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Oligonucleotides as therapeutic tools for brain disorders: Focus on major depressive disorder and Parkinson's disease

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Abstract

Remarkable advances in understanding the role of RNA in health and disease have expanded considerably in the last decade. RNA is becoming an increasingly important target for therapeutic intervention; therefore, it is critical to develop strategies for therapeutic modulation of RNA function. Oligonucleotides, including antisense oligonucleotide (ASO), small interfering RNA (siRNA), microRNA mimic (miRNA), and anti-microRNA (antagomir) are perhaps the most direct therapeutic strategies for addressing RNA. Among other mechanisms, most oligonucleotide designs involve the formation of a hybrid with RNA that promotes its degradation by activation of endogenous enzymes such as RNase-H (e.g., ASO) or the RISC complex (e.g. RNA interference - RNAi for siRNA and miRNA). However, the use of oligonucleotides for the treatment of brain disorders is seriously compromised by two main limitations: i) how to deliver oligonucleotides to the brain compartment, avoiding the action of peripheral RNAses? and once there, ii) how to target specific neuronal populations? We review the main molecular pathways in major depressive disorder (MDD) and Parkinson's disease (PD), and discuss the challenges associated with the development of novel oligonucleotide therapeutics. We pay special attention to the use of conjugated ligand-oligonucleotide approach in which the oligonucleotide sequence is covalently bound to monoamine transporter inhibitors (e.g. sertraline, reboxetine, indatraline). This strategy allows their selective accumulation in the monoamine neurons of mice and monkeys after their intranasal or intracerebroventricular administration, evoking preclinical changes predictive of a clinical therapeutic action after knocking-down disease-related genes. In addition, recent advances in oligonucleotide therapeutic clinical trials are also reviewed.

Keywords: Oligonucleotide therapeutics; ASO; miRNA; brain delivery; depression; Parkinson's disease

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Abbreviations

5-HT serotonin

5-HT_{1A}R serotonin$_{1A}$ receptor

α-SYN α-synuclein

Antagomir anti-microRNA

ASO antisense oligonucleotide

BBB brain-blood barrier

DA dopamine

DRN dorsal raphe nucleus

ER endoplasmic reticulum

FDA Food and Drug Administration

GxE gene x environment

GWAS genome-wide association studies

HPA hypothalamus-pituitary-adrenal

icv intracerebroventricular

MDD major depressive disorder

mRNA messenger RNA

miRNA microRNA

MAT monoamine transporters

NE norepinephrine

ncRNA non-coding RNAs

PD Parkinson's disease

PFC prefrontal cortex

PMO phosphorodiamidate morpholino oligonucleotide

PNA peptide nucleic acids

SERT serotonin transporter

shRNA short-hairpin RNA

siRNA small interfering RNA

SNc substantia nigra pars compacta

TCA tricyclic antidepressants

UPR unfolded protein response

1. Disorders associated with derangements of brainstem monoamine systems

The brainstem monoamine nuclei contain neuronal groups synthesizing dopamine (DA), norepinephrine (NE), serotonin (5-hydroxytryptamine, 5-HT), acetylcholine, histamine, and orexin, which give rise to a widespread innervation of the brain. Unlike cortical and subcortical glutamatergic projection neurons showing a short- or long-distance precise connectivity with other neuronal groups (Gabbott et al., 2005), the monoamine cell groups have an anatomically expanding structure. They are composed by relatively low neuronal numbers (e.g., the human dorsal raphe nucleus (DR) contains around 250,000 neurons, out of a total of 10^{11} neurons in the whole brain) whose axons branch widely to innervate the rest of the brain with a very high axonal density (>10^6 serotonergic nerve endings/mm^3 have been reported in rat neocortex) (Artigas, 2013). Since the discovery of DA, NE, and 5-HT as brain neurotransmitters six decades ago, derangements of these systems have been associated with the pathophysiology of neurological and psychiatric disorders (**Figure 1**). Hence, Parkinson's disease (PD) involves (but not only) the loss of DA neurons from the substantia nigra pars compacta (SNc), whereas major depressive disorder (MDD) is associated (but not only) to a hypo-function of the 5-HT and/or NE systems. On the other hand, from a therapeutic perspective, these monoamine systems are the target for most drugs used in neuropsychiatric disorders (except, for example, benzodiazepines and mood stabilizers), including precursors such as L-DOPA, reuptake inhibitors such as antidepressant drugs, and receptor agonists and antagonists in the case of antipsychotic drugs.

The anatomically expanding organization of monoamine systems makes that molecular, neurochemical, and functional changes occurring at the cell body level in the brainstem can be rapidly translated into activity changes in interconnected brain regions thanks to the dense network of monoamine axons. This anatomical feature is particularly useful for oligonucleotide therapeutics since modulations of messenger RNA (mRNA) expression in discrete brainstem nuclei may have a strong impact on monoamine function in distant brain areas. Here we review the use of oligonucleotides as therapeutic tools for brain disorders, paying special attention to the selective targeting of mRNA expressed in 5-HT and DA neurons for the treatment of MDD and PD, respectively.

2. Neurobiology of MDD: A perspective on current molecular pathways

MDD is one of the most prevalent and debilitating medical condition worldwide. Moreover, the risk of suicide among the patients with MDD is approximately 20-fold higher than in the general population. About a quarter of a billion incident cases of MDD were estimated in 2017 and MDD has prevailed as one of the three leading causes of years lived with disability (YLD) for nearly three decades (James et al., 2018). MDD is a complex disorder resulting from the interaction of genetic, neurobiological, cultural, and environmental risk factors (e.g. stress- or immune-related pathology) (Nestler et al., 2002). Genome-wide association studies (GWAS) and linkage studies have provided an overwhelming literature on candidate genes, often inconsistent and not replicated in subsequent studies, possibly because none of the GWAS so far has incorporated environmental risk factors (Flint and Kendler, 2014; Wray et al., 2018; McIntosh et al., 2019). However, burgeoning advances in functional genomic technologies have led to the identification of a vast number of novel genes that are potentially implicated in the MDD pathophysiology. In this way, several key genes linked to serotonergic neurons, neurogenesis, neuronal plasticity, synaptogenesis, and transcription factors, as well as cytokine signaling and glucocorticoid receptor signaling, among others, have been involved in MDD (Thakker et al., 2006; Lin and Tsai, 2019). Many of these candidate genes have not yet been functionalized and require validation *in vivo*. Moreover, most candidate genes found in gene x environment (GxE) interactions involve the regulation of stress and the hypothalamus-pituitary-adrenal (HPA) axis (Gonda et al., 2019). Genetic variation and environmental exposure have both been shown to impinge on epigenetic factors, and GxE interactions may be mediated by such changes. Investigating epigenetic factors such as DNA methylation, histone modifications and post-transcriptional regulation by non-coding RNAs (ncRNA) such as microRNAs (miRNA) in MDD may thus allow an integrated view of how genetic and environmental factors alter risk (Sun et al., 2013; Vialou et al., 2013; Peña et al., 2014). In this context, the oligonucleotides may be a useful tool to examine the role of candidate genes in the pathophysiology and treatment of MDD. An extensive review of MDD neurobiology is beyond the scope of the present review. We summarize below

different views on MDD pathophysiology that emerged after several decades of research, in order to provide the reader with information about possible targets for oligonucleotide therapeutics.

Classical theories on MDD postulated a deficiency of 5-HT and/or NE brain systems, and were prompted by the observation that the first clinically-effective antidepressant drugs discovered by serendipity, the tricyclic antidepressants (TCA) and the monoamine oxidase (MAO) inhibitors increased 5-HT/NE activity by blocking their neuronal reuptake or preventing their degradation, respectively. These theories have dominated the field for decades and have fostered the development of 5-HT-enhancing drugs based on the selective blockade of 5-HT and/or NE neuronal transporters, the selective serotonin reuptake inhibitors (SSRI) and the serotonin and noradrenaline reuptake inhibitors (SNRI). Interestingly, despite the improved selectivity of SSRI and SNRI, first-generation TCA such as amitriptyline appear to have a superior efficacy (Cipriani et al., 2018). Notwithstanding the limitations of the theory - at least, as originally formulated - numerous GxE alterations of the monoaminergic systems - in particular of 5-HT - have been reported in MDD (Berton and Nestler, 2006; Krishnan and Nestler, 2008). Hence, several studies have focused on the monoamine transporter genes, most often the gene encoding the serotonin transporter gene *SLC6A4*. A first study (Caspi et al., 2003) examined GxE interactions between stressful life events and the serotonin transporter gene-linked polymorphic region (5-HTTLPR) variant in the *SLC6A4* gene contributing to MDD. Since then, several other variants have been associated with MDD via complex GxE interactions. The 5-HTTLPR variant in the *SLC6A4* gene is normally represented as short and long alleles, where the short and long alleles are associated with lower and higher SLC6A4 gene expression activity, respectively (Gibb et al., 2006). Interestingly, GxE interactions between the 5-HTTLPR variant and childhood trauma are linked with a tendency to suicidal behaviors for patients with a low-expressing 5-HTTLPR genotype; however, patients with intermediate-expressing and high-expressing genotypes did not have this tendency (Roy et al., 2007; Cicchetti et al., 2010; but not Culverhouse et al, 2018).

In addition, several studies reported that differences in serotonin$_{1A}$ receptor (5-HT$_{1A}$R) expression are associated with MDD and antidepressant response. Hence, individuals with

high 5-HT_{1A}R expression in 5-HT neurons, including those with a functional C(-1019)G polymorphism in the promoter region of *HTR1A* gene, are more susceptible to MDD and suicide, and respond poorly to antidepressant therapy (Stockmeier et al. 1998; Lemonde et al. 2003; Fakra et al. 2009; Neff et al. 2009; Sullivan et al. 2009). This association is likely mediated by a decreased ability of 5-HT neurons to release 5-HT under stressful conditions, since serotonergic activity is negatively modulated by intrinsic levels of 5-HT_{1A} autoreceptor, whose knockdown increases 5-HT release and stress resiliency (Bortolozzi et al., 2012; Ferrés-Coy et al., 2013a). Subsequent studies also revealed potential GxE interactions between the rs6313 single nucleotide polymorphisms (SNP) in the *HTR2A* gene, and cumulative types of lifetime stressful life events in a family-based study design of both parents and offspring, who had committed a suicide attempt (Ben-Efraim et al., 2013). The *HTR2A* gene encodes serotonin$_{2A}$ receptor (5-HT_{2A}R) and has been indicated as a potential marker for antidepressant therapy (Lin and Chen, 2008).

As reported, stress is intimately linked to MDD since adverse life events can precipitate depressive episodes in vulnerable individuals (Kendler et al., 1999; de Kloet et al., 2005), and childhood abuse or neglect increases the risk of depression in adult life (Hammen, 2005; Pechtel and Pizzagalli, 2011). Stress deeply affects brain neurotransmitter systems, activates adrenal-glucocorticoids systems, and alters the innate immune system and inflammatory cytokines, among others (Duman et al., 2016). Indeed, most MDD-like animal models based on stress such as social defeat stress or chronic mild stress, induce a series of behavioral abnormalities parallel to MDD symptoms, including anhedonia, decreased motivation and sleep disturbances (Willner, 2016). Furthermore, MDD and stress-related pathology are also associated with impaired hippocampal neurogenesis and neuroplasticity, which are reversed by antidepressant treatments with different mechanisms of action (Duman et al., 2000; Malberg et al., 2000; Santarelli et al., 2003; Sahay and Hen, 2007; Pittenger and Duman, 2008; Snyder et al., 2011; Eisch and Petrik, 2012; Mahar et al., 2014). Early studies in rodents showed that stress caused atrophy of hippocampal neurons, reduced neurogenesis in the dentate gyrus, and decreased neuronal synapses in the prefrontal cortex (PFC) and hippocampus (Gould et al 1997, 1998; McEwen, 1999; Sapolsky, 2000; Duman and Aghajanian, 2012). In contrast, chronic stress causes hypertrophy of neurons in the amygdala and nucleus accumbens (Christoffel et al., 2011),

effects that could contribute to disrupted emotion, motivation, and reward behaviors that are regulated by these brain regions, thus demonstrating the involvement of a broad neurocircuitry in MDD pathophysiology. Brain-imaging studies of depressed patients provide strong and consistent evidence of decreased volume of cortical and limbic brain regions - such as the PFC and the hippocampus - suggesting neuronal atrophy related to the length of illness and time of treatment (Sheline, 1996; Drevets et al., 1997; Lucassen et al., 2001; Campbell et al., 2004; Geerlings and Gerritsen, 2017).

Likewise, stress and adrenal glucocorticoids influence neuronal systems at many levels, but neurotrophic factors - mainly brain derived neurotrophic factor (BDNF) - have been of particular interest with respect to atrophy of neuronal connections (Krishnan and Nestler, 2008). BDNF is critical for neuronal survival and guidance during development, but also for activity-dependent synaptic plasticity in the adult brain (Duman and Voleti, 2012). Hence, stress and glucocorticoid exposures decrease the expression of BDNF in the hippocampus and PFC, and BDNF levels are decreased in postmortem brains of depressed patients (Duman and Aghajanian. 2012). As might be expected, BDNF substantially affects dendrite complexity and spine density. Indeed, BDNF heterozygous deletion mutant mice, as well as mice with a knock-in of a human loss-of-function BDNF gene variant (Val66Met), exhibit decreased dendrite length and branching in the hippocampus and PFC (Chen et al., 2006; Chiaruttini et al., 2009). Brain-imaging studies demonstrate that human carriers of the Met polymorphism have decreased hippocampal volume and impaired executive functions (MacQueen and Frodl, 2011) and, if exposed to early-life stress, are more vulnerable to depressive symptoms (Gatt et al., 2009). Finally, stress and MDD also disrupt synaptic signaling pathways including BDNF–tropomyosin related kinase B (TrkB) receptor signaling, leading to reductions of the downstream signal such as regulated kinase (ERK) and Akt pathways in the hippocampus and PFC (Duric et al., 2010). These pathways positively influence synaptic maturation and stability via regulation of synaptic protein synthesis and glutamate receptor cycling (Collingridge et al., 2010). In addition, the reduction of synaptic density and proteins in response to stress in the PFC of depressed patients is associated with increased expression of GATA1 transcriptional repressor of synaptic proteins (Kang et al., 2012). Therefore, a deep understanding of the synaptic

processes underlying MDD would help to develop new treatment strategies based on oligonucleotide therapeutics, especially for those undruggable protein targets.

