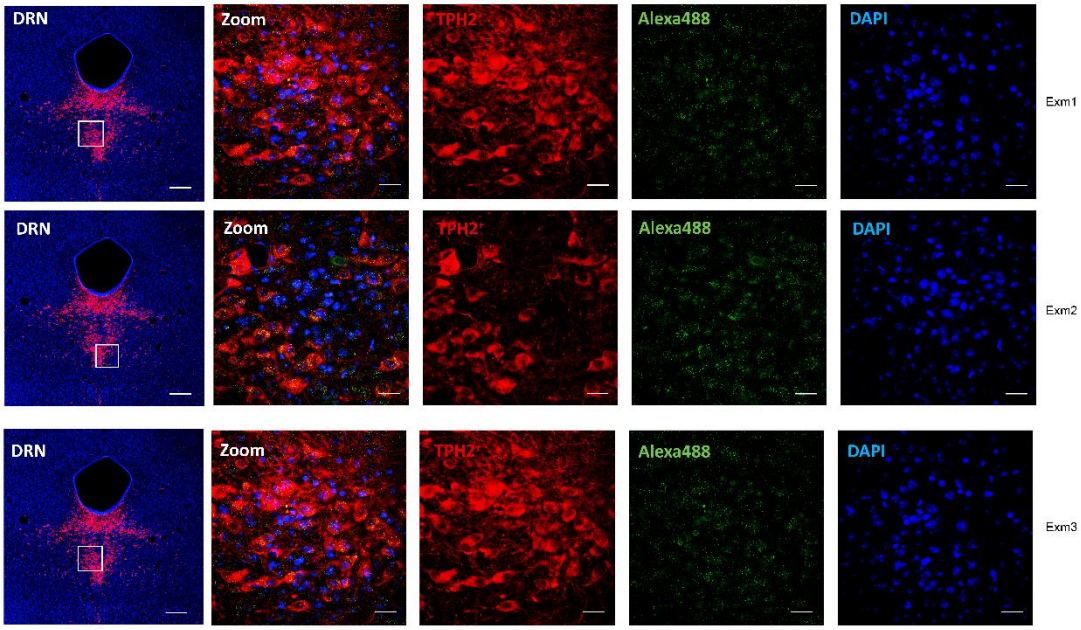
miCure-135-3
Control
miCure-135-3
SERT
β-actin
70 kDa
42 kDa
B
5HT1AR (50-55 kDa) - %
*
Band Intensity (corrected by β-actin)
sertraline-conjugated-control
miCure-135-3
Control
miCure-135-3
5-HT1A-R
β-actin
55 kDa
42 kDa
C
sertraline-conjugated-control
miCure-135-3
mPFC
8-OH-DPAT 1mg/kg i.p.
Extracellular 5-HT (% of baseline)
Time (h)
*
D
nPFC
Cit 10uM
Extracellular 5-HT (% of baseline)
p = 0.05
Time (h)
E
*
Immobility time (s)
sertraline-conjugated-control
miCure-135-3
F
**
Immobility time (s)
Sertaline-cojugated-control
miCure-135-3

Fig. 14. Acute intranasal administration of miCure-135-3 (2500 µg) effects the serotonergic function and evokes anti-depressant-like responses. Immunoblot image and quantification demonstrate the reduction in the protein levels of SERT (A) and 5-HT1A (B) in the dorsal raphe of treated mice compared to control (n = 6-7) *p < 0.05; Reduced extracellular serotonin in mPFC of controls, but not in treated mice after 8-OH-DPAT administration (1mg kg-1, i.p.) (n = 5–6), significant effect of group (P < 0.05) versus controls (C); Local selective serotonin reuptake inhibitor (Citalopram 10 µM) infusion by reverse-dialysis led to an increase of extracellular 5-hydroxytryptamine (5-HT) in the PFC of sertraline conjugated control (2500 µg) treated mice as compared to miCure-135-3 (2500 µg) treated mice (D); Two-way analysis of variance showed a P value of 0.05 versus control groups (n = 6). Single intranasal miCure-135-3 administration (2500 µg) evoked a reduction in immobility time in the tail suspension test (n = 8 -14) *p < 0.05 (E). Mice underwent a 28-day protocol that induces a depression-like behavior (see '*Materials and Experimental Procedures*' section) followed by a single intranasal administration of control or miCure-135-3. The results demonstrate that a single intranasal miCure-135-3 administration (2500 µg) evoked a reduction in immobility time in the tail suspension test 3 days following treatment (n = 8-10) *P < 0.05 versus control (F).

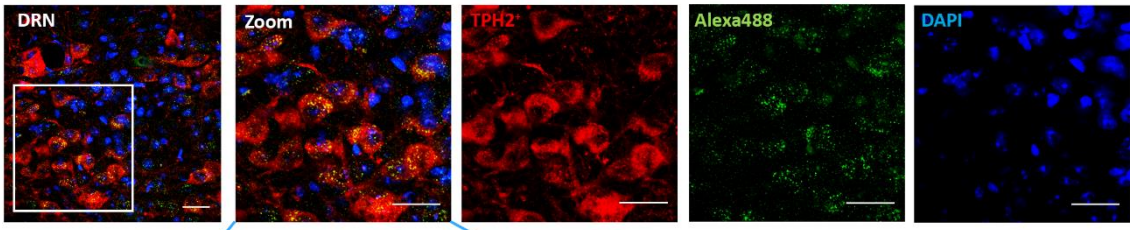
3.10. Selective accumulation of miR-135 mimetics following intranasal administration

To examine the brain distribution of miR-135 mimetics following intranasal administration, an alexa488-labeled miCure-135-3 was synthetized. The labeled molecule was delivered to mice using intranasal administration and 6 hours following administration brain tissue was collected. A Confocal fluorescence microscopy revealed that alexa488-labeled miCure-135-3 was located inside TPH2-positive 5-HT neurons in the midbrain of mice following signle intranasal administration (Figure.15A-C). Confocal analysis showed that alexa488-labeled miCure-135-3 was not found in brain areas adjacent to the application site (olfactory bulb) or to brain ventricles (hippocampus and striatum) (Figure.15D), supporting the hypothesis that the expression of surface SERT is necessary for the uptake and internalization of the oligonucleotide and that miCure-135-3 is accumulated selectively in the DRN.

