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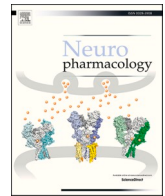
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Serotonin 5-HT_{1A} receptor biased agonists: The challenge of translating an innovative neuropharmacological concept into therapeutics

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ABSTRACT

Serotonin 5-HT_{1A} receptor agonists are prime candidates for CNS drug discovery due to their involvement in physiological and pathological processes relevant to neurology and psychiatry. However, the lack of target specificity of many previously characterized agonists has long been a barrier to pharmacological and therapeutic progress. Some of the obstacles may be overcome through the recent concept of biased agonism, which has attracted considerable attention to the development of novel chemical entities at 5-HT_{1A} receptors, by specifically targeting intracellular signalling pathways that may themselves be linked to specific brain regions and therapeutic indications. There is now abundant translational data demonstrating distinct molecular and functional pharmacological signatures between different 5-HT_{1A} receptor agonists, opening new opportunities for research in neurology and psychiatry. Nevertheless, important limitations need to be overcome, including understanding the precise molecular basis for biased agonism, the need for improved translatable models, and the currently limited clinical data on biased agonists. Here, we review the current limits of our knowledge of 5-HT_{1A} receptor biased agonists and the limitations of available pharmacological tools, counterbalanced by the translational possibilities and therapeutic perspectives opened by novel, highly selective 5-HT_{1A} receptor drug-candidates.

1. Introduction

The vast majority of current neurotherapeutic strategies uses small heterocyclic molecules for clinical indications in neurology and neuropsychiatry. For pharmacological treatment of CNS indications, all drugs currently target ten families of neurotransmitters or related compounds, i.e., acetylcholine, dopamine, GABA, glutamate, histamine, ion channels, melatonin, noradrenaline, opioids and serotonin; using nine pharmacological mechanisms, i.e., receptor agonist, partial agonist, antagonist, reuptake inhibitor, neurotransmitter releaser, enzyme inhibitor, ion channel blocker, positive allosteric modulator and enzyme modulator (Zemach et al., 2023).

Among these mechanisms, direct receptor stimulation remains the prerogative of orthosteric agonists. Indeed, the recent history of neuropharmacology has tended towards the identification of increasingly selective agonist compounds, in step with advances in characterisation of the molecular structures of receptors. Serotonergic neurotransmission is a perfect illustration of this progress and the quest

for greater specificity. In the 1970s–1980s, two families of receptors were identified: S1 and S2, distinguished by their binding affinity for [³H]serotonin and [³H]ketanserin, respectively (Biegon et al., 1986). Then, with the advent of molecular biology and the cloning of distinct genes, 14 serotonin receptor subtypes gradually emerged, divided into 7 families (Barnes et al., 2021). While numerous selective antagonists have been synthesized for these different receptors and have contributed to their characterization, the choice has always been more limited for agonists.

In classical receptor theory, and as often taught in pharmacology courses, an agonist activates a specific neurotransmitter receptor. However, the therapeutic use of serotonergic agonists remains very limited in neurology or psychiatry (Staron et al., 2018), an important limitation being their relatively limited specificity. In other words, the agonists often have a range of receptor interactions that is too broad. While the affinity of many agonists for the primary receptor target may be high (e.g., with a binding dissociation constant, K_d, between 1 and 10 nM), as the systemic concentration of the compounds approaches this

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range, they often also bind to other receptors. Thus, many typical pharmacophores for G protein-coupled receptor agonists interact with multiple secondary receptor targets. A recent example of this is illustrated by the (re)discovery of the potent serotonergic properties of psychedelics such as psilocybin and lysergic acid diethylamide (LSD). Such compounds are, in fact, broadly acting serotonergic agonists that activate a swath of 5-HT receptors, notably including 5-HT_{2A}, but also 5-HT_{2B}, 5-HT_{2C} and 5-HT_{1A} receptors, with a mixture of potential therapeutic and side-effect profiles (Holze et al., 2024). Multireceptor actions also typify supposedly more specific serotonergic agonists: in the field of 5-HT_{1A} receptor neurotransmission, notable examples are buspirone, an approved non-benzodiazepine anxiolytic (Buspar®), and gepirone, which was recently approved as an antidepressant (Exxua®). Both of these compounds are members of the azapirone drug class which, in addition to 5-HT_{1A} receptor agonism, also shows antagonist activity at dopamine