Some recent clinical and preclinical evidence also suggest that neuroinflammation is a key factor that interacts with three neurobiological correlates of MDD: reduced brain 5-HT function, dysregulation of the HPA axis and alteration of hippocampal neurogenesis (Kiecolt-Glaser et al., 2015; Miller and Raison, 2016; Syed et al., 2018). The link between inflammation and MDD is also supported by several observations, including i) increased circulating pro-inflammatory cytokines found in treatment-resistant MDD patients, ii) imbalance in kynurenine metabolite pathways found in blood of MDD patients, iii) anti-inflammatory compounds having antidepressant effects, and iv) PET imaging showing activated microglia in depressed individuals (Troubat et al., 2020). Some key genes such as *A2M*, *FoxO1*, and *TGF-β1* involved in the signaling of cytokines, TGF-β1, and glucocorticoid receptor show significant GxE interactions with stressful life events, such as childhood sexual, physical or emotional abuse, thus contributing to the development of MDD (Cattaneo et al., 2018). In addition recent advances in the understanding of the molecular mechanisms that drive tissue damage in different inflammatory diseases, have facilitated the identification of novel targets and offered interesting therapeutic perspectives based on oligonucleotides for the treatment of MDD patients. Among these, the P2x7-NLRP3 inflammasome cascade is a key mechanism in MDD pathophysiology. The recognition of damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) by cell-surface receptors activates several intracellular signaling pathways, such as nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK). Both, NF-κB and MAPK, control the expression of many inflammatory and anti-inflammatory genes and the NLRP3 inflammasome, which cleaves pro-IL-1β and pro-IL-18 cytokines into their mature forms (Tang et al., 2012). NLRP3 is expressed in both microglial cells and peripheral immune cells. Under physiological conditions, inflammasome-induced IL-1β expression is essential as a trophic factor to promote long-term potentiation and memory formation (Yirmiya, et al., 2002). However, at high levels, IL-1β becomes excitotoxic, alters synaptic function and affects monoaminergic and glutamatergic transmission (Huang et al., 2011; Yang et al., 2019a). Interestingly, the antidepressant-like ability of ketamine to reverse lipopolysaccharide-induced depressive-

like behaviors in mice correlates with hippocampal over-expression of NLRP3 and IL-1β (Li et al., 2019).

Finally, the recognition of MDD as a complex brain disorder that involves many transcriptional changes caused an increased interest in miRNAs, since they possess the ability to serve as key modulators of entire gene networks. A growing body of evidence supports that miRNAs has an important role in regulating several genes and brain pathways that are associated with the MDD pathophysiology and treatment (Dwivedi, 2011; Issler and Chen, 2015; Artigas et al., 2018; Fiori et al., 2018; López et al., 2018; Peña and Nestler, 2018). However, despite the relatively large number of studies conducted, the findings are inconsistent, and the number of miRNAs linked to MDD replicated in more than one study remains low. The reason for the inconsistency is not fully understood, perhaps involving heterogeneity of the tissue samples. In section 4.2.3 of this review, we will discuss the growing field investigating the role of key miRNAs in MDD pathophysiology and treatment under the hypothesis that targeting miRNAs directly could be therapeutically beneficial for MDD.

2.1 Overcoming current limitations in MDD treatments

Standard antidepressant drugs (SSRI and SNRI) are pharmacological refinements of first-generation antidepressant drugs (e.g. TCA), and are based on the same therapeutic mechanism, the inhibition of neuronal reuptake of 5-HT and/or NE. These agents show a slow onset of clinical action and limited efficacy to remit MDD symptoms due to the existence of pre- and postsynaptic adaptive mechanisms following monoamine reuptake inhibition (Berton and Nestler, 2006; Artigas, 2013). Controlled clinical trials in selected patient populations show response and remission rates of 60 and 40% after 6-8 week treatments (Stahl, 2000). However, real world records are worse, showing that nearly 80% of MDD patients attending a psychiatric consultation are chronic or recurrent, and show response and remission rates of 43% and 28%, respectively, after 8 weeks of treatment with the SSRI citalopram. Remission rates after 4 sequenced treatments during 1-year increase to 67% (Rush et al., 2006; Trivedi et al., 2006). Thus, nearly one third of MDD patients treated with standard antidepressant doses for sufficient periods of time do not respond

adequately, a situation that worsens quality of life and increases suicide risk of MDD patients.

During the last 15 years, there have been a number of key observations indicating that a fast and more effective antidepressant action is possible, even in patients resistant to standard treatment, provided that adequate neural and regional targets are used. Last year, the US Food and Drug Administration (FDA) approved the use of the non-competitive NMDA receptor (NMDAR) antagonist esketamine (the S-isomer of ketamine) for the treatment of MDD patients resistant to conventional treatment strategies (https://www.fda.gov/news-events/press-announcements/fda-approves-new-nasal-spray medication-treatment-resistant-depression-available-only-certified). The intravenous or intranasal administration of single ketamine or esketamine doses evokes immediate (in hours) and persistent (for up to 1 week) antidepressant effects in treatment-resistant MDD and bipolar patients, after an initial dissociative (psychotic) phase (Zarate et al., 2006; Lapidus et al., 2014; Canuso et al., 2018; Daly et al., 2018; Popova et al., 2019). These observations have opened new ways for the development of glutamatergic antidepressant agents that may overcome current limitations of monoamine-based antidepressants. Several preclinical studies have examined ketamine effects on potential targets/pathways linked to MDD, including AMPA receptor, the mammalian target of rapamycin (mTOR), eukaryotic elongation factor 2 (eEF2), and glycogen synthase kinase-3 (GSK-3), among others (Li et al., 2010; Duman and Aghajanian, 2012; Duman and Voleti, 2012; Niciu et al., 2014; Zanos et al., 2016, 2018; Moda-Sava et al., 2019). However, despite these studies, the exact mechanism of ketamine´s antidepressant action is still unclear (neuronal activation or inhibition? - Abdallah et al., 2018), which makes it difficult to develop agents capable of modulating the different glutamatergic targets of ketamine. Overall, oligonucleotides are tools that offer a rapid means of target validation to decipher the function(s) of glutamatergic pathways involved in MDD and its treatment.

Similarly, $GABA_A$ receptor ($GABA_AR$) allosteric modulators, such as SAGE-547 or brexanolone, have been reported to exert fast (in 3 days) antidepressant actions in post-partum depression after a single intravenous injection, an effect lasting until the end of the trial (day 39) (Kanes et al., 2017; Meltzer-Brody et al., 2019). Recently, the $GABA_AR$

modulator SAGE-217 showed also relatively rapid antidepressant effects (in 2 week) after oral dosage in a population of MDD patients in a phase 2 controlled trial (Gunduz-Bruce et al., 2019). Overall, these clinical observations with ketamine and $GABA_AR$ allosteric modulators are expanding the antidepressant field in terms of new targets beyond monoamine transporters/receptors, and have led many pharmaceutical companies to the development of new glutamatergic and GABAergic agents with potential antidepressant properties (Wilkinson and Sanacora, 2019). Other agents, endowed with pro-psychotic actions, such as the muscarininc receptor antagonist scopolamine (Drevets et al., 2013) or serotonergic hallucinogens such as psilocybin (Carhart-Harris et al., 2016; Nutt et al., 2020) show also antidepressant properties, further increasing the expectations of getting fast-acting antidepressant actions via different neuronal mechanisms.

Finally, oligonucleotides may also play a role in MDD treatment, by targeting RNAs involved in the synthesis/degradation of monoamine and amino acid targets, as well as of signaling pathways involved in MDD treatment. Hence, the cell- or tissue-specific regulation of monoamine transporters/receptors, trophic factors (e.g., BDNF), signaling pathways involved in neuroplasticity (e.g., mTOR) or pro-inflammatory cytokines etc. by oligonucleotides might be considered as a highly selective alternative to drug development. In this regard, the antidepressant-like effects produced by the use of small interfering RNA (siRNA) directed to classical targets, such as SERT or $5\text{-}HT_{1A}R$ (Thakker et al., 2005; Bortolozzi et al., 2012; Ferrés-Coy et al., 2013a,b, 2016; Artigas and Bortolozzi, 2017), can be viewed as a first step in this direction. This also offers the possibility of modifying the expression of genes for whose encoded proteins linked to MDD there are no appropriate pharmacological treatments or existing drugs lack regional selectivity in the brain (Fullana et al., 2019a,b) (see also section 4.2.1).

3. Current understanding of molecular pathways in PD

PD is second to Alzheimer's disease as the most common age-related complex, idiopathic neurological disorder. It is estimated that PD affects 1-2% of people over 65 yr., with increasing incidence with age (Reeve et al., 2014). Albeit most studies in PD patients

have focused on motor symptoms including tremor, bradykinesia, and muscle rigidity along with impaired gait and posture (Alexander, 2004; Jankovic, 2008), in recent years there has been more recognition on neuropsychiatric symptoms. Among them, depression and anxiety are the most frequently reported (Reijnders et al., 2008; Aarsland et al., 2011). Depression is known to have a major impact on the prognosis of PD: depressed PD patients score lower on scales assessing motor function and activities of daily living, exhibit more cognitive symptoms, and report a lower quality of life (Weintraub et al., 2003). In addition to depression and anxiety symptoms, PD patients also exhibit frontostriatal-mediated executive dysfunction, including deficits in attention, speed of mental processing, verbal disturbances, impairment of working memory as well as impulsivity (Solari et al., 2013; Shepherd et al., 2013). Two major histopathological hallmarks of PD are the accelerated cell loss of DA neurons in the SNc and striatal depletion of DA levels, and the presence of cytoplasmic aggregates of the presynaptic α-synuclein (α-SYN) protein in Lewy bodies and neurites, collectively referred to as Lewy-related pathology. In addition to α-SYN, these structures also contain other proteins and a crowded environment of membranes including vesicles and dysmorphic organelles, e.g. mitochondria, autophagosome-like structures and endoplasmic reticulum (ER), supporting the hypothesis of an impaired organelle trafficking as a potential driver of pathogenesis in PD (Jiang and Dickson, 2018; Shahmoradian et al., 2019).

Recent evidences also suggest that α-SYN not only accumulates in Lewy bodies (Braak et al., 2003), but may also act as a disease-propagating agent causing its own misfolding and aggregation into anatomically connected neurons throughout the brain (Henderson et al., 2019; Zheng et al., 2019). While the cell-to-cell transmission of α-SYN explains much of the pathology and progression of symptoms seen in PD, certain neuronal populations – particularly monoamine neurons (DA, 5-HT and NE) – are clearly more vulnerable than others, which results in their specific neurodegeneration while preserving others nearby (Henrich et al., 2018; Henderson et al., 2019). Likewise, the α-SYN mRNA expression profile appears to correlate with the observed differential vulnerability between brain regions, suggesting that anatomic connectivity and α-SYN expression contribute greatly to the transport and spread of the pathogenic protein (Henderson et al., 2019).

Despite all the progress on understanding the α-SYN role in PD, the etiology of PD remains unknown. Environmental factors including pesticides, organic solvents, air pollutants as well as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have been implicated in PD (Goldman, 2014). The discovery of mutations in the α-SYN gene (*SNCA*) in familial PD as well as duplications and triplications in the α-SYN gene, and subsequent demonstration that Lewy-related pathology contained α-SYN, confirmed the etiologic importance of α-SYN to PD (Polymeropoulos et al., 1997; Spillantini et al., 1998). Genetic studies of families with PD led to the identification of several other genetic loci implicated in autosomal dominant or recessive forms of PD (Klein and Westenberger, 2012). Furthermore, genetic factors involved in idiopathic PD can be ascertained from GWAS. A meta-analysis of currently available GWAS implicated 28 loci and an additional locus was identified in an autopsy-confirmed cohort of PD (Nalls et al., 2014; Beecham et al., 2015). The top genetic loci are associated with genes that implicate distinct cellular processes affecting specific cellular compartments, notably cytoplasm, cytoskeletal components, mitochondria, endosomes, lysosomes and ER. Perturbations in proteostasis and mitochondrial quality control, and other basic cellular processes, converge in producing PD. It is increasingly recognized that impairment of several major cellular functions associated with energy metabolism, protein degradation, and stress response play important roles in α-SYN deposition and programmed cell death of DA neurons in PD (Moon and Paek, 2015; Varma and Sen, 2015).

Although several new treatments for PD have been developed (George et al., 2009; Sardi et al, 2018), none of them effectively halts the progression of PD. Indeed, many promising neuroprotective therapies in PD-like animal models failed to demonstrate efficacy in human clinical trials (Charvin et al., 2018). Furthermore, the US National Institutes of Health (NIH) have recently proposed identifying the disease subtype as one of the top three priorities for clinical research in PD, given the different patient symptom manifestation and progression (Sieber et al., 2014). Understanding the genetic-molecular differences among individuals and defining PD subtype populations may ultimately allow for a better definition of the target population of each treatment strategy assessing disease-modifying therapies. Given the pressing need for the development of new rational therapies for PD, in this review, we provide basic conceptual information on the major molecular pathways

underlying PD, which may assist in the design of more effective therapeutic strategies, focusing on the use of therapeutic oligonucleotides **(Figure 2)**.

3.1 Mitochondrial dysfunction and oxidative stress

The role of mitochondrial dysfunction in PD includes evidence for reduced complex I activity, due in part to mitochondrial accumulation of α-SYN, change in membrane potential quality control, disruption of calcium homeostasis and enhanced release of cytochrome *c* (Bose and Beal, 2016). Mitochondrial deficits and subsequent oxidative stress are a main hypothesis for DA neuronal death in PD (Pissadaki and Bolam, 2013). Furthermore, the energy deficiency associated to mitochondrial dysfunction and/or the presence of α-SYN aggregates become obstacles to normal axonal transport, which in turn alters synaptic plasticity (Bellucci et al., 2016). In addition, a number of PD-related genes are tightly associated with mitochondrial dysfunction (Valente et al., 2004; Strauss et al., 2005). Likewise, there is increasing evidence of mutations or deletions in mitochondrial DNA in both idiopathic and familial PD supporting the idea that mitochondrial defects play a key role in the development of PD (Gu et al., 2002; Podlesniy et al., 2019).

Some of the gene mutations linked to mitochondrial dysfunction include:

- *PINK1 (PARK6)*. PTEN-induced putative kinase-1 (PINK1) is a 63 kDa serine/threonine-protein kinase, which is localized in the mitochondria, and protects neurons from stress-induced mitochondrial damage. A *PINK1* gene mutation has been observed in several families with PD, in which it causes an increase in cell vulnerability (Dias et al., 2013). Several *in vitro* and *in vivo* studies indicated that loss-of-function mutations in *PINK1* are linked to altered Ca^{2+} transporters, resulting in mitochondrial Ca^{2+} overload and increased production of reactive oxygen species that ultimately induced cell death (Gispert et al., 2009; Miller and Muquit, 2019). To ameliorate the consequences of mitochondrial dysfunction leading to PD pathology, several therapeutic approaches including small-molecule activation of PINK1 are being explored (Miller and Muquit, 2019).