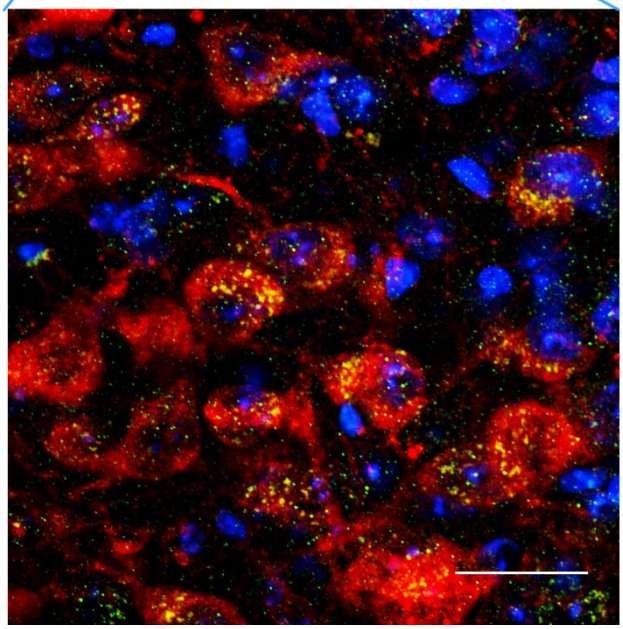
A


DRN
Zoom
TPH2
Alexa488
DAPI
Exm1
DRN
Zoom
TPH2
Alexa488
DAPI
Exm2
DRN
Zoom
TPH2
Alexa488
DAPI
Exm3

B


DRN
Zoom
TPH2
Alexa488
DAPI

C



D

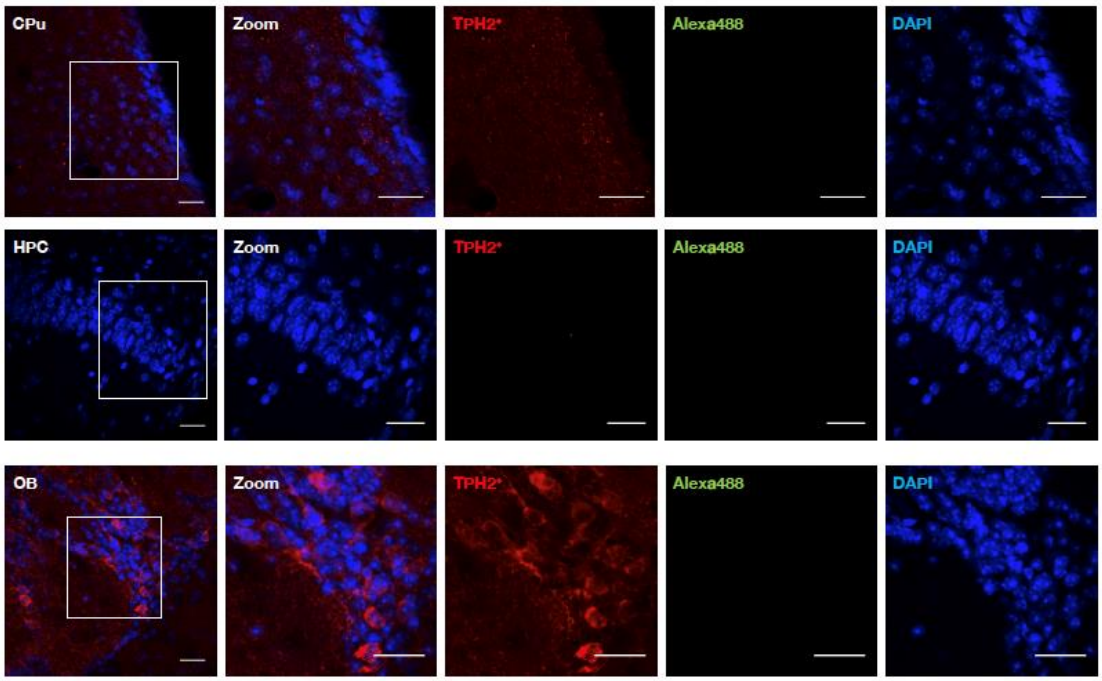


Fig. 15. Localization of miCure-135-3 in the dorsal raphe nucleus following intranasal administration. Selective accumulation of alexa488-labeled miCure-135-3 (green) in tryptophan hydroxylase2-positive (TPH2-positive) neurons following intranasal administration (1000 µg). Confocal images show co-localization of alexa488-labeled miCure-135-3 (yellow) in dorsal raphe (DR) 5-HT neurons (TPH2-positive, red). Cell nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; blue). Each row represents a different mouse (n=3,A); High-magnification photomicrographs of the frames depicted in 'exm1' (A) were provided to show the co-localization of alexa488-labeled miCure-135-3 in dorsal raphe nucleus (DR) 5-HT neurons (B&C). Scale bars: left = 100 µm, right = 25 µm. In contrast, alexa488-labeled miCure-135-3 was not found in brain areas adjacent to the application site (olfactory bulb) or to brain ventricles (hippocampus and striatum) (D).

3.11. Temporal changes in gene expression following miCure-135-3 administration

To assess the duration of molecular phenotype effects resulting from acute intranasal administration, miCure-135-3 was intranasally administered to wildtype mice. Western blot analysis was conducted to investigate the compound's impact on reducing protein levels of SERT and 5-HT1A. Immunoblotting was performed on samples collected at intervals of 3, 5-, 7-, 10-, and 14-days post-administration. The results indicated a significant reduction in SERT protein levels within the dorsal raphe at 3- and 5-days post-administration, followed by restoration to control levels at 7 days, which

were maintained at 10- and 14-days post-administration (see Figure 16.A). Notably, DRN-5-HT_{1A} protein levels were consistently reduced at all time points examined (strong tendency post 5 days) (see Figure 16.B).

In the projection areas, a distinct pattern emerged where SERT protein levels in the PFC of miCure-135-3-treated mice displayed a decrease compared to the control group at 3, 5, 7, and 10 days post-administration, returning to similar levels as the control at 14 days (see Figure 16.C). Conversely, SERT protein levels in the hippocampus remained unaffected at 3 and 5 days post-administration, but a reduction was observed at 7, 10, and 14 days (see Figure.16E). In the prefrontal cortex, 5-HT_{1A} protein levels exhibited a reduction at 3 days post-administration, followed by restoration at 5 days and no further changes in subsequent time points (see Figure 16.D). In contrast, 5-HT_{1A} protein levels in the hippocampus mirrored the pattern seen with SERT protein levels: no effect at 3- and 5-days post-administration, with a notable reduction at 5, 7, and 14 days (see Figure 16.F).

Additional investigations were carried out on the protein levels of other known targets of miR-135. The results revealed a significant reduction in mineralocorticoid levels in the amygdala at 3 days post-administration, whereas no such reduction was observed in the DRN or hippocampus (see Figure 16.G). CPLX1 protein levels were reduced in both the PFC and HPC, while no reduction was detected in the DRN (see Figure 16.H). Similarly, CPLX2 levels displayed a reduction in the PFC but not in the HPC. However, technical issues hampered the detection of this protein in the DRN (see Figure 16.I).

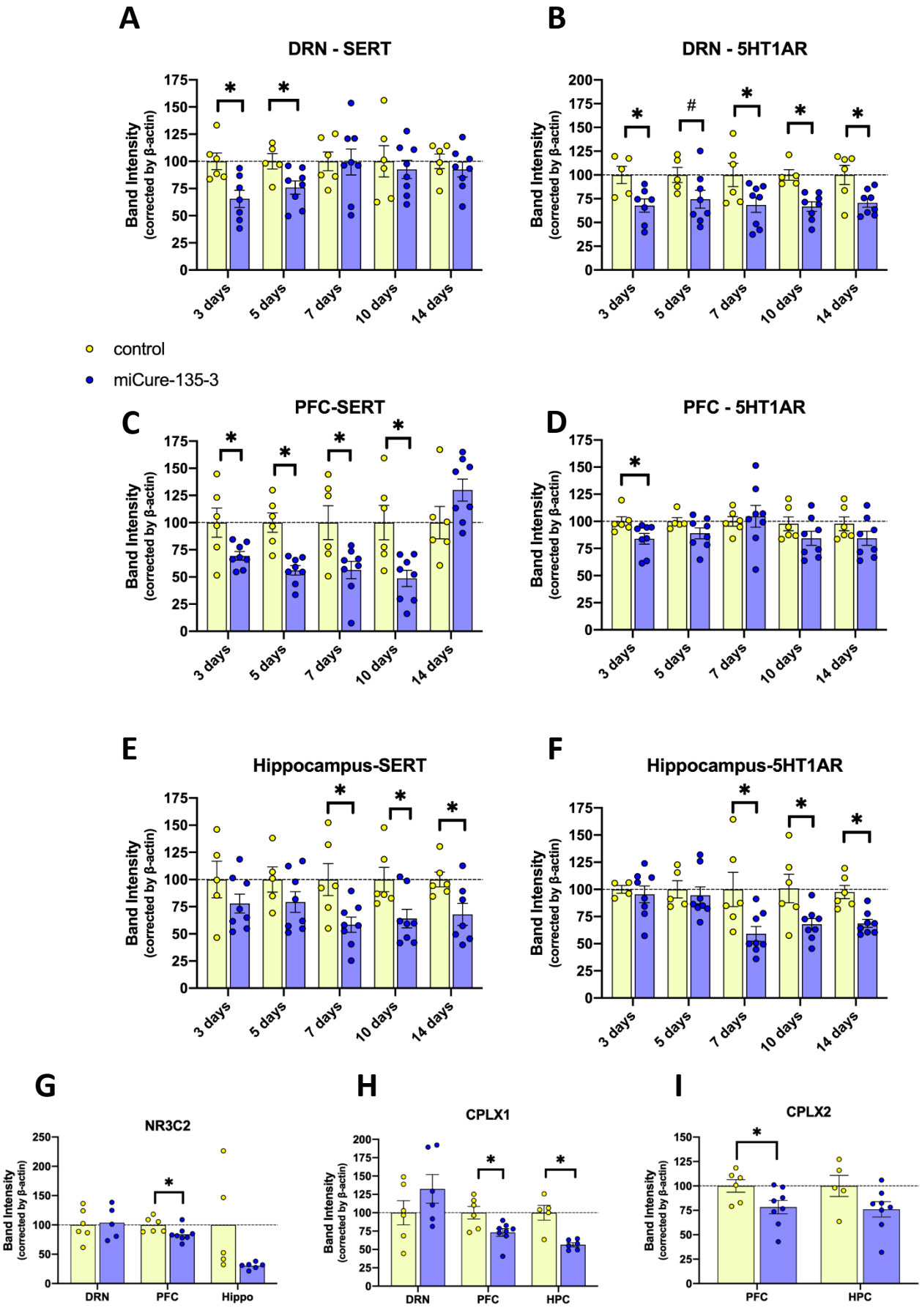

A
DRN - SERT
B
DRN - 5HT1AR
Band Intensity (corrected by β-actin)
3 days
5 days
7 days
10 days
14 days
control
miCure-135-3
C
PFC-SERT
D
PFC - 5HT1AR
E
Hippocampus-SERT
F
Hippocampus-5HT1AR
G
NR3C2
DRN
PFC
Hippo
H
CPLX1
HPC
I
CPLX2