- *DJ-1 (PARK7).* DJ-1, a dimer consisting of 189 amino acids, is localized in the cytoplasm, nucleus, and mitochondria, and has been linked to early-onset PD. DJ-1 deletions and point mutations result in autosomal recessive PD (Ariga et al., 2013). *In vitro* studies demonstrated that DJ-1 has neuroprotective and chaperone functions regulating the activity of certain cell survival-related genes (PI3K/Akt pathway) and transcription factors (e.g. p53, NF-kβ and Nrf2; Canet-Avilés et al., 2004). DJ-1-deficient mice showed motor deficits and decreased DA D2 receptor function (Chandran et al., 2008). In addition, the DJ-1 protein has been linked to several chaperones, including mitochondrial HSP70/Grp75, that can help in the degradation of misfolded α-SYN. Due to its ability to protect from oxidative stress, DJ-1 is an interesting target for therapeutic interventions aimed at either increasing the activity of DJ-1 or stabilizing the active form of the protein (Repici and Giorgini, 2019).
- *Mitochondrial DNA.* Increases in age-related mitochondrial DNA mutations have been found in PD brain tissue (Gu et al., 2002). Interestingly, a PD-like mouse model was developed using a mitochondrial targeted restriction endonuclease (Mito-PstI) to selectively cleave mitochondrial DNA double-strand in DA neurons. Using this strategy, mice showed a L-DOPA reversible motor phenotype, progressive neurodegeneration of DA neurons, and striatal DA depletion (Pickrell et al., 2011). Replenishment of mitochondrial DNA might improve neuronal bioenergetic function and prevent further cell death, although it needs additional development to become a potential therapy for PD (Keeney et al., 2009).

3.2 Autophagy-lysosomal pathway dysfunction

The autophagy-lysosomal system is a catabolic process responsible for degrading dysfunctional organelles or misfolded proteins, an essential role in maintaining cellular energy and metabolic homeostasis. Autophagy-lysosomal degradation involves the orchestrated action of a large number of cellular factors (macro-autophagy, micro-autophagy and chaperone-mediated autophagy) that are distinguished by distinct routes of delivery to target lysosomes (Pan et al., 2008). Defects in any step or essential factor, such as formation of autophagosome, lead to accumulation of unwanted proteins e.g., α-SYN (Webb et al., 2003). Certainly, substantial evidence from human post-mortem studies

reveals that autophagy mechanisms become impaired in PD brain, being a critical mechanism for Lewy-related pathology (Dehay et al., 2010). While normal monomeric α-SYN is degraded by chaperone-mediated autophagy, macro-autophagy is involved in the clearance of high molecular weight α-SYN species (oligomers and fibrils). Therefore, the burden of toxic α-SYN aggregates in Lewy-related pathology may compromise the macro-autophagy pathway by interfering with autophagosome formation (Xilouri et al., 2016). In addition, genetic variants in certain lysosomal enzymes are associated with familial PD (Park et al., 2015; Schapira, 2015). These include:

- *GBA1*. *GBA1* encodes glucocerebrosidase (GCase), a lysosomal enzyme that catalyses the hydrolysis of glycolipid glucocerebroside to ceramide and glucose. To date, mutations in the *GBA1* gene constitute numerically the most important risk factor for PD. The type of PD associated with *GBA1* mutations is almost identical to idiopathic PD, except for a slightly younger age of onset and a tendency to more cognitive impairment (Migdalska-Richards and Schapira, 2016). *GBA1* mutations are associated with alterations in lipid levels, leading to accumulation of autophagosomes and of α-SYN oligomeric and fibril forms (Velayati et al., 2010). In addition, mutant GCase is co-localized with α-SYN in Lewy bodies (Goker-Alpan et al., 2008). Current therapeutic approaches for GBA-related PD are based on either small molecule chaperones to increase GBA activities (e.g. ambroxol) or glucosylceramide synthase inhibitors to reduce GBA substrate. Several of these drugs are in clinical trials, none of which yet utilizes oligonucleotide therapeutics, although some of them are under development (Doxakis, 2020).
- *ATP13A2 (PARK9).* ATP13A2 is a late endosomal/lysosomal P5-type transport ATPase that is emerging as a critical regulator of lysosomal functions (van Veen et al., 2014). Overexpression of ATP13A2 suppresses α-SYN toxicity, highlighting the central role of ATP13A2 in PD (Gitler et al., 2009, but not in Daniel et al., 2015). A mouse model lacking ATP13A2 showed α-SYN accumulation, predominantly in the hippocampus, as well as age-related motor deficits (Schultheis et al., 2013). To date, there are no reports showing oligonucleotide therapeutics targeting the ATP13A2 gene.

- *LRRK2 (PARK8*). Leucine-rich repeat kinase 2 (LRRK2) is a 268 kDa multi-domain protein. Mutations in *LRRK2*, the most common genetic cause of late-onset PD, are associated with defects in autophagy. Post-mortem tissue from PD patients shows several point mutations in *PARK8*, with significant DA neurodegeneration, with or without the presence of Lewy-related pathology (Zimprich et al., 2004; Gaig et al., 2007; Manzoni, 2012). Gain-of-kinase-function variants in LRRK2 are known to significantly increase the risk of PD (Greggio et al., 2006; West et al., 2005; Whiffin et al., 2020), suggesting that inhibition of LRRK2 kinase activity is a promising oligonucleotide-based therapeutic strategy.

3.3 Ubiquitin-proteosome system (UPS)

The UPS is the most efficient disposal system of cells, and is mainly responsible for protein catabolism in the cytosol and nucleus, playing a key role in several basic cellular processes (McNaught et al., 2001). Since the UPS is a major route for α-SYN degradation, it is conceivable that the blockage of this pathway could contribute to accumulation of α-SYN and eventual Lewy-related pathology. Indeed, ubiquitinated α-SYN co-localized with proteasome subunits in Lewy bodies in PD, and both structural and functional defects in the 26/20S proteasome have been reported in PD brains (Bennett et al., 1999; McNaught et al., 2001). Furthermore, several other proteins, such as parkin and UCH-L1, along with UPS, are involved in the degradation of misfolded α-SYN.

- *Parkin (PARK2)*. Parkin is an E3 ubiquitin ligase that plays an important role in UPS as well as mitochondrial quality control. Mutations in the parkin gene are associated with autosomal recessive juvenile onset Parkinsonism, while heterozygous mutations may play a role in sporadic PD (Dawson and Dawson, 2010). Mutant parkin (e.g. p.T240R) impairs UPS protein degradation, leading to abnormal accumulation of proteins. Several reports have suggested that parkin gene therapy could be effective in a subset of PD patients with mutations in the α-SYN gene (Mochizuki, 2007). However, contrary to these expectations, the overexpression of mutant and wild-type parkin in rats was linked to progressive DA loss in SNc, α-SYN pathology and mild motor deficits, an effect contrary to expectations (Van Rompuy et al., 2014).

- *UCHL1 (PARK5)*. UCHL1 is a member of a gene family whose products hydrolyze small C-terminal adducts of ubiquitin to generate ubiquitin monomers (Maraganore et al., 2004). A rare gene variant of UCHL1 has been linked with familial PD and a recent meta-analysis demonstrated moderate evidence of an association between a common variant and sporadic PD (Ng ASL et al., 2020). In addition, an increasing number of studies reported on the dysregulation of ncRNA UCHL1 which acts by directly promoting translation UCHL1 protein leading to perturbation of the UPS in PD brains (Riva et al., 2016). Transgenic mice with p.I93M UCHL1 mutation had significant reduction in SNc DA neurons and decreased DA concentration in the striatum, as well as motor deficits (Setsuie et al., 2007). Overall, the evaluation of UCHL1 diagnostic significance and therapeutic potential could also address the setting of novel treatments in PD where no cure is available to date.

3.4 ER stress and unfolded protein response (UPR)

The ER plays a key role in properly folding proteins. Disturbances in the ER homeostasis due to an excessive protein synthesis lead to an accumulation of misfolded/unfolded proteins in the ER lumen. Stressed ER activates UPR, an adaptive signaling pathway, in order to restore protein folding. Three ER stress sensors induce independent and convergent UPR signaling branches helping to maintain homeostasis, or eventually trigger cell death under chronic stress conditions (Mercado et al., 2013). These sensors are PERK (protein kinase R-like ER kinase), IRE1 (inositol requiring enzyme), and ATF6 (activated transcription factor 6). These are inactive in normal conditions and are bound by BIP, an ER chaperone and master regulator of UPR. Release of BIP from these stress sensors during ER stress results in their activation leading to downstream changes in translation and transcription (Kim et al., 2008; Walter and Ron, 2011). There is compelling evidence that UPR activation markers such as phosphorylated PERK and eukaryotic initiation factor (eIF2α), as well as BIP protein levels. are increased in the postmortem SNc tissue of PD patients, and they co-localize with α-SYN in Lewy bodies (Hoozemans et al., 2007; Slodzinski et al., 2009). Moreover, several studies in PD-like animal models revealed a clear activation of markers of UPR branches including activating transcription factor 6 (ATF6), X-box binding protein 1 (XBP1) and C/EBP homologous protein (CHOP)

(Salganik et al., 2015; Gully et al., 2016; Coppola-Segovia et al., 2017). Therefore, the ER/UPR cellular pathway has become a main therapeutic target in PD. Pharmacological modulation of the UPR is challenging considering the physiological importance of the pathway in various organs. However, the selective targeting of ER stress responses in affected tissue/cellular populations using oligonucleotide therapy strategies to improve ER proteostasis could emerge as a solution to overcome side effects.

Taken together, substantial progress in the intracellular molecular pathways considered to be affected in PD highlights that three genes are of great interest in the development of oligonucleotide-based therapies. These include the *SNCA, LRRK2 and GBA* genes. **Table 1** shows the status of potential oligonucleotide therapeutics for PD (see also section 4.3).

4. Challenges and opportunities in oligonucleotide therapeutics for MDD and PD

4.1 Basic information underlying oligonucleotide therapeutics

Remarkable advances in understanding the role of RNA in health and disease have expanded considerably in the last decade. RNA is becoming an increasingly important target for therapeutic intervention. The mRNA molecule conveys information from the genome, and its intrinsic property to complementary base-pair with itself or other molecules represents a powered tool to modify its splicing, translation or abundance for treating diseases (Doxakis, 2020; Li et al., 2020a). Oligonucleotides, including antisense oligonucleotide (ASO), siRNA, short-hairpin RNA (shRNA), miRNA, and anti-microRNA (antagomir) are perhaps the most direct therapeutic strategies for addressing RNA.

Since the first US FDA approved oligonucleotide, Vitravene, in 1998 to treat cytomegalovirus retinitis in immunocompromised patients, oligonucleotide programs have attained increasing importance in drug development (Khorkova and Wahlestedt, 2017; Doxakis, 2020; Li et al., 2020a). More than 200 clinical trials of diverse oligonucleotide therapeutics are registered at http//www.ClinicalTrials.gov and over 10,000 patients have received these compounds since 2016. In part, this is mainly due to the discovery of i) new oligonucleotide chemistries and ligand-oligonucleotide conjugations, ii) new delivery agents, iii) a better understanding of mechanisms of action of oligonucleotides, as well as

iv) an improved knowledge of the molecular mechanisms of the diseases, which have opened opportunities to address novel therapeutic targets that were previously considered non-druggable (**Figure 3**).

4.1.1 Oligonucleotide chemistries

Notably, despite the marked difficulties in delivering oligonucleotides to the brain, there is a growing interest in developing non-viral oligonucleotides for brain disorders (Southwell et al., 2012; Doxakis, 2020). This interest is based on several aspects, including the high target specificity of oligonucleotides, the ability to address previously inaccessible drug targets, a limited systemic exposure and toxicity as well as extended half-life that allows for infrequent dosing. In addition to down regulation of disease-relevant genes, oligonucleotides offer multiple approaches for precise gene upregulation and splice editing, goals not easily achieved by traditional small molecule therapies or monoclonal antibodies. At the same time, oligonucleotide effects are reversible; thus, oligonucleotides do not have many of the problems associated with viral gene therapy – another approach capable of modulating gene expression – which involves genome integration, inability to be delivered repeatedly, and possible host rejection (Khorkova and Wahlestedt, 2017; Smith and Zain, 2019; Alarcón-Arís et al., 2020).

Conversely, for non-viral oligonucleotides to be effective, they must be able to reach the affected brain regions or cells in appropriate concentrations. Furthermore, they must be stable, therefore require additional protective measures (end-blocking, backbone modification, ribose sugar modification, etc.), and maintain effectiveness over time for feasible treatment throughout the course of the disease. Brain diseases often pose problems that limit efficient and effective drug delivery linked to the brain-blood barrier (BBB). Multiple studies have shown that less than 1% of systemically administered oligonucleotides reach the brain (Juliano et al., 2008), and once in the brain compartment, a major limitation is delivery to selected neuronal populations or cell types. Other difficulties encountered in the application of oligonucleotides to treat brain disorders are their

unconventional pharmacokinetic and pharmacodynamic properties, still poorly known, but with far-reaching implications for dosing regimens and clinical efficacy.

To overcome these challenges, several chemical strategies are being implemented. The phosphorothioate backbone is one of the first effective modifications that increases both exo- and endonuclease resistance and facilitates association with carrier proteins in the blood, leading to slower excretion through kidneys, a longer half-life and greater absorption (Juliano, 2016). Other backbone modifications, including phosphorodiamidate morpholino oligonucleotide (PMO), tetramethyl phosphoryl guanidine and peptide nucleic acids (PNA) were proposed, alone or in combination with the sugar modifications (Khorkova and Wahlestedt, 2017). In general, these modifications improve the pharmacological profile of oligonucleotides by decreasing the charge on the oligonucleotide backbone, which may facilitate their uptake into cells and, therefore, increase their potency. In addition, modifications on both sugar and backbone such as PNA and PMO have also been developed to enhance oligomer stability (for more information, see Doxakis, 2020; Li et al., 2020a; Roberts et al., 2020). Based on a backbone of morpholine rings connected by phosphorodiamidate bonds, neutrally charged PMO exhibits very safe chemistry, improving hybridization affinity and reducing binding to nonspecific proteins, which in turn reduces toxicity. However, they are rapidly cleared by the kidney and, therefore, a delivery system is needed to allow better uptake of PMO (Amantana et al., 2007; Iversen et al., 2009).

Newer oligonucleotide chemical modifications, including anhydrohexitol nucleic acids (HNA), cyclohexane nucleic acids (CeNA), and D-altritol nucleic acids (ANA) improve duplex stability with complementary DNA and RNA oligonucleotides (Verbeure et al., 2001; Le et al., 2016). However, the toxicity and *in vivo* safety profile of these chemistries remain to be determined.

Nucleobase modifications have also been introduced. Since the nucleobases provide the main recognition site for Watson–Crick base pairing through specific hydrogen bonding interactions, the scope of nucleobase modification is limited, improving only the binding affinity for the complementary oligonucleotide, but not the resistance to nucleases (Dhuri et al., 2020). Among nucleobase modifications, cytosine analogs have been widely used.

Oligonucleotides containing cytosine-guanosine dinucleotide have been observed to activate the Toll-like receptor and cause immune stimulation (Krieg, 2007). Hence, 5-methyl cytosine-based analogs were used to minimize immune stimulation. Similarly, another cytosine analog, 5-propynil cytosine, increases the binding affinity and efficacy of oligonucleotides (Rapireddy et al., 2011).