Fig. 16. Temporal Changes in Gene Expression in DRN and Projection Areas Following miCure-135-3 Administration. Immunoblot quantification demonstrate a decrease in the protein levels of SERT in the dorsal raphe of treated mice compared to control at 3- and 5-days post administration, whereas no changes were observed at 7-, 10- and 14-days post administration (A). A significant decrease in Htr1a protein levels were detected in the DRN in all time points examined except for 5-days post administration where a strong tendency was calculated (p=0.07) (B). The reduction in the protein levels of SERT in the prefrontal cortex of treated mice compared to control in 3-, 5-, 7-, and 10-days post administration is shown in (C). PFC Htr1a protein levels were reduced in 3 days post administration (D). A similar pattern in the HPC was demonstrated where both SERT (E) and Htr1a (F) protein levels were unchanged at 3- and 5-days post administration and were significantly reduced at 7-, 10- and 14-days post administration. The effect on the protein levels of additional miR-135 targets genes was tested using western -blot and quantification of the immunoblots revealed a reduction in the levels of mineralocorticoid in the amygdala (G), cplx1 in the hippocampus and prefrontal cortex (H) and cplx2 in the PFC (I) n= *$p < 0.05$; #p= 0.06

4. Discussion

Starting from the approval of the initial oligonucleotide-based treatment, Fomivirsen (ASO-based drug), in 1998, a total of 14 more oligo therapeutics, including 5 RNAi therapies (siRNA-based drugs), have gained approval in the US or the EU by June 2022. This increasing number of approved oligo-based drugs, coupled with advancements in enhancing their pharmaceutical attributes, has fueled a surge in interest within the field. This heightened enthusiasm is mirrored by a substantial upswing in investment levels. For example, the market capitalization of public oligonucleotide companies demonstrated an increase of 94.2% between 2015-2020[342]. As a result, the number of clinical trial initiations in which oligos are being tested has almost doubled between 2020 to 2021. However, one of the major obstacles preventing the extensive application of oligonucleotide therapeutics is the complexity in securing effective delivery to target organs and tissues beyond the liver. On top of that, most of the current oligo programs in clinical phases target rare, gene-specific related diseases. At present, no RNA therapeutic has entered clinical trials for mood disorders treatment. However, as far as targeting tissues outside the liver, some programs are employing intrathecal delivery, allowing for swift administration to the CNS. Moreover, several molecules are undergoing clinical trials to address complex multifactorial neurological conditions. The nature of the development programs in the preclinical phases is similar. The majority of these programs concentrate on monogenetic diseases. Consequently, the use of ASOs or siRNAs, which enable precise targeting of individual transcripts, is more prevalent than the adoption of miRNA mimics that target multiple transcripts.

Depression is a complex disorder which involves biological, psychological, and environmental factors. A great body of knowledge was accumulated in over half of a century showed that addressing this complex human disorder will require the

orchestration of multiple levels of molecular, cellular, and circuit functions, that are probably governed by several genes. The mechanism of action of miRNAs bestows them with the capacity to serve as key regulator of entire pathways: cellular or indication-specific related (e.g., tumors), hence, mimicking their action with synthetic miRNA, can serve as a suitable modality for the treatment of complex multigenic diseases. The levels of miR-135 were shown to be downregulated in the brain and plasma of depressed patients[239,343] and pre-clinical studies done by few research groups have demonstrated the involvement of this miRNA in various models of anxiety and depression, through its regulation of different genes within the known depression related network (e.g., PFC, amygdala, DRN)[239,241,244,246,247,344,345]. The rationale that guided us in our approach was to replenish the levels of miR-135 in order to restore homeostasis to the system that was affected by MDD, and thus result in antidepressant effect, or responsiveness to drug (this hypothesis it yet to be tested).

Here we demonstrated that synthetic version of miR-135 (miRNA mimic) has the capacity to replicate the molecular and behavioral effects previously seen in a transgenic mice model overexpresses miR-135 specifically in the serotonergic neurons of the DRN[239]. Administration of miR-135 mimics directly to the DRN, ICV or via intranasal administration, affected the functionality of the serotonergic system, the expression levels of target genes and demonstrated antidepressant and anxiolytic like effects in mice models. These results are in line with the proposed role of miR-135 in articles demonstrating miR135 involvement in anxiety and in DRN, PFC and amygdala[239,241,244,246,247,344,345].

We first designed synthetic versions of miR-135 and tested their efficacy in a reporter assay *in-vitro*. We tested duplexes with minimal sequence modifications in the passenger strand and we also incorporated chemical modifications (2´-O-Me) in a couple of nucleotides in both passenger and guide strands aimed to increase the duplex stability.

The chemical modifications were placed at the end of each strand, in the seed area or in the bulge area (see table.5.). The effect of the different locations of the modification on the ability of miR-135 to inhibit its targets was tested. One of the synthesized mimetic miR-135 molecules (referred to as duplex 11 in table.5) was shown to silence miR-135 targets (e.g., Htr1a and Slc6a4) significantly better than the other molecules synthesized and tested in the human cell line (see Figure.5A&B). This mimic miRNA is comprised of a guide sequence which is completely identical to the endogenous miR-135b guide strand while the passenger sequence is 100% complementary to the guide strand and it is distinct from the native passenger sequence. The full complementary sequence should result in increased stability of the duplex *in-vivo*. Interesting to note that miR-135 guide sequence has two areas that are considered as Htr1a seed match (see Figure.17) and the results showed that duplex 17, that has chemical modification in the seed match shared to both Htr1a and Slc6a4, did abolish the mimic's ability to decrease the 3'UTR levels of Slc6a4 whereas the Htr1a levels were decreased significantly, but to a lower extend when compared to duplex 11, a duplex in which both Htr1a seed matches were not chemically modified.

Fig. 17. Nucleotide base pairing of miR135b with Slc6a4 3´ UTR (D), and with Htr1a 3 UTR (F). Extracted from Issler et al., 2014.

Further *in-vivo* experiments with 'naked' (i.e., with no carrier vehicle nor biomolecule conjugation) duplex 11 showed limited efficacy when was administered directly into the DRN. The modest effectiveness of the 'naked' duplex is to be expected, considering prior demonstrations that, despite their significant size and negative charge, dsRNA can partially traverse neuronal cell membranes[346,347]. Unlike systemic exposure,

there is diminished nuclease activity in the CSF, leading to the observed impact on 5-HT_{1A} levels for up to 4 days after administration (Figure. 5F). Direct brain administration is considered a highly invasive approach for the treatment of psychiatric disorders hence, we were exploring less invasive routes of administration. As expected, systemic administration of 'naked' duplex 11, resulted in lack of target engagement (i.e., reduction in the levels of the target genes) and no effect on behavioral phenotypes (i.e., antidepressant/anxiolytic like effects) was recorded. (Data not shown, experiments were conducted before the initiation of this dissertation). The absence of an effect is likely attributed to the very short half-life of dsRNA in the bloodstream, characterized by high nuclease activity, as well as the duplex's incapacityto cross the BBB due to its size.

Oligonucleotide delivery is challenging, especially when aimed at targeting the CNS, as they cannot cross the BBB due to their size. The vast majority of ASOs and siRNAs drugs aimed to target the CNS are locally infused intrathecally (ITH). On top of that, recent data suggests that ITH route is suitable for targeting cortical areas whereas targeting deep brain areas requires ICV route of administration. As discussed in the introduction, carrier vehicles are often used to deliver oligonucleotides as they protect from nuclease attack and renal clearance together with bestowing the oligos with increased membrane transfer. Lipid nanoparticles were employed to encapsulate the oligos, facilitating the systemic delivery (via intravenous administration) of synthetic miR-135. No effects were demonstrated following this administration (data not shown). One possible explanation to the absence of effects could be that encapsulation with LNPs predominantly results with accumulation of the dsRNA in the liver[313]. Biomolecule conjugation was also shown to be an effective approach for siRNA delivery. For example, the utilization of GalNAc technology showed hepatocyte-specific uptake and has been used in few approved drugs and programs in advanced clinical phases. However, the GalNAc conjugate depends on the fact that system delivery naturally ends up in the liver,

making it not easily adaptable for non-hepatic pathologies. Beside GalNAc, conjugation of cholesterol was demonstrated to be effective delivery in the case of miR-29 mimic (Remlarsen), in which the cholesterol moiety is used to facilitate the uptake of the compound by skin fibroblasts in a phase II study on keloid disorder. Cholesterol and other lipophilic moieties can render the dsRNA more hydrophobic and significantly enhance its pharmacokinetic properties. This augmentation increases the duplex's resistance to nuclease and boosts cellular internalization by promoting interaction with the cellular membrane, without interfering with its silencing activity. A recent study published by Alnylam demonstrated that a single intrathecal (IT) administration of siRNA with a palmitic acid conjugated at the 5′-end of its sense strand (referred to as C16 conjugated siRNA), aimed to target SOD1 in rodents, exhibited extensive distribution in the CNS. This corresponded with a dose-dependent knockdown of the target mRNA. Remarkably, comparable outcomes were observed in NHPs treated with C16-conjugated siRNA targeting the amyloid beta precursor protein (APP), where a single intrathecal administration was sufficient to induce an 80 percent reduction of the target gene, three months after administration[348]. But as our aim is to replenish the reduced levels of miR-135 in the DRN of depressed patients, the more selective targeting of neuronal subpopulation together with a noninvasive route of administration (i.e., intranasal) proposed by Artigas and Bortolozzi[349], deemed more suitable to our drug development.

Bortolozzi et al., demonstrated that a covalent conjugation of a small molecule drug to an siRNA can guide the compound selectively to a subtype of neuronal population. In this study, Sertraline (an SSRI that targets the serotonin transporter) was conjugated to siRNA that targets the 5-HT_{1A} mRNA (C-1A-siRNA). Following acute ICV administration, a selective accumulation of C-1A-siRNA in serotonergic neurons was shown without detrimentally impacting the siRNA gene silencing activity as it was reflected in significant reduction in the levels of 5-HT_{1A} in dorsal and median raphe

nuclei, but with no observed changes in post-synaptic 5-HT_{1A} density or other genes, showing a selective effect on pre-synaptic 5-HT_{1A} (autoreceptors). As expected, the selective 5-HT_{1A} autoreceptors reduction suppressed the negative feedback mechanisms mediated by those receptors hence, resulted with antidepressant-like effect. Remarkably, these effects were replicated following acute intranasal administration, which marked it as the first to demonstrate the viability of employing RNAi approaches for the treatment of mood disorders[338]. The same research group demonstrated the potential of their approach by using sertraline conjugation to a siRNA directed against the serotonin transporter (SERT). They showed a very selective accumulation of the conjugated compound in the serotonergic neurons of the dorsal raphe following intranasal administration in mice. A reversible reduction of SERT levels was recorded to coincide with elevation of 5-HT levels and antidepressant-like effects. Important to note that the control compound which was comprised of sertraline-conjugated nonsense-siRNA didn't induce any of these effects. Moreover, the compound showed a better profile than Fluoxetine (an SSRI) both in term of earlier onset, as tested by measuring the 5-HT levels following chronic treatment, and by showing that unlike fluoxetine, the conjugated compound promotes neuroplasticity in the hippocampus, which is widely recognized to be linked with clinical antidepressant activity[340]. Bortolozzi and colleagues expand their target population by using the triple monoamine reuptake inhibitor indatraline (IND) conjugated to siRNA and ASO to target a-synuclein. The results show selective targeting of serotonergic and noradrenergic neurons in mice[350] and a decrease in α-synuclein protein levels within the midbrain monoamine nuclei of NHP[339]. The group also used anti TASK3- siRNA to which they conjugated sertraline or reboxetine, to facilitate targeting the serotonin and norepinephrine neurons, respectively, and showed a selective accumulation and target engagement in the intended targets[351]. We have synthesized duplex 11 with sertraline conjugated to the 5' end of the passenger strand (miCure-135-