4.1.2 Oligonucleotide delivery to the brain

Oligonucleotides are usually administered intravenously, subcutaneously, intranasally, intravitreally, intrathecally, transvascularly, by enema or by submucosal injection. Oral administration is the least preferred option due to the relatively high molecular weight of the oligonucleotides (5–10 kDa for single-stranded) and their very low transcellular permeability. Oral administration is favored if the mRNA target is located in the gastrointestinal tract. Several obstacles need to be overcome before oligonucleotides reach their target. The relative importance of each obstacle/barrier depends on the chemical and physical properties of the oligonucleotides and their associated conjugates. An extensive review of the mechanisms of oligonucleotide delivery to different tissues is beyond the scope of the present article, and we will focus on their selective delivery to the brain.

There have been several attempts to convey adequately oligonucleotides across the BBB. Perhaps the most promising ones involve the conjugation of oligonucleotides with cell penetrating peptides (CPPs). However, the advancement of these delivery systems to the clinic has been limited due to toxicity caused by excessive cationic loading, as well as multiple non-specific interactions with serum and tissue proteins (Boisguerin et al., 2015; Evers et al., 2015). Another interesting strategy involves the use of nanoparticles, not only to protect oligonucleotides from endonucleases, but also to achieve delivery across the BBB. Nanoparticles (also called nanocarriers) consist of a group of diverse assemblies (lipid nanoparticles, polymers, exosomes, among others) that protect oligonucleotides by encapsulation, and as a consequence of their relatively large size, their diffusion through the extracellular matrix of the brain parenchyma is restricted, while ‘free’ oligonucleotides spread easily throughout the brain (Chauhan et al., 2011; Cheng et al., 2012). Among them,

lipid nanoparticles are readily cleared by the cells of the reticuloendothelial system and, for this reason, are often conjugated with ligands such as monoclonal antibodies or transferrin for delivery by receptor-mediated transcytosis across the BBB (Zhang et al., 2003; Soni et al., 2008). More recently, glucose-installed nanocarriers capable of crossing the BBB by active translocation of glucose-transporter1 (GLUT1) expressed in endothelial cells of brain capillaries were successfully used to deliver oligonucleotides (Min et al., 2020). In addition, exosomes have received great interest for oligonucleotide delivery into the brain from their intrinsic ability to cross biological barriers, including the BBB, high stability in the circulation, and minimal immunogenicity and toxicity (Alvarez-Erviti et al., 2011; Lee et al., 2017; Cui et al., 2019; Kim et al., 2019). However, an important consideration to keep in mind with the use of nanocarriers is the presence of an intact BBB, any loss of its integrity due to infection, inflammation, etc. could lead to toxicity from the delivery of the vehicle itself.

Direct conjugation of oligonucleotides with antibodies is another potentially interesting strategy owing to their ability of highly specific recognition and high affinity binding to biological targets. The high molecular weight of the resulting conjugates (~150 kDa) also allows for a prolonged presence in the blood; conversely, the increase in molecular weight might hamper translocation through the endothelial barrier including BBB. The use of antigen-binding fragments instead of antibodies for oligonucleotide conjugation probably facilitates their brain penetration, although a study directly comparing these two approaches is needed to further conclude on this subject (Cortinhas et al., 2020).

Adequate bio-distribution of therapeutic oligonucleotides to the brain is essential to achieve long-lasting effects on gene silencing or gene upregulation for the treatment of brain disorders. In this regard, the transvascular cerebral administration of therapeutic oligonucleotides represents an attractive strategy. However, the inability to effectively cross the BBB has limited the potential utility of systemic administrations for brain oligonucleotide delivery (Boursereau et al., 2015). To overcome some of these limitations and capitalize on the vast capillary network of the brain, approaches that aim to transiently disrupt BBB have gained considerable attention as a way to improve transvascular delivery of therapeutics to the brain (Hersh et al., 2016; Mendoça et al., 2021). Therefore,

pharmacological disruption using hyperosmolar agents, such as mannitol, results in osmotic shrinkage of endothelial cells and opening of tight junctions, leading to a temporary disruption of the BBB for productive brain delivery of therapeutic oligonucleotides (Hersh et al., 2016; Godinho et al., 2018).

Alternatively, physical methods such as focused ultrasound (FUS) has also emerged as a potential new means of effective drug delivery to the brain (Chen et al., 2019). Recent research has shown that, under burst-type energy exposure with the presence of microbubbles, this modality can transiently permeabilize the BBB and increase the bioavailability of therapeutic agents, including chemotherapeutic agents, peptide therapeutics, monoclonal antibodies, and nanoparticles only in the area where the FUS energy is directed. Preliminary results have confirmed the safety and efficacy of this approach for brain tumor treatments and show significant promise for additional indications, increasing the potential of FUS for oligonucleotide delivery to the targeted brain region (Negishi et al., 2015; Lipsman et al., 2018). However, until the long-term effects of repetitive disruptions of the BBB on brain homeostasis are determined, these approaches would appear useful as a single treatment (e.g. malignant brain tumors) rather than for repetitive treatments in chronic disorders such as MDD and PD.

Early research with conjugated siRNAs focused on the attachment of a cholesterol moiety to improve cellular uptake and tissue retention (DiFiglia et al., 2007; Wolfrum et al., 2007). Due to their high hydrophobicity, when locally injected in the brain, cholesterol–conjugated siRNAs display a steep gradient of diffusion from the site of injection, limiting gene knockdown to defined structures around the injection site (Alterman et al., 2015; Nikan et al., 2016). Fine tuning the hydrophobicity of conjugated oligonucleotides using other lipid modalities, such as docosahexaenoic acid (DHA), a polyunsaturated fatty acid, has enabled broader distribution from the site of injection while still allowing higher retention than unconjugated siRNA (Nikan et al., 2017). Although lipid conjugation offers a great means for modulating the spread of distribution within the brain it does not provide cell-type-specific delivery after local injections because oligonucleotides are internalized by both neurons and glia (Nikan et al., 2016, 2017).

Ultimately, there is a surge of interest in delivery of oligonucleotides to specific cell types in the brain using ligand-oligonucleotide conjugates. This cellular selectivity may be achieved by conjugating oligonucleotides to a ligand that interacts selectively with a receptor/transporter expressed in the cellular surface. Ideally, this strategy requires receptors/transporters expressed only in a single cell population in high density, allowing rapid and extensive internalization, and for which high affinity ligands are readily available. Some ligand candidates that demonstrate high affinity and selectivity for brain receptors include anisamide, a ligand that binds to the sigma receptor, and trivalent conjugates provided selective delivery of ASO molecules to the brain (Nakagawa et al., 2010). Likewise, anandamide, a ligand for cannabinoid receptors, provides effective functional delivery of siRNA to several neuronal types (Willibald et al., 2012). However, none of these studies allowed the delivery of oligonucleotides to specific neuronal populations. In contrast, we successfully developed a strategy to supply *in vivo* oligonucleotides selectively to brainstem monoamine neurons (DA, 5-HT and NE). This was achieved by conjugating oligonucleotides with inhibitors of monoamine transporters (MAT) such as sertraline, reboxetine and indatraline, showing nM affinity for MAT, and which are exclusively expressed in monoamine neurons at high densities (Bortolozzi et al., 2012; Artigas and Bortolozzi, 2017; Artigas et al., 2019). MAT inhibitors allow the selective accumulation of oligonucleotides in monoamine neurons after internalization in deep Rab-7-associated vesicles (Ferrés-Coy et al., 2016; Alarcón-Arís et al., 2018; Fullana et al., 2019a) (**Figure 4**). Therefore, although the conjugated ligand-oligonucleotide approach is still under development, it offers a promising path forward for oligonucleotide therapeutics.

As an example of the broad therapeutic potential of oligonucleotides in brain disorders, we review the progress of development of oligonucleotide-based compounds for the treatment of MDD and PD.

4.2 MDD

4.2.1 siRNA

Twenty years after the first description of RNA interference (RNAi), the first RNAi drug Onpattro® was approved by the US FDA for the treatment of hereditary transthyretin amyloidosis in 2018 (Adams et al., 2018). RNAi is a potent and specific post-transcriptional regulation mechanism that occurs naturally in all eukaryotic cells, and has emerged as a powerful therapeutic strategy for precision medicine. siRNA duplex is synthetic, chemically modified short double-stranded RNA that usually contain 21-23 nucleotides in length. To be functional, siRNAs need to be incorporated into a protein complex known as the RNA-induced silencing complex (RISC), prior to the cleavage of antisense active strand and sense RNA sequence. After RISC assembly and maturation, a specific mRNA recognized and bound by the small RNA, based on sequence complementarities, leading to site-specific mRNA cleavage and degradation (**Figure 3**).

At the time of writing this review paper, relatively few studies have examined the potential antidepressant effects induced by non-viral siRNA in preclinical models. As mentioned in section 1, due to their anatomically expanding structure, the monoaminergic systems offer the possibility that siRNA-induced changes in mRNA expression in these discrete brainstem nuclei are immediately translated into functional neurotransmitter changes in interconnected brain areas involved in MDD through the dense axonal network. An early study (Shishkina et al., 2004) using siRNA to knockdown α2-adrenoceptors in neonatal mice, reported a decreased anxiety-like behavior in the elevated plus maze, but the study did not examine whether this effect was linked to an antidepressant-like action. Subsequently, Thakker et al., (2005) examined the effects of the intracerebroventricular (icv) administration of a siRNA targeting SERT using minipumps (0.4 mg/day, 20 days). This treatment evoked a 35-40% decrease of SERT mRNA in the raphe nuclei together with a downregulation of SERT binding sites in all brain regions examined. In parallel, mice treated with SERT-siRNA and the SSRI-citalopram showed a comparable reduction of immobility time in the forced swim test, suggesting that SERT-siRNA may induce clinical antidepressant effects in MDD patients (Thakker et al., 2005). Likewise, a second study examined the neurobiological effects of knocking-down SERT expression after local application in mouse DR of small amounts (10 µg/day, 4 days; see details in Ferrés-Coy et al., 2013b) of the same SERT-siRNA sequence used by Thakker et al., (2005). The SERT-siRNA effects were compared to those produced by SSRI-fluoxetine administration (20

mg/kg/day, 14 days). The partial reduction of SERT expression (40-50%) decreased immobility time in the tail suspension test, displaying an antidepressant potential. Moreover, short-term SERT-siRNA treatment modified mouse brain variables considered to be key markers of antidepressant action: reduced expression and function of 5-HT_{1A} autoreceptors, elevated extracellular 5-HT concentration in forebrain, and increased neurogenesis and expression of plasticity-related genes (e.g. BDNF) in hippocampus. Remarkably, these effects occurred much earlier and were of greater magnitude than those evoked by long-term fluoxetine treatment. These findings highlight the critical role of SERT in the serotonergic function, and show that the reduction of SERT mRNA expression regulates 5-HT neurotransmission more potently than pharmacological blockade of SERT (Ferrés-Coy et al., 2013b).

Somatodendritic 5-HT_{1A} autoreceptors play a critical role in attenuating serotonergic activity (Celada et al., 2001) and, even more, after antidepressant administration (Artigas 2013). Therefore, another study examined the effects of the local application of a pool of siRNAs targeting 5-HT_{1A}R in the DR (at 2.5 or 10 µg/µl; Ferrés-Coy et al., 2013a). Intra-DR application of the 5-HT_{1A}R-siRNAs selectively reduced 5-HT_{1A}R mRNA and 5-HT_{1A} autoreceptor density as assessed by [^{3}H]8-OH-DPAT autoradiography. Mice treated with the 5-HT_{1A}R-siRNAs showed an antidepressant-like behavior, as indicated by a marked reduction of the immobility time in the tail suspension test and in the forced swim test. This behavioral phenotype was accompanied by a greater increase of 5-HT release in medial PFC during the tail suspension test. Hence, simply reducing the 5-HT_{1A} autoreceptor density/sensitivity can produce antidepressant-like effects in rodents by allowing 5-HT neurons to increase their activity and terminal release -an effect promoting stress resiliency- after stress-induced excitatory inputs to the DR (Ferrés-Coy et al., 2013a).

In an attempt to develop translational strategies to target genes expressed in 5-HT neurons, the sequences of siRNAs targeting SERT or 5-HT_{1A}R mRNA were covalently linked to sertraline (selective SERT inhibitor), and the sertraline-conjugated siRNA molecules were administered in mice via icv or intranasally (Bortolozzi et al., 2012; Ferrés-Coy et al., 2016). The conjugation with sertraline allowed a selective accumulation of siRNA sequences in 5-HT neurons through an internalization mechanism dependent on

endosomal activation. Interestingly, despite the ~50% reduction of SERT expression induced by initial doses of SERT-siRNA, the conjugate kept accumulating in 5-HT neurons, showing that the remaining SERT protein was functionally active (Ferrés-Coy et al., 2016). Both, icv or intranasal administration of sertraline-conjugated siRNAs targeting 5-HT_{1A}R or SERT mRNA at doses of 30-100 µg/day evoked antidepressant-like effects in mice comparable to those described previously after local infusion of naked siRNA sequences into the DR (Bortolozzi et al., 2012; Ferrés-Coy et al., 2013a,b, 2016) (**Figure 5a**).

More recently, a study examined the potential antidepressant actions of silencing TASK-3 (KCNK9), a member of two-pore domain (K2P) potassium channel family (Fullana et al., 2019a). The full deletion of TASK3 in mice markedly reduced REM sleep and elicited antidepressant-like effects, suggesting that TASK3 channel blockers may be a new antidepressant drug class (Gotter et al., 2011). TASK3 channels are abundantly expressed in the cerebral cortex, hippocampus, thalamic and hypothalamic nuclei, and cerebellum, as well as in brainstem 5-HT and NE neurons of the DR and locus coeruleus (LC), respectively. TASK3 channels may have therapeutic potential in neuropsychiatric disorders due to their ability to control the resting membrane potential and neuronal excitability (Bayliss and Barrett, 2008; Russo et al., 2012; Borsotto et al., 2015). Given that the constitutive activation of TASK3 channels contributes to hyperpolarize neuronal membranes, we hypothesized that TASK3 knockdown would render 5-HT and NE neurons more sensitive to excitatory inputs (e.g., such as after knocking-down 5-HT_{1A} autoreceptors), therefore enhancing their activity and the stress resiliency. Therefore, a TASK3-siRNA was conjugated to sertraline or reboxetine to promote their selective accumulation in 5-HT and NE neurons, respectively, and the conjugates were intranasally administered at 75 µg for 7 days. Following neuronal internalization, conjugated TASK3-siRNA reduced TASK3 mRNA and protein levels in 5-HT and NE neurons from DR and LC, respectively. Sertraline-TASK3-siRNA desensitized 5-HT_{1A} autoreceptors, induced a robust antidepressant-like behavior, enhanced the hippocampal plasticity, and potentiated the fluoxetine-induced increase on extracellular 5-HT. Similar antidepressant responses were detected for reboxetine-TASK3-siRNA, yet of lower magnitude (Fullana et al., 2019a) (**Figure 5b**). These results further support the notion that TASK3 channels may be a new

target for antidepressant drug development. Given the widespread distribution of TASK3 channels, their selective knockdown in monoamine neurons by conjugated siRNAs is an advantage over the use of drugs, which may cause unwanted side effects due to their interaction with channels expressed in brain areas unrelated to MDD, such as the cerebellum or sensory cortical areas, among others.