1) and checked its efficacy following intraDRN administration. In this set of experiments, we demonstrated that the conjugation of sertraline did not interfere with the silencing activity of the synthetic miRNA. We showed that a low dose of 30ug was sufficient to induce reduction in both 5-HT_{1A} autoreceptor protein levels, as was shown by abolishing the effect caused by presynaptic 5-HT_{1A} agonist (8-OH-DPAT) (Figure.7A-D), and in the SERT mRNA levels as shown by *in-situ* hybridization (Figure.7H). On top of that, an antidepressant like effect was recorded to coincide with an increase of 5-HT levels in the projection area (Figure.7F&G). Our first attempt with intranasal administration of the conjugated molecule (miCure-135-1) was partially successful and we did see a reduction in Htr1a and an antidepressant-like effect, but these results were not robust and in repeated attempts were not reproducible even when a very high dose was applied via intranasal administration (Figure.8).

As robust efficacy had to be achieved via a non-invasive route of administration, we sought to optimize the chemical modifications used in the duplex, in order to increase its stability and efficacy. 16 different duplexes based on the previously chosen sequence were tested in a two-step *in-vitro* screening. First, we tested the efficacy of the duplexes in reducing the target genes using a reporter assay, similarly to what we did in the first round. Later, we tested the pattern of immune activation induced by the new modifications using human peripheral blood mononuclear cells (hPBMCs). Immunostimulation is a major challenge in oligonucleotides therapeutic. The receptors engaged in identifying RNAs are Toll-like receptors (TLR), Protein kinase R (PKR), and several helicases[352]. The primary outcomes of dsRNA-mediated immunostimulation are the activation and release of cytokines and interferons. In terms of efficacy, 6 duplexes showed a significant or a strong tendency toward a reduction in the levels of 5HT1 a and SERT 3'UTRs across 5 repetitions (Figure.9A&B). As it is known that the modifications have the potential to hinder the silencing activity of synthetic miRNAs, it is not surprising

that the most efficacious mimics have the least number of modifications on the guide strand: (i) a single 2'-O-Me at the 3' end. (ii) i+ additional thymidine with 2'-O-MOE at the 5' end (iii) ii+ 2 Adenine overhang with 2'-O-MOE modification and phosphorothioate linkage between them, at the 3' end of the strand. On the other hand, heavy phosphorothioate on the backbone resulted in adversity as seen in dying cells or very low effectiveness (Figure.9A&B miCure-135-5/6). The selected mimics were further tested for immune activation, and it was shown that four out of the six mimics that were tested had induced immune activation which reflected by the secretion of IFN-alpha-2a and TNF alpha. The secretion of IFN-Alpha is induced by the activation of Toll-like receptor 3 (TLR3), which is known to be induced by unmodified and unformulated dsRNA [353]. The secretion of both cytokine (TNF and INF alpha) can result from the dsRNA binding to RIG-I (retinoic acid-inducible gene I) which is a RIG-I-like receptor dsRNA helicase enzyme. Typically, RIG-I recognizes short, uncapped double-stranded or single-stranded RNA[354,355]. miCure-135-1 which caused immune activation differs from miCure-135-2 by one 2'-O-MOE at the 5' end, and from miCure-135-3 by the same modification and an additional overhang adenine with 2'-O-MOE and phosphorothioate. 2'-O-MOE was proposed previously to reduce immune activations induced by dsRNA[356]. On the other hand, the same three guide strands used in miCure-135-1&2&3 were found to be immunoreactivite when combined with a different passenger strand that had additional phosphorothioate linkages on both ends, this emphasizes the notion that the location of chemical modifications along the duplex can determine whether it will induce an immune activation. The safety of miCure-135-3 was further validated by examining its effect on cellular viability following direct administration into mice DRN, with no observed effects on neurons, microglia, or serotonergic-neurons viability (Figure.12).

Following the toxicity screening, miCure-135-1 had to be discarded and we focused our development efforts mainly on miCure-135-3. We demonstrated the capacity

of this synthetic miR-135 to regulate Htr1a and SERT levels, as well as its efficacy in mitigating depressive-like behavior in mouse models, following intraDRN (Figure13A) and ICV (Figure.13D) administrations. But as described earlier, one of the key goals in the development of a drug for the treatment of mood disorder is to find an administration route which will be readily acceptable in the clinic. We showed in our studies that acute intranasal administration of miCure-135-3 could be effective in mimicking the effects achieved following intraDRN administration. The administration of intranasal sertraline-conjugation-miR-135 triggered diminution in depressive-like behavior and led to notable reductions in SERT and 5-HT_{1A} protein levels in the DRN (Figure.14). Intranasal administration was used for many decades systemic administration of drugs, however, employing this noninvasive technique to swiftly deliver medications directly from the nasal mucosa to the brain and spinal cord is a relatively novel approach. Pharmacokinetic and pharmacodynamic studies in animals have shown specific brain targeting with impacts on behaviors mediated by the CNS shortly after intranasal administration [357]. For instance, the effects of cocaine are noticeable within minutes of administration, even prior to being detectable in the bloodstream, making the case that a rapid, extracellular pathway into the brain following intranasal administration does exist[358]. The precise pathways and mechanisms by which drug reaches neurons after intranasal administration have not been fully elucidated. In general, the pathways could be divided into intracellular and extracellular pathways. Depending on the characteristics of a compound, a portion of it may be transported intracellularly, commencing at the olfactory epithelium and utilizing the sensory neurons to reach regions within the brain, the first being the olfactory bulb. This transportation to the neurons could occur via receptor-mediated or non-specific fluid phase endocytosis. Alternatively, other substances might gain access to the lamina propria, from where they can progress through extracellular pathways such as olfactory blood vessels, olfactory lymphatic vessels, or the olfactory submucosa. The latter route

has the potential to reach the olfactory bulb or CSF circulation as fast as 30 minutes[350,357,359,360]. The group led by Bortolozzi proposes that sertraline/indatraline-conjugated siRNA molecules, detected in the brain within an hour after administration, likely distributed through the extracellular pathway to the CSF. There, the numerous axons of monoamine cell bodies, which are densely populated throughout the brain, may internalize the compound through endocytosis[361,362]. In support of this hypothesis the same group demonstrated that Indatraline-conjugated-ASO reaches the brainstem monoamine nuclei 10–20 minutes after intranasal administration[350]. Moreover, the association of conjugated siRNA[363,364] or ASO[350] With Rab5 and Rab7 present in the monoamine neurons, they facilitate endocytosis and intracellular trafficking processes through early and late endomembrane compartments. Lastly, fluorescence-tagged oligos were not detected in brain regions outside the target areas, even those adjacent to the administration site[338,350,364] , those observations were replicated also in our studies where we showed that the covalent binding of sertraline to miR-135-mimic allows its selective accumulation into raphe serotonergic neurons (Figure.15) whereas no fluorescence signal was detected in the control areas, including olfactory bulb, 6 hours after intranasal administration.

Intranasal administration of miR-135 mimic in mice evoked several changes in both molecular and behavioral levels which are predictive of antidepressant activity such as (i) reduction in the mRNA/protein levels of the presynaptic Htr1a and serotonin transporter – both proteins are known targets of antidepressants, (ii) the effect on serotonergic function as it was demonstrated in 5-HT levels in DRN projection area, (iii) reduced depressive-like behavior in mice models. Those observations following a non-invasive drug administration reinforced the potential of miR1-135 mimic as a drug for MDD. In order to further support the development, we characterized the drug like properties of miCure-135-3, we checked (i) the duration of the observed effects (ii) the

stability of the molecule in different matrices (iii) the selectivity of the effect in different brain areas. We examined the duration of the effects on molecular and behavioral phenotype following acute intranasal administration. The reduction in DRN protein levels of 5-HT_{1A} was significant up to 14 days following single administration whereas the DRN SERT levels were recovered 7 days following single administration. Interesting to note that the effect on the behavior was seen only on the third day following administration. To further understand the kinetics of the miR-135 mimic metabolic stability, the compound has been studied in serum of mouse, rat, monkey and human and CSF of monkeys and human. The half-life of the molecule is relatively short in serum (±2h) for all species, probably due to high activity of RNA nucleases in the serum. The metabolite pattern was shown to be a bit longer in CSF, with pattern difference between human and monkey CSF (data not shown). These results suggest that the synthetic miR-135 is rapidly transported via CSF and then be taken up by the cells where it is less exposed to nucleases and remains intact to perform its inhibitory action up to weeks following its administration. The relatively short half-life is unsurprising given that miCure-135-3 incorporates only a few chemical modifications aimed at enhancing its stability. In the stability experiment, the control sequence contains additional phosphorothioate groups, which provide protection against nucleases.

Next, the effect of the molecule on the target genes in the projection areas of the DRN was examined (Figure.16). We focused on two main areas that are known to be involved in MDD: prefrontal cortex and hippocampus. As expected, a significant reduction of serotonin transporter was detected in both areas with a delayed onset in the hippocampus. 5HTT is a terminal transporter hence its levels in the projection areas of raphe can be affected by the reduced translation levels that occur in the serotonergic neurons' soma located in the raphe. On the other hand, the reduction of 5-HT_{1A} in the projection areas was not expected. 5-HT_{1A} is a somatodendritic receptor hence its

reduction in the dorsal raphe is not supposed to be reflected in the projection areas like hippocampus and PFC. Prior research has indicated that the levels of 5HTT can impact the levels of Htr1a. By employing a model of 5HTT knockout mice, it was observed that SERT−/− mice exhibited markedly elevated basal levels of 5-HT compared to SERT+/+ mice in both the striatum and the frontal cortex. This phenomenon can be attributed to the desensitization of presynaptic 5-HT1A receptors in those mice as subjecting these mice to the 5-HT1A agonist -8-OH-DPAT led to a decrease in physiological responsiveness[365–367] and this desensitization of 5-HT_{1A} receptors in male dorsal raphe and hypothalamus and amygdala in addition in female. The density of dorsal raphe 5-HT_{1A} receptors was lower in 5-HTT −/− mice. Conversely, no notable alterations were observed in those receptors within the hippocampus or the frontal cortex[366,367]. Similar results were found in slice preparation from SERT −/− and SERT +/+ mice, where a reduction in the sensitivity to 5-HT_{1A} agonists was found in the neurons in the DRN but not in the hippocampus [368]. Interestingly, a possible support for the reduction observed in the PFC rises from PET studies demonstrating that 5-HT_{1A} receptor binding potential was lower in the mPFC of individuals carrying the short 5-HTTLPR genotypes (SS or SL) compared to those with the long genotypes (LL). Further validation with binding assay or with qPCR is needed to better understand the reduction of 5-HT_{1A} receptors levels in the projection areas. Another possible explanation for the reduction of 5-HT_{1A} observed in the mPFC could be that the sertraline conjugate delivery is not as selective as we see in the distribution experiment. To answer these questions, we have designed together with Axolabs GBH, a bioanalytical assay based on peptide nucleic acid (PNA) that binds to the antisense strand of the miR-135 mimic and using anion exchange HPLC. This method enables us to quantify the levels of the compound in different tissues. We are also interested in investigating the possibility of partitioning the mouse brain into distinct regions such as the midbrain, cortex, and the remaining regions. This would allow us to

compare the levels of the compound detected in these various areas, with the anticipation of observing a higher concentration of the duplex in the midbrain. This assay will also serve to quantify the compound in other organs throughout the body. However, its primary application will involve establishing a pharmacokinetic/pharmacodynamic (PK/PD) relationship across various doses and time intervals. In this context, the pharmacodynamic aspect will involve assessing the levels of the target genes.