4.2.2 shRNA

shRNAs are RNA transcripts with a loop structure and a stem consisting of a paired sense and antisense strand. Unlike siRNAs that preferentially accumulate in the cytoplasm, viral vectors (e.g. lentiviruses - LV, adeno-associated virus - AAV) are required to deliver shRNAs to the cell nucleus, and then they are translocated to the cytoplasm and converted to siRNA by the same RNAi machinery that processes miRNAs and siRNAs (**Figure 3**). Since host cells can continuously synthesize shRNAs, they have several advantages over siRNAs including longer-lasting effects, lower dose requirement, and less off-target effects (Rao et al., 2009). However, viral vectors have certain issues involving genome integration, potential risk of inducing mutations, the inability to be delivered repeatedly, and possible host rejection by immunogenicity (Jiao et al., 2020).

As previously described, dysregulation of the serotonergic system is crucial in MDD. P11 protein (also known as S100A10) is involved in the control of gene transcription, and in the 5-HT signaling. Different studies showed that the 5-HT receptors such as 5-HT_{1B}R, 5-HT_{1D}R and 5-HT_4R, all interacted with the p11 protein. In addition, p11 levels are regulated by antidepressant treatments and, conversely, p11 protein modifies responses to antidepressants and/or regulates the depression-like behaviors in rodents (Svenningsson et al., 2006, 2013). Using a shRNA targeting p11 mRNA cloned in an AAV vector, and injected into the mouse nucleus accumbens, Alexander et al., (2010) showed that p11 knockdown increases depressive-like behaviors; conversely, increased p11 expression in nucleus accumbens of p11 knockout mice reverses depressive-like behaviors.

Other studies reported the use of shRNAs against i) glyoxalase-1 (critical enzyme for the detoxification of methyl-glyoxal, a cytotoxic product of glycolysis), ii) corticotrophin

releasing factor receptor type 1 (CRFR1), or iii) protein kinase C epsilon (PKCε) cloned in LV vector. These shRNA oligonucleotides showed anxiolytic-like effects, but not antidepressant-like effects, after local administration in the cingulate cortex or in the amygdala of rodents (Hovatta et al., 2005; Sztainberg et al., 2010; Lesscher et al., 2008).

4.2.3 miRNA and antagomir

miRNAs are double-stranded short noncoding RNAs, generally 20-25 nucleotides in length, that regulate gene expression through binding to the 3′ untranslated region (UTR) of a targeted mRNA. They are relatively conserved from an evolutionary point of view, and furthermore, miRNAs are predicted to regulate more than half of all protein-coding genes in mammals, which emphasize their importance in transcriptional regulation in health and in pathological related pathways (Friedman et al., 2009, Esteller, 2011). Each miRNA can hybridize to large numbers of RNAs, including mRNAs and long noncoding RNAs, with a major collective biological outcome (**Figure 3**). Understanding the critical role of miRNAs in cellular and molecular biology and unveiling the mechanisms of dysregulation of miRNAs under disease conditions has made these molecules very attractive propositions with respect to drug development. On the other hand, single-stranded antagomirs incorporating either locked nucleic acid (LNA) or 2'-O-methy (2'-OMe) residues can inhibit specific miRNAs, and thus upregulate targeted mRNA expression offering a great opportunity for precision medicine (Cheng et al., 2015; Nakamori et al., 2019).

Accumulating data support the association between several psychiatric disorders, including MDD, and changes in miRNA regulation or function. Such changes were identified in post-mortem brain tissue samples, cerebrospinal fluid (CSF) or peripheral blood of MDD patients (Issler and Chen, 2015; Artigas et al., 2018; Lopez et al., 2018). Likewise, the involvement of miRNA pathways in the underlining biological mechanism of depressive behavior was described in several MDD-like animal models (see **Table 2**). Here, we review some of the key miRNAs associated with the serotonergic system, as well as the stress response and neuroplasticity with the potential to become new tools for the treatment of MDD.

As mentioned above (section 2), the research in the field of MDD has shifted away from biochemical theories that link changes in function of a single neurotransmitter to depressed mood. However, the 50 year-old "5-HT hypothesis" of clinical depression (Coppen, 1967) is still of interest. A major reason for the longevity of this theory is the fact that SSRIs and other 5-HT-enhancing drugs are useful antidepressant drugs, extensively used in the clinic. Hence, it is not surprising that a great deal of research was conducted in order to decipher the role of miRNAs within the serotonergic system. In an extensive study, Issler et al., (2014) reported lower levels of miR-135 in blood and brain samples from MDD patients compared to normal subjects, and identified miR-135 as a strong modulator of the serotonergic system. Both *in vitro* and *in vivo* studies showed that miR-135 targets (among others) and directly regulates both SERT and 5-HT_{1A}R transcripts. As SERT is the main target for SSRIs and the activation of 5-HT_{1A} autoreceptors decreases the synaptic 5-HT concentration, miR-135 therapeutic potential was recognized. Therefore, miR-135 overexpression specifically in 5-HT neurons evokes antidepressant- and anxiolytic-like effects in mice; conversely, miR-135 knockdown increased anxiety-like effects and decreased antidepressant drug responses. In addition, acute and/or chronic administration of SSRI-fluoxetine increased miR-135 levels in the raphe nuclei of mice (Issler et al., 2014).

In another study, exposure to stressful conditions in adolescence induced anxiety-like behaviors in adult rats, an effect associated with reduced miR-135 levels and in parallel, increased 5-HT_{1A}R expression was found in the medial PFC. Treatments with SSRIs alleviated the anxiety behaviors and reversed miR-135 and 5-HT_{1A}R expression levels (Liu et al., 2017). Likewise, *in silico* predicted targets and *in vivo* studies demonstrated a direct regulation of the mineralocorticoid receptor by miR-135 (Mannironi et al., 2013). The same research group also reported that pharmacological inhibition of miR-135 in the amygdala increased anxiety-like behaviors, which were partly explained by a direct regulation of two key regulators of synaptic vesicle fusion, complexin-1 (CPLX1) and complexin-2 (CPLX2) by miR-135 (Mannironi et al., 2018). Taken together, there is overwhelming evidence in humans and animal models to support the involvement of miR-135 in regulating the expression of several key transcripts (e.g. SERT, 5-HT_{1A}R, CPLX1/2, etc.) in brain circuits involved in MDD, highlighting miR-135 as a promising candidate for future research aimed at elucidating its therapeutic potential in MDD. To this end, miCure Therapeutics designed

several synthetic miR-135 double strand oligonucleotides of different chemical backbone combined with different sugar modifications that aimed to increase potency and reduce innate immune toxicity. The synthetic miRNAs were tested *in vitro* and *in vivo* for their ability to regulate SERT and 5-HT_{1A}R transcripts, as well as their potency in alleviating depressive-like behavior in mouse models. Moreover, the toxicity profile of the potent miRNA candidates was tested in a cytokine activation assay. As mentioned above, one of the biggest hurdles for progressing miRNAs in the clinic is finding an appropriate delivery system that would guide intact miRNAs to their right action site in the brain. To overcome this challenge, we tested ligand-oligonucleotide conjugates, namely sertraline conjugate with synthetic miR-135 after intranasal administration. To date, we confirmed the enrichment of the sertraline conjugated-synthetic miR-135 (c-miR-135) in raphe 5-HT neurons of mice. Moreover, intranasal c-miR-135 administration induced parallel antidepressant-like behavioral responses with significant reductions of SERT and 5-HT_{1A}R protein levels in DR (miCure Therapeutics's c-miR-135 under development).

Previous studies also indicated that SERT is a target of miR-16 (Baudry et al., 2010). Local infusion of miR-16 into DR of mice mitigated the behavioral deficits induced by chronic stress, suggesting the therapeutic effect of the SSRIs may be mediated via altering the expression of miR-16 (Baudry et al., 2010). In an additional study, reduced miR-16 levels were found in CSF from drug-free MDD patients compared to control subjects. The miR-16 levels were negatively correlated with MDD severity (as assessed by the Hamilton Depression Rating Scale), and positively associated with CSF 5-HT levels (Song et al., 2015). Furthermore, icv administration of an antagomir (anti-miR-16) to rats evoked depressive-like behaviors, and lower CSF miR-16, higher CSF 5-HT and increased raphe SERT protein levels than control rats (Song et al., 2015).

Others miRNAs in different related pathways outside the serotonergic system, including miRNAs in synaptic plasticity and in stress, have been involved in MDD pathophysiology, being potential targets to develop new therapeutic agents. Hence, Lopez et al., (2014) reported a downregulation of miR-1202 in depressed individuals. miR-1202 is primate-specific, preferentially enriched in brain tissue and targets the expression of the gene encoding metabotropic glutamate receptor-4, which was upregulated in MDD patient

samples. Interestingly, a recent study showed that all three microRNAs (miR-135, miR-16 and miR1202) have been found to be reduced in the serum of depressed individuals, also suggesting their possible use as MDD biomarkers (Gheysarzadeh et al, 2018). Moreover, miR-144-5 targets the protein C (PKC) and β-catenin pathways associated with MDD (Wang et al., 2015a), and miR-144-5 is involved in the response to mood stabilizer treatment and stress responses (Zhou et al., 2009). Others downregulated miRNAs: miR-30a-5p, miR-34a-5p and miR-221 were identified in blood of MDD patients. These miRNAs target different genes such as serine protein kinase (AKT), corticotrophin-releasing hormone receptor (CRHR1) and glutamate transporters (SCL1A2) found to be altered in pathway related to MDD (Wan et al., 2015). In addition, post-mortem PFC tissue from MDD patients showed increased levels of miR-124-3p as compared to controls. Interestingly, miR-124-3p target genes are glutamate receptor-3, glutamate receptor-4, and glucocorticoid receptor (NR3C1), which are involved in stress response and neuroplasticity (Roy et al., 2017). MiRNA expression analysis profile in rat PFC following chronic corticosterone exposure revealed differential expression of 26 miRNAs. Target prediction analysis for the miRNAs that showed the biggest alteration following corticosterone - miR-124 and miR-218 - revealed critical genes that earlier been reported to be associated with MDD and stress-related disorders, include: glucocorticoid receptors (NR3C1 and NR3C2), and BDNF, among others (Dwivedi et al., 2017).

Since the critical role of BNDF in MDD pathophysiology (Dwivedi et al., 2009), expression of BDNF and miRNAs that target BDNF gene have been studied in MDD patients and in MDD-like mouse models (**Table 2**). Li et al., (2013) found that two putative BDNF associated miRNAs, miR-182 and miR-132 were upregulated along with decreased levels of BDNF in MDD patients and that there was a significant negative correlation between the Self-Rating Depression Scale score and serum BDNF levels, and a positive correlation between the Self-Rating Depression Scale score and miR-132 levels. Likewise, chronic stress induced increased levels of miR-124a and miR-10b in the rat hippocampus coincides with lower BDNF levels (Bahi et al., 2014; Jiang and Zhu, 2015). Hippocampal miR-124a overexpression exacerbated stress-induced depressive-like behavior. In contrast, inhibition of miR-124a signaling in the hippocampus using a lentiviral-mediated silencer

expression ‘‘rescued’’ the anti-depressant effect seen in BDNF-overexpressing animals (Bahi et al., 2014).

Based on these studies, miRNAs could be used as molecular targets to develop new therapeutic agents for brain disorders. Interestingly, in cancer biology, several therapeutic approaches have been used either to reduce or overexpress miRNA associated with proliferation. For example, miRNA oligonucleotides have been generated, which can directly compete with endogenous miRNAs. This strategy has been successfully used to knockdown specific miR-21, which is overexpressed in several types of cancer (Si et al., 2007). The other strategy to reduce miRNA expression is to employ ASOs, which requires a sequence with perfect complementarity to the target endogenous miRNA. miRNAs can also be overexpressed and/or miRNA mimics can be used to increase specific miRNA expression. Several of these strategies are in pipeline for neurodegenerative diseases (Meng et al., 2014), but it will be interesting to see if these strategies are useful in the complex disorders such as MDD.

4.3 PD

4.3.1 ASO

ASOs are single-stranded DNA (ssDNA)-based compounds designed to anneal to target RNA transcripts through Watson-Crick base pairing. Depending upon the base and backbone chemistries, ASOs can modify gene expression through inducing a variety of mechanisms including suppressing translation, altering splicing, modifying polyadenylation or degrading RNA by activating RNase-H (**Figure 3**).

Multiple lines of evidence support a pivotal role of α-SYN protein in the pathogenesis of PD. The observation that multiplication of the α-SYN gene leads to α-synucleinopathy and neurological disease in humans is a solid premise to develop oligonucleotide therapies aimed at inhibiting α-SYN synthesis (**Table 1**). One of the first approaches using ASO compounds designed against α-SYN was developed by IONIS pharmaceuticals. They reported an ASO-induced reduction of α-SYN levels in rodent models received intrastriatal

α-SYN preformed fibrils (Cole et al., 2016). Currently, Biogen described a phase 1 study of intrathecal administration of BIIB101 ASO targeting α-SYN (NCT04165486) in patients with multiple system atrophy to evaluate the safety and tolerability of the compound (Doxakis, 2020).

Recent studies using PD-like animal models reported benefits of chemically-modified ASO molecules targeting α-SYN as a disease modifying therapy for PD and other synucleinopathies. Among these, an ASO with amino-bridged nucleic acid-modified bases at each end and a DNA core was designed to downregulated α-SYN mRNA and protein levels in transgenic mice expressing the human *SNCA* gene with the A53T mutation. ASO administration (100 μg, icv) decreased α-SYN levels ~40-50% for 2 weeks and ameliorated the motor deficits (Uehara et al., 2019). Furthermore, we also reported that 1233-ASO conjugated to the triple MAT inhibitor, indatraline, was selectively accumulated in 5-HT, DA and NE neurons after intranasal administration in wild-type mice, leading to a reduction of ~30% of α-SYN expression in monoamine nuclei. In parallel, the mice showed an increased monoamine neurotransmission in cortical and subcortical brain regions (Alarcón-Arís et al., 2018). The sequence 1233-ASO was developed such that the target mRNA displays homology to murine, rhesus macaque and human α-SYN. Remarkably, indatraline-1233-ASO administration to aged nonhuman primates (1 mg/day for 28 days, icv) specifically decreased α-SYN protein levels in monoamine brain areas, providing an initial demonstration of specific neuronal delivery of conjugated ASO in monkeys, whose brain anatomy is much closer to the human brain than that of rodents (Alarcón-Arís et al., 2020). In addition, indatraline-1233-ASO was highly effective in reducing the synthesis and accumulation of human α-SYN in DA and NE neurons of double mutant human A30P*A53T* α-SYN transgenic mice (Pavia-Collado et al., 2021). Likewise, the indatraline-1337-ASO sequence-designed specifically to target human α-SYN transgene cloned in the adeno-associated-viral vector (AAV5)- reduced α-SYN synthesis in DA neurons of mice overexpressing human α-SYN and its accumulation in the brain connectome over a sustained period of time (2 weeks after the end of treatment). The mice treated with indatraline-1337-ASO (100 μg/day for 28 days, using minipumps) also showed

an improvement of α-SYN pathology-associated deficiencies on DA neurotransmission (Alarcón-Arís et al., 2020) (**Figure 5c**).

LRRK2 mutations are the most common cause of autosomal dominant PD for 5-15% of dominant familial PD and 1-3% of idiopathic PD (Healy et al., 2008). Given that kinase activity is currently the most appealing therapeutic target in LRRK2-PD, several studies are focused on the discovery of kinase inhibitors, two of which are in clinical trials (DNL201, NCT03710707 and DNL151, NCT04056689). An alternative approach is the use of ASO to decrease LRRK2 expression and, thereby, its function. Indeed, it was reported that the single administration of a LRRK2 ASO (700 μg, icv) reduced LRRK2 mRNA and protein levels, ameliorating α-SYN pathology (Zhao et al., 2017). Currently a phase 1 clinical trial (NCT03976349) has begun to assess the safety, tolerability, and pharmacokinetics of LRRK2 ASO BIIB094 through intrathecal administration in PD patients.

Heterozygous mutations in GBA are the single largest risk factor for PD (Migdalska-Richards and Schapira, 2016). The precise mechanisms by which GBA mutations predispose to α-synucleinopathies and affect disease progression are still poorly understood. Several therapeutic strategies including gene therapy and small molecules are under development for patients carrying this particular PD risk. Chamishi Therapeutics was founded in 2019 with a specific focus on developing ASO therapeutics for GBA-linked PD.

4.3.2 siRNA

In addition to ASO therapeutics for α-SYN, siRNA compounds have been also developed against α-SYN as a viable therapeutic strategy for PD. Naked siRNAs targeting α-SYN transcripts as well as nanoconjugates including i) anionic liposomes loaded with siRNA-protamine complex, ii) polyethylenimine-siRNA, iii) peptide-siRNA complex and iv) rabies virus glycoprotein-exosome-siRNA induce a robust reduction of both α-SYN mRNA and protein in neuronal cultures and in PD-like mouse/rat models, which delay α-SYN pathology development (Hayashita-Kinoh et al., 2006; Lewis et al., 2008; Cooper et al., 2014; Helmschrodt et al., 2017; Schlich et al., 2017; Acharya et al., 2019; Spencer et al., 2019; Zharikov et al., 2019).

An early study in nonhuman primates, also showed that the intra-SNc administration of siRNA targeting α-SYN significantly suppressed α-SYN expression (~40-50%) and had no overt adverse consequences (McCormack et al., 2010). Likewise, we reported that intranasal administration of an indataline-conjugated siRNA against α-SYN specifically reduced α-SYN mRNA and protein levels (~30%) in monoamine neurons of normal mice, without causing monoaminergic neurodegeneration (Alarcón-Arís et al., 2018).

4.3.3 shRNA

Using shRNA cloned in AAV vectors to knockdown α-SYN (>70% reduction), several reports indicated degeneration of DA neurons and striatal denervation in rats and nonhuman primates (Gorbatyuk et al., 2010; Khodr et al., 2014; Collier et al., 2016; Benskey et al., 2018). These data are important in the context of ongoing attempts to develop PD therapeutics targeting α-SYN expression using shRNA. Since α-SYN protein is essential for synaptic homeostasis and neurotransmission, its depletion would dramatically alter brain function, arguing for the need to maintain a threshold for α-SYN knockdown.

Furthermore, instead of targeting α-SYN directly, other approaches have focused on reducing inflammation through class II transactivator-targeting shRNAs that attenuate MHCII expression, peripheral immune cell infiltration, and α-SYN-induced neurodegeneration (Williams et al., 2018). In addition, silencing of Nurr1 by shRNAs was designed as a potential strategy for the management of levodopa-induced dyskinesias (Sellnow et al., 2015). However, since Nurr1 has multiple functions including protection of DA neurons against neurotoxins and suppressing neuroinflammation, there is still much research needed before the Nurr1-based PD therapeutics can be considered for the treatment of PD.

4.3.4 miRNA and antagomir

Altered expression of certain miRNAs in the brains of PD patients suggests that they could have a pivotal role in the pathogenesis of PD, thus offering a great therapeutic

potential (Junn and Mouradian, 2012). Initially, miRNA-7 was identified to bind to the 3′-UTRs of α-SYN mRNA and downregulate its expression (Junn et al., 2009). Since then, five additional miRNAs, miR-153, miR-34b, miR-34c, miR-214, and miR-1643, were also found to bind to the 3′-UTR of α-SYN mRNA and inhibit its expression (Doxakis, 2010; Kabaria et al., 2015; Wang et al., 2015b; Nakamori et al., 2019). Among them, miR-7, miR-34b, and miR-34c were shown to be decreased in PD brains (Minones-Moyano et al., 2011), suggesting that decreased expression of these particular miRNAs in PD brains can result in elevated α-SYN levels, thus contributing to PD pathogenesis.

In addition, miRNA-205 has been reported to bind to the 3′-UTR of LRRK2 mRNA and repress its expression. Decreased levels of miR-205 were found in the frontal cortex and striatum from PD patients, together with a parallel elevation of LRRK2 expression (Cho et al., 2013). This observation suggests that downregulation of miR-205 might contribute to the pathogenic increase of LRRK2 in PD brains.

Since neuroinflammation may play a key role in the pathogenesis of PD (Gelders et al., 2018), strategies using miRNAs to inhibit neuroinflammatory pathways have also been explored. In this regard, miRNA-155, a well-known modulator of inflammatory response, is reportedly upregulated in the SNc of a PD-like mouse model. On the other hand, deletion of the miR-155 gene prevented microglial activation and mitigated the loss of nigral TH-positive neurons of PD-like mice (Thome et al., 2016). Overall, although there is no established therapy or ongoing clinical trials using miRNAs, targeting miRNAs seems to be a potential therapeutic intervention for PD.

4.3.5 Others: aptamers

Aptamers are short, single-stranded DNA or RNA molecules that can bind to a wide range of target proteins with high affinity and specificity. They are considered as “chemical antibodies” and are widely used as a substitute for antibodies. Compared with the conventional antibodies, aptamers have unique features such as i) they are non-immunogenic and non-toxic, ii) they show high thermal stability and maintain their structures over repeated cycles of denaturation/renaturation; and iii) they can be easily

generated by chemical synthesis, and can be easily labeled and adjusted (Qu et al., 2017). With the advancements of these technologies, aptamers could bind to target proteins to interrupt their accumulation, subsequently blocking or preventing neurodegenerative processes. Moreover, aptamers can discriminate between different conformations of the same target protein, therefore, resulting in more precise diagnosis tools to discriminate - for instance - between different forms of misfolded α-SYN in PD patients (Zheng et al., 2018).

Since the approval by US FDA of the Macugen's apatamer in 2004, the number of studies on the applications of aptamers has been rapidly increased (Bunka and Stockley, 2006). Since α-SYN oligomers are reported to be cytotoxic and are likely to play a crucial role in PD pathogenesis, several DNA aptamers against α-SYN have been designed to have a better affinity for the oligomeric form rather than monomeric or fibrillary forms (Tsukakoshi et al., 2012). Among them, some were used as aptasensors in order to detect α-SYN oligomers for clinical applications and high-throughput screening of small molecules for structure-activity relationship studies (Bouvier-Müller and Ducongé, 2018).

5. Conclusions and outlook

This is a very exciting time for the field of oligonucleotide therapeutics as a range of new approaches and potential new clinical applications have emerged, including substantial achievements in the central nervous system. Indeed, oligonucleotides with diverse mechanisms of action represent viable candidates for the therapy of brain disorders, including MDD and PD. One of the main arguments for oligonucleotide-based therapeutics has always been the ability to address targets that are not 'druggable' with conventional agents. High target specificity and a relatively single design of classical ASOs and siRNAs, as well as the discovery of antagomirs for miRNAs that allow up-regulation of gene expression, ensure continued interest in the development of oligonucleotides. The advantages of non-viral oligonucleotide delivery over gene therapy include reversibility of the treatment and lack of need for the viral vectors with their inherent liabilities.

However, in this promising landscape, the delivery to the brain compartment remains a key obstacle. The issue is not simply to get oligonucleotides to the brain, but getting them

to selective brain cells and to intracellular sites where they actually function. Fortunately, challenging delivery issues are being addressed using a large variety of innovative approaches that have been described in this review. Among these, currently ligand-oligonucleotide conjugates seem to offer the greatest potential for future development. In fact, the use of high affinity, highly selective ligands, e.g. MAT inhibitors (sertraline, nomifensine, indatraline) allow the possibility of very precise targeting of particular genes expressed in monoamine neurons. For brain disorders such as MDD and PD, where monoamine systems play a key role in neuropathology and treatment, the molecular changes occurring at the cell body level in the brainstem can be rapidly translated into functional changes in the interconnected brain regions thought to be the monoamine connectome. This anatomical feature may be particularly useful for oligonucleotide therapeutics since modulations of mRNA expression in discrete brainstem nuclei would have a strong impact on monoamine function in distant brain areas, offering unprecedented value.

Conflict of interest statement

F. Artigas and A. Bortolozzi are inventors of the patent WO/2011/131693 issued for the selected delivery of oligonucleotides to specific neuron populations. A. Bortolozzi is also inventor of the patents WO/2014/064257 and WO/2014/064258 for conjugated oligonucleotide to decrease α-synuclein expression in monoaminergic neurons as a potential treatment of PD. S. Manashirov is employed by miCure Therapeutics, LTD. Alon Chen has no conflicts of interest.

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Table 1. Current status and patents focusing on oligonucleotide therapeutics for PD

Genes	**Potential oligonucleotide therapeutic**	**Current Status**
SNCA	Knockdown: ASO, siRNA, shRNA, miRNA	Preclinical phase: ISIS Pharmaceuticals *(Carlsbad, USA)*, Patent WO2012068405: New oligonucleotide library to decrease α-SYN expression University Of Medicine & Dentistry of New Jersey (Somerset, USA), Patent WO2010129791: Use of miRNA to decrease α-SYN expression Isis Innovation (Oxford, GB), Patent WO2007135426: Agents (siRNA, DNA, ribozyme) to decrease α-SYN expression levels Children's Memorial Hospital (Chicago, USA), Patent WO2006039253: siRNA to decrease α-SYN expression nLife Therapeutics (Granada, Spain), Patent WO2014064257, WO2011131693: Conjugated oligonucleotide to decrease α-SYN expression in monoaminergic neurons
LRRK2	Knockdown: ASO	Clinical phase 1: Biogen/IONIS ASO BIIB094, ClinicalTrials.gov

		Identifier: NCT03976349
GBA	Knockdown: ASO	Preclinical phase: The Silverstein Foundation for Parkinson's with GBA together with Q-State Biosciences have launched Chamishi Therapeutics, a company focus on developing ASO therapies for PD patients carrying mutations in the GBA gene

Table 2. Studies on the involvement of miRNAs in MDD-like animal models

miRNAs	Samples	Target genes	Effects	Reference
miR-18a	Rats, Paraventricular nucleus (PVN)	Glucocorticoid receptor (GR, NR3C1)	Strain of stress-sensitive rats (F344) expressed lower levels of the NR3C1 protein and higher levels of miR-18a in the PVN compared to Sprague-Dawley rats.	(Uchida et al., 2008)
miR-16	Mice, Raphe nucleus (RN)	Serotonin transporter (SERT)	*In vitro* validation of SERT as miR-16 target. Fluoxetine infusion into RN increased miR-16 levels, and decreased SERT levels.	(Baudry et al., 2010)
miR-34c	Mice, Central amygdala (CeA)	Corticotrophin releasing factor receptor type 1 (CRFR1)	Acute restrained stress upregulated miR-34c expression. Overexpression of miR-34c in the CeA had an anxiolytic effect. CRFR1 was shown to be target of miR-34c.	(Haramati et al., 2011)
miR-16	Mice, Hippocampus	SERT	MiR-16 mediated the adult neurogenesis in the mouse hippocampus. Fluoxetine decreased hippocampal miR-16 levels, which in turn increased SERT expression.	(Launay et al., 2011)
miR-16	Rats, Hippocampus	BDNF	Depression-like behaviors were induced in rats by maternal deprivation (MD), and chronic unpredictable stress (CUS). MD, but not CUS, increased miR-16 levels and reduced hippocampal BDNF mRNA expression.	(Bai et al., 2012)
miR-504	Rats, Nucleus accumbens (NAc)	DA D1 and D2 receptors	MD induced depression-like behaviors, which were paralleled an increase of miR-504 levels and a reduction of DA D1 and D2 receptor expression in the NAc.	(Zhang et al., 2013)
miR-212	Rats, Blood, and dentate gyrus (DG)	BDNF	Electroconvulsive therapy increased miR-212 levels in DG and blood.	(Ryan et al., 2013)
miR-451	Rats, Hippocampus	Not applicable	MiR-451 displayed an altered expression due to maternal separation (MS) that was reversed by antidepressant treatments.	(O'Connor et al., 2013)
miR-135	Mice, Dorsal raphe nucleus	SERT 5-HT_{1A} receptor	MiR-135 knockdown increased SERT and 5-HT_{1A} receptor expression, evoking anxiety-like behaviors and attenuating response to antidepressants	(Issler et al., 2014)
miR-19b	Mice, Amygdala	β-adrenergic receptor-1	MiR-19b levels in the amygdala were increased following chronic social defeat stress (CSDS). MiR-19b was shown to target the β-adrenergic receptor-1.	(Volk et al., 2014)
miR-375-3p, miR-375-5p, miR-200b-3p, miR-672-5p, and miR-466-5p	Mice Sperm, Hippocampus	β-catenin-1	Exposure to traumatic stress in early life using a mouse model of unpredictable maternal separation combined with unpredictable maternal stress induced anxiety-like effects and upregulation of sperm miRNAs in the F1 generation. Cultured cells transfected with a miR-375	(Gapp et al., 2014)