Unlike ASOs and siRNAs, microRNAs are partially complementary to their target mRNAs hence one miRNA has the capacity to target few mRNAs. The proposed mechanism of action by which miR-135 excreted his antidepressants effects goes beyond the regulation of the target genes within the serotonergic system (i.e., Htr1a and SERT). Here we demonstrated that miR-135 mimic reduces the protein levels of the mineralocorticoid receptor in the prefrontal cortex of mice whereas no reduction was observed in the DRN nor hippocampus. Those results are partially in-line with a study that showed that NR3C2- 3'UTR has two seed matches of miR-135 and a direct regulation of this gene was confirmed in luciferase mutation studies and in *in-vitro* studies using cell culture. On top of that, model of acute stress revealed a rapid increase in the levels of mineralocorticoid receptors coincides with a rapid decrease of miR-135 in the amygdala of the stressed mice. Mineralocorticoid receptors are plentiful within the limbic structures of the brain, such as the prefrontal cortex and hippocampus, and they exert inhibitory control on the secretion of cortisol[369]. A diminished GR sensitivity, associated with elevated cortisol secretion and compromised cognitive function, has been previously documented in patients with MDD[370–373]. However, the role of MR in depression is less understood. Still, a handful of postmortem studies have suggested a reduction in MR levels in the prefrontal cortex and hippocampus of patients with MDD[374,375].

The findings we have observed remain preliminary and a temporal analysis of miR-135 effect on MR expression should be conducted. This course of investigation is

warranted due to prior evidence indicating miR-135 is involved in the regulation of early stress response[345]. Notably, our study has unveiled a downregulation of MR in the prefrontal cortex (PFC) 3 days after administration, aligning with the onset of an antidepressant-like effect. Likewise, the spatial analysis of MR reduction across different brain areas (e.g., amygdala) should be conducted to further understand the relationship between miR-135 expression, MR, and depression. Further validation of the silencing efficacy of the miR-135 mimic was achieved through an assessment of CPLX-1 and CPLX-2. Notably, a decline in their protein levels was observed in the prefrontal cortex (PFC), along with a reduction in CPLX-1 levels in the hippocampus, subsequent to acute intranasal administration in mice. It is noteworthy that CPLX-1 and CPLX-2 protein levels have been found to rise in the amygdala following acute stress, a response attributed to the diminished miR-135 that governs the expression of both transcripts. CPLX-1 and CPLX-2 play integral roles in moderating the release of neurotransmitters at synapses. They associate with SNARE (Soluble NSF Attachment Protein Receptor) complexes, influencing both spontaneous and calcium-induced neurotransmitter exocytosis[376,377]. Moreover, postsynaptic complexins are involved in the regulation of AMPA receptor exocytosis and membrane insertion during processes such as long-term potentiation (LTP)[378]. The role of Complexins in MDD is poorly understood. Reduced Complexin-1 and Complexin- protein levels were reported in the prefrontal cortex and anterior cingulate cortex of depressed patients[379,380] but it is not determined whether and how exactly reductions in Complexin levels could contribute to the symptoms of depression. Reduced levels of Complexin-1 and Complexin-2 were also reported in the hippocampus in patients suffering from bipolar disorder[381,382]. Interestingly, three evolutionarily conserved regions, which correspond to miR-135-binding sites, are found in the 3′UTRs of both Cplx1 and Cplx2[244] and elevation in the levels of both gene in the amygdala of mice was associated with increased anxiety-like behavior following a

pharmacological inhibition of miR-135 in the amygdala. The regulation of complexin-1 and -2 by miR135 was demonstrated to be required for prolong spine remodeling following induction of NMDAR-long term depression (LTD) which causes long-lasting synaptic depression and spine lose[383].

To summarize, our results serve as a strong proof of concept of the potential of miR-135 mimic to be developed as an antidepressant drug, via its effects on the serotonergic system. We also have a lead candidate with a specific sequence and chemical modifications that increase the efficacy of the compound and reducing its innate immune activation. There are still many hurdles to overcome before a clinical study could commence. Drug development demands robust and reproducible results and moreover, a correlation between administrated dose and effect should be established. A quantitative pharmacokinetics (PK) studies should be conducted to understand the distribution of the molecule and its selective targeting. Upon successful PK data, a dose response study should be conducted to establish a clear PK/PD relationship. As mentioned before, a bioanalytical study was developed with Axolabs and the first studies should be concluded soon. In case the levels of the compound measured in the brain would suggest that improved accumulation should be reached, the molecule could undergo further chemical modification to increase its stability. For instance, to increase half-life, oligos are often designed with a phosphorothioate (PS) linkage which bestows the oligonucleotides with resistance to nucleases and makes them more readily binding to plasma proteins. On the other hand, PS can potentially cause a reduction of the binding affinity with target sequence and recent data suggests that miRNAs are less tolerant than siRNAs to chemical modifications as reflected in reduced silencing activity. On top of that, chemistry-related toxicities with high PS content for instance, were demonstrated. Thus, the gain in stability associated with the chemical modifications needs to be weighed against reduced efficacy and increased safety risks. An additional challenge would be finding a translatable

biomarker- a readout for efficacy that can be used in both preclinical and clinical setups. One of the more common models used in the field of depression is to use positron emission tomography (PET) scanning (microPET in case of rodents) which allows for measuring the binding potential of serotonin 5-hydroxytryptamine1A (5HT1a).

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Affidavit

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Sharon Manashirov

Surname, first name

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