			mimic showed a downregulation of β-catenin-1.	
miR-132	Mice, Hippocampus	Not applicable	Oleanolic acid reversed depression-like phenotype, and restored CMS-induced miR-132 downregulation.	(Yi et al. 2014)
miR-206	Rats, Hippocampus	BDNF	MiR-206 was decreased in ketamine-treated rats. Hippocampal BDNF was identified as a direct target gene of miR-206.	(Yang et al. 2014)
miR-124	Rats, Hippocampus	BDNF	CSDS induced increased miR-124 levels in hippocampus. Knockdown of miR-124 expression abolished the depression-like phenotype in CSDS rats.	(Bahi et al., 2014)
miR-10B	Rats, Hippocampus	BDNF	CUS induced depression-like behaviors that coincided with higher hippocampal miR-10B and lower BDNF expression levels. A direct regulation of BDNF 3'UTR by miR-10B was demonstrated *in vitro*.	(Jiang and Zhu, 2015)
miR-24-2-5p, miR-27a-3p, miR-30e-5p, miR-362-3p, miR-139-5p, miR-28-3p, miR-326-3p, miR-99b-5p, miR-126a-3p, miR-708-5p	Rats, Blood, medial prefrontal cortex (mPFC)	Not applicable	Lower expression of miR-24-2-5p, miR-27a-3p, miR-30e-5p, miR-362-3p predicted a vulnerability to CSDS. After exposure to chronic stress miR-139-5p, miR-28-3p, miR-326-3p, miR-99b-5p expression levels decreased in animals exhibiting resilience to defeat. Levels of miR-126-a-3p and miR-708-5p were elevated in the mPFC of rats vulnerable to CSDS.	(Chen et al., 2015)
miR-9	Rats, Striatum, NAc	DA D2 receptor	Maternal deprivation and CUS decreased miR-9 levels and increased DA D2 receptor mRNA expression in the striatum and NAc.	(Zhang et al., 2015)
miR-18a	Mice, Frontal lobe	Not applicable	Chronic unpredictable mild stress (CUMS) significantly reduced miR-18a levels, which were reversed by antidepressant duloxetine.	(Pan and Liu, 2015)
miR-124	Mice, Hippocampus	Histone deacetylase 4-5 (HDAC4-5), glycogen synthase kinase 3 (GSK-3)	CUMS reduced hippocampal miR-124 and induced depressive-like behaviours that was reversed by miR-124 overexpression	(Higuchi et al., 2016)
miR-17-92	Mice, Adult neuronal progenitors	Glucocorticoid pathway related genes	MiR-17-92 knockout mice or miR-17-92 overexpressing mice showed elevated or reduced depression-like behaviors, respectively.	(Jin et al., 2016)
miR-15-5p, miR-144-3p, miR-582-5p, miR-879-5p	Mice, mPFC	GABA transporter type 3 (GAT-3), vesicular GABA transporter (VGAT), glutamate descarboxilase 67 (GAD-679	CUMS induced depression-like phenotype, and reduced the expression of several miRNAs in the mPFC.	(Ma et al., 2016)
miR-383-5p, miR-764-5p	Rats, Serum	Not applicable	CUMS-induced depression like-behavior was reduced after electro-acupuncture intervention. This effect was paralleled by a reverse expression pattern of miR-383-5p and miR-764-5p.	(Duan et al., 2016)
miR-155	Mice, Hippocampus	Inflammatory cytokines IL-6 and TNF-	MiR-155 knockout mice showed reduced anxiety- and depression-	(Fonken et al., 2016)

			like behaviors.	
miR-124	Mice, Hippocampus, cell line	GR	MiR-124 antagomir reversed the depression-like response induced by chronic corticosterone administration.	(Wang et al., 2017)
miR-9-5p, miR-128-1-5p, miR-382-5p, miR-16-5p, miR-129-5p, miR-219a-5p	Mice, PFC	Not applicable	CMS induced significant increases in these miRNA pathways.	(Buran et al., 2017)
miR-135a, miR-16	Rats, PFC	5-HT_{1A} receptor	The inescapable stress of early adolescence induced depression-like behavior, decreasing prefrontal miR-135a and increasing hippocampal miR-16.	(Liu et al., 2017)
miR-455-3p, miR-30e-3p	Rats, Ventral hippocampus (vHPC)	Not applicable	MiR-435-5p was significantly higher in the vHPC of the rats that were resilient to the CSDS compared to vulnerable rats. MiR-30e-3p was significantly lower in vulnerable rats compared to resilient.	(Pearson-Leary et al., 2017)
miR-124a	Rats, Basolateral amygdala (BLA)	GR	CUMS induced a depressive-like behavior that was positively correlated with BLA miR-124a levels and negatively correlated with BLA GR levels.	(Xu et al., 2017)
miR-135a	Mice, Amygdala	Complexin-1,2	Knockdown of miR-135a in the mouse amygdala increased the anxiety-like responses.	(Mannironi et al., 2018)
miR-134	Rats, BLA	Not applicable	CUMS caused a depression-like behavior leading decreased miR-134 levels in the BLA of rats.	(Yu et al., 2018)
miR-134	Rats, Dorsal hippocampus	BDNF, synaptophysin (SYN) and postsynaptic density protein 95 (PSD95)	CUMS induced depressive-like behaviors and increased miR-134 levels.	(Shen et al., 2019)
miR-30	Mice, DG	Rcor1, MII3, Ppp3r1, Gnb1, Gpr125, Nrp1	CSDS induced depression-like phenotype and a downregulation of miR-30 levels in the DG.	(Khandelwal et al., 2019)
miR-99a	Mice, Hypothalamus	FK506-binding protein 51 (FKBP51)	Both ovariectomy plus CMS reduced miR-99a levels, and increased FKBP51 levels in the hypothalamus.	(Yang et al., 2019b)
miR-301b	Mice, Hippocampus	Neuronal pentraxin II (NPTX2)	Upregulation of miR-301b impaired cognitive function in mice with depressive-like behavior. NPTX2 mRNA is a direct target to miR-301b.	(Tang et al., 2019)
miR-26a-2	Mice, Dorsal raphe nucleus (DRN)	5-HT_{1A} receptor	MiR-26a-2 downregulated 5-HT_{1A} receptor mRNA expression. MiR-26a-2 levels were upregulated in the DRN by antidepressant imipramine. Transgenic mouse model overexpressing miR-26a-2 in 5-HT neurons displayed improved behavioral resiliency to social defeat. MiR-26a-2 knockdown in 5-HT neurons increased anxiety-like behaviors.	(Xie et al., 2019)
miR-144-3p, miR-879-5p, miR-15b-5p, miR-582-5p	Mice, NAc	VGAT, GAT-3	CUMS-induced depression-like responses resulted in increased levels of several miRNAs, and reduction of the target mRNAs in the NAc.	(Ma et al., 2019)
miR-124	Mice, PFC	Sirtuin-1 (SIRT1), CAMP responsive element binding protein 1 (CREB1)	CUMS induced depression-like behaviors and up-regulated miR-124, while down-regulated SIRT expression in the PFC. MiR-124	(Gu et al., 2019)

			overexpression exacerbated depression-like behaviors and decreased SIRT1 levels.	
miR-214-3p	Mice, mPFC	β-catenin-1	CSDS induced depression-like behavior that coincides with elevated levels of miR-214-3p and reduced β-catenin-1 expression. Antagomir-214-3p improved depressive-like behavior in mice.	(Deng et al., 2019)
miR-124	Mice- Hippocampus	Signal transducer and activator of transcription (STAT3)	CUMS-induced depression resulted in decreased miR-124 levels in the hippocampus. STAT3 expression is direct target to miR-124.	(Lou et al., 2019)
miR-18a-5p	Rats, Hippocampus, Serum.	5-HT_{1A} receptor	CUS induced a depressive-like behavior, reduced miR-18a-5p expression in the hippocampus and in serum.	(Zurawek et al., 2019)
miR-139-5p	Mice, Human blood derived exosomes	Not applicable	Tail vein injection of blood exosomes isolated from MDD patients into normal mice resulted in depressive-like behaviors. Blood exosomes isolated from healthy volunteers injected to CUMS-treated mice alleviated their depressive-like behaviors. CUMS mice also showed significantly increased blood and brain levels of exosomal miR-139-5p.	(Wei et al., 2020)
miR-15b	Mice, NAc	Vesicle associated membrane protein 1 (VAMP1), syntaxin binding protein 3A (STXBP3A)	Anti-miR-15b-5p significantly rescued CUMS-induced depression and synapse downregulation.	(Guo et al., 2020)
miR-138	Mice, Hippocampus	SIRT1	CUMS induced upregulation of miR-138 that positively correlated with decreased SIRT1 expression.	(Li et al., 2020b)
miR-146a, miR-30c miR-223	Rats, Plasma	Not applicable	Increased levels of miR-146a, miR-30c and miR-223 in plasma were found following 1 week of repeated exposure to social stress.	(Jacobsen et al., 2020)

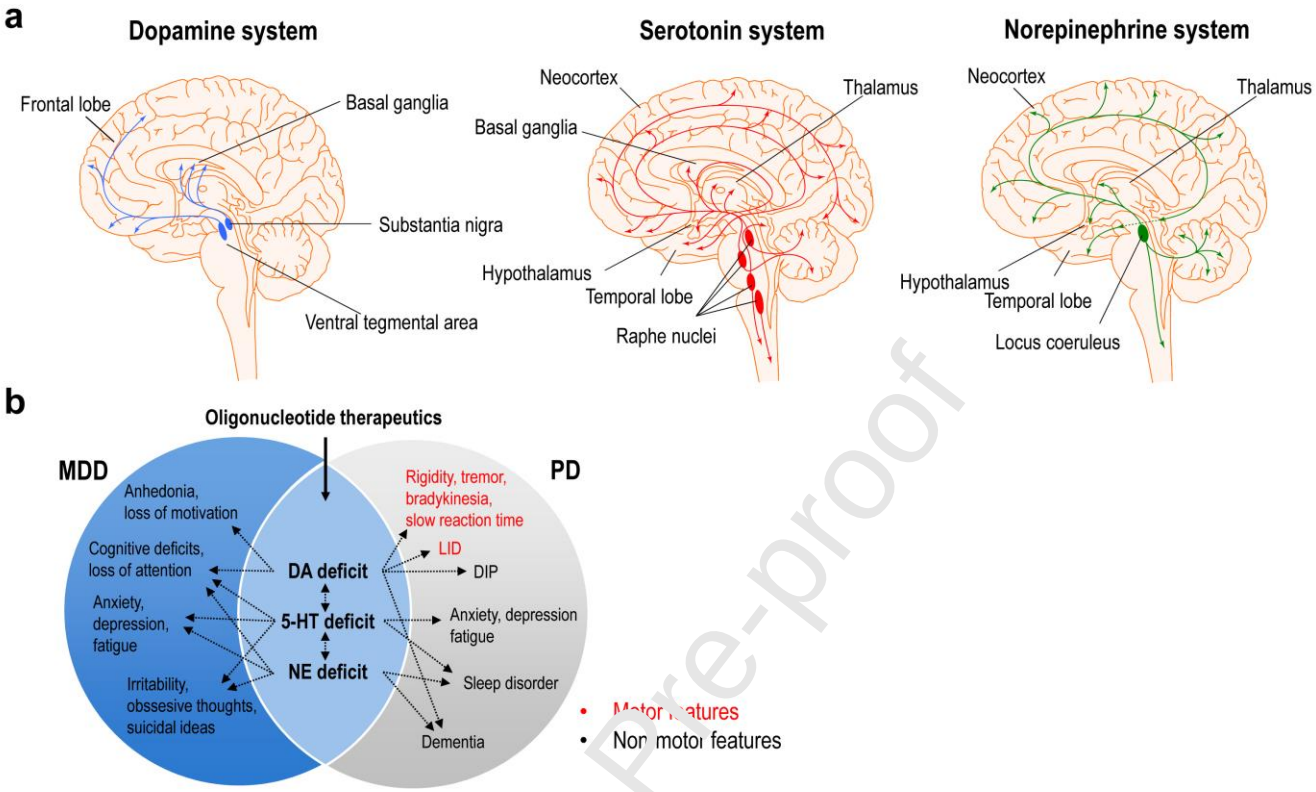

Figure 1. Brainstem monoamine systems. a) Schematic representation of the brainstem monoamine nuclei containing neuronal groups synthesizing dopamine (DA), serotonin (5-hydroxytryptamine, 5-HT), and norepinephrine (NE), which give rise to a widespread innervation of the brain. **b)** Veen diagram showing the complex interplay between monoamine systems, as well as their involvement in clinical outcomes of MDD and PD. Mutually influential and/or synergistic interactions are indicated with arrowheads. Since monoamine dysfunction is central in the pathophysiology of MDD and PD, MAT ligand-conjugated oligonucleotides play a key role in the treatment of MDD/PD by targeting non-druggable RNAs involved in synthesis, degradation, or signaling pathways of these neuronal populations, as well as in the mechanisms stopping neurodegeneration or promoting cell survival. DIP, dopamine-induced psychosis; LID, levodopa-induced dyskinesia.

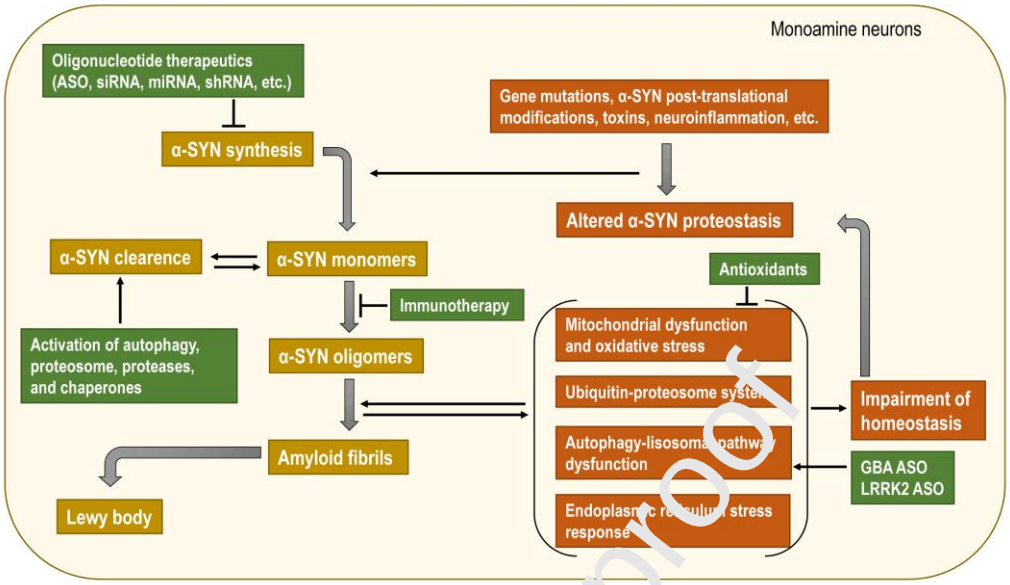

Figure 2. Molecular pathways involved in PD and associated therapeutic strategies. Schematic diagram showing interactions between major molecular pathways that are implicated in the pathogenesis of PD together which current or emerging therapeutic strategies (green boxes). Although α-synuclein (α-SYN) is depicted as a principal player in PD pathophysiology, perturbations in proteostasis, mitochondrial quality control, an other basic cellular proccesses (orange boxes) converge in producing PD. Intracellular levels of α-SYN are tightly regulated by the balance between the rates of synthesis, clearance and aggregation of the protein. Alterations of α-SYN proteostasis, including gene mutations, post-translational modifications, toxins, neuroinflammation, etc. may increase intracellular α-SYN levels and induce its accumulation (brown boxes). Increased phosphorilated α-SYN monomers interact to form oligomers that grow to form amyloid fibrils and Lewy bodies. Cellular damage induced by toxic forms of α-SYN includes impairment of axonal transport, mitochondrial dysfunction, ubiquitin-proteosome disturbances, autophagy-lisosomal pathway dysfuntion and endoplasmic reticulum stress response, which ultimately induce neurotoxicity. Impairment in these processes can also induce cell death in the absence of altered α-SYN, and thus may precede and cause or contribute to α-SYN dysfunction. Toxic forms of α-SYN can be transferred between cells and spread the disease to other brain regions. Targeted mechanisms to confer neuroprotection include decreasing α-SYN synthesis using oligonucletides (ASO, siRNA, miRNA, etc.); increasing the clearance of α-SYN by activating autophagy, the proteasome, proteases or chaperones; or blocking α-SYN aggregation and spreading using immunotherapy. In addition, antioxidants can be used to decrease cellular stress.

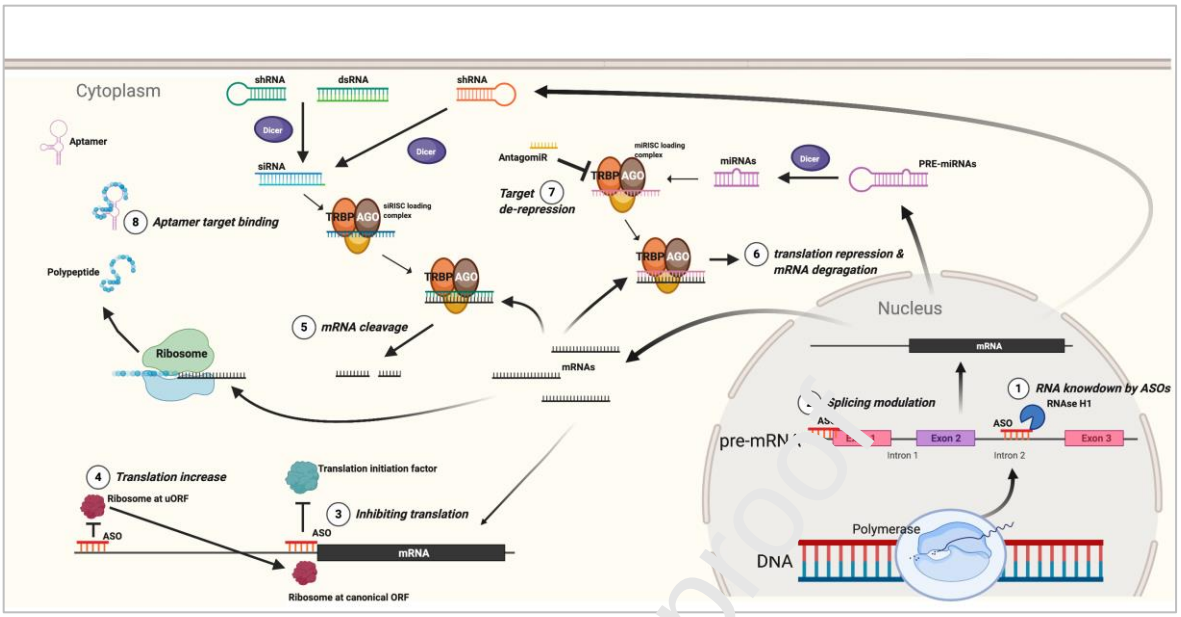

Figure 3. Schematic representation of oligonucleotide's mechanisms of action. **1.** ASO can bind anywhere in the pre-mRNA (messenger RNA), then RNAse-H is recruited to cleave the target RNAs, an action resulting in RNA knockdown. **2.** ASO causes an exon to be preferentially included or excluded resulting 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, similar to antibodies. TRBP - TAR RNA-binding protein, Ago – Argonaute.

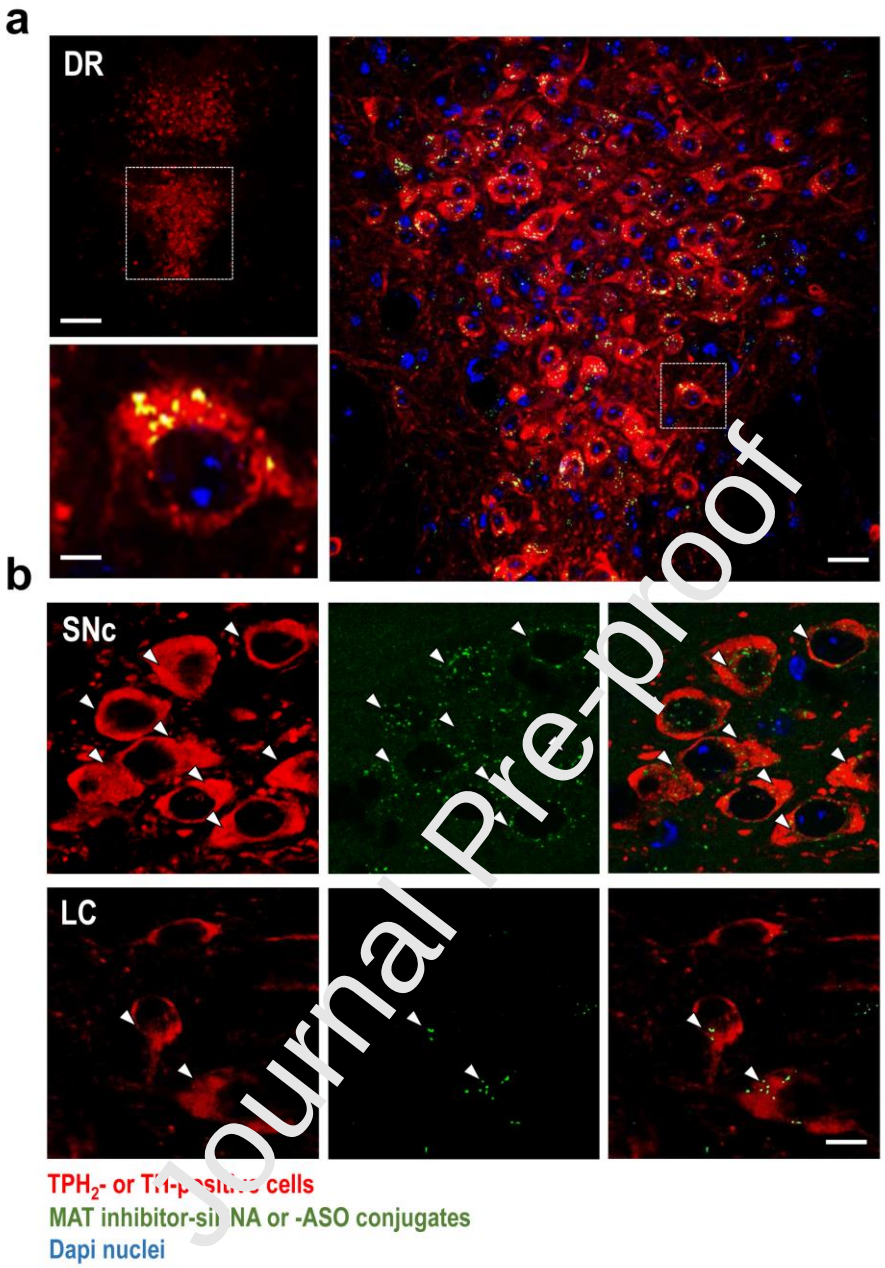

Figure 4. Selective delivery of conjugated MAT inhibitor-oligonucleotides to monoamine neurons of mice after intracerebroventricular or intranasal administration. a) Confocal images showing the co-localization of conjugated sertraline-siRNA with tryptophan hydroxylase (TPH_2)-positive cells in the mouse dorsal raphe nucleus (DR). Mice received 1 µl sertraline-siRNA (30 µg/µl for 4 days) into the lateral ventricle, and were sacrificied 12 h post-administration. Frames indicate the amplified area. Scale bars (from low to high magnification): 250 µm, 40 µm and 5 µm. **b)** Confocal images showing the co-localization of conjugated indatraline-ASO with tyrosine hydroxylase (TH)-positive cells in substantia nigra compacta (SNc) or of reboxetine-siRNA with TH-positive cells in locus coeruleus (LC) of mice identified with white arrowheads. Animals were intranasally treteated with ligand-oligonucleotide conjugates at 30 µg/day for 4 days and sacrificied 6 h after last administration. Scale bar: 10 µm (for a more detailed information on the administration of conjugated oligonucleotides see Ferrés-Coy et al., 2016; Alarcón-Arís et al., 2018; Fullana et al., 2019a).

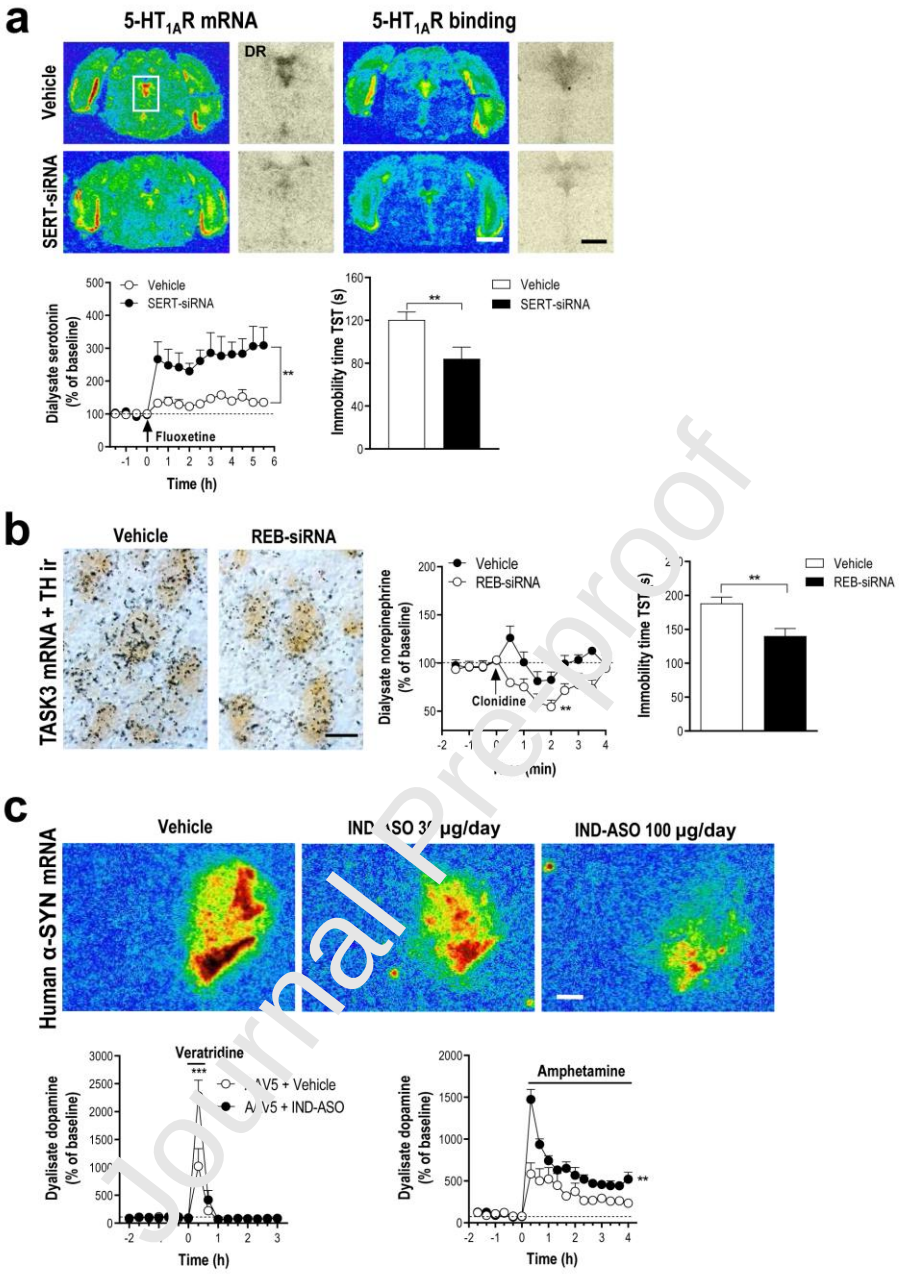

Figure 5. Reduction of MDD/PD-related gene expression selectively in mouse 5-HT, NE and DA neurons induced by MAT inhibitor-oligonucleotide conjugates. a) Representative coronal brain sections showing the reduction of 5-HT_{1A} receptor (5-HT_{1A}R) expression in the dorsal raphe nucleus (DR) of mice, as assessed by *in situ* hybridization and [^{3}H]-8-OH-DPAT binding. Scale bars: white = 2 mm, black = 500 µm. Mice (n = 6/group) were treated with vehicle or a single dose of sertraline-siRNA targeting 5-HT_{1A}R (SERT-siRNA, 30 µg, intracerebroventricular) and sacrificed a 48 h post-administration. Selective 5-HT_{1A}R knockdown in DR increased the effects of SSRI fluoxetine on extracellular serotonin (5-HT) levels in medial prefrontal cortex (mPFC), and evoked an antidepressant-like response in the tail suspension test (TST), as denoted by the reduced immobility time. **b)** Photomicrographs showing tyrosine hydroxylase (TH)-positive neurons expressing TASK3 potassium channel mRNA (^{33}P-oligonucleotide silver grains) in the locus coeruleus (LC) of mice treated with PBS- and conjugated reboxetine-siRNA targeting TASK3 (REB-siRNA, 75 µg/day for 7 days, intranasal, n = 8/group). Scale bar: 10 µm. Selective TASK3 knockdown in LC reduced

the effect of α_2-adrenoreceptor agonist clonidine on extracellular norepinephrine (NE) levels in medial prefrontal cortex (mPFC), and mice displayed a reduced immobility in the tail suspension test (TST) compared to vehicle-treated mice. **c)** Coronal brain sections showing human wild-type α-synuclein (α-SYN) mRNA expression in substantia nigra compacta (SNc) of adenoasociated viral vector-injected (AAV5) mice and treated with vehicle or indatraline-ASO targeting α-SYN (IND-ASO, 100 μg/day for 28 days, intracerebroventricular, n = 6/group). Scale bar: 1 mm. Reduction of human α-SYN in SNc induced by IND-ASO improved DA neurotrasmision in caudate-putamen of PD-like mice. $P < 0.01$ compared to control group (Data from Bortolozzi et al., 2012; Fullana et al., 2019a; Alarcón-Arís et al., 2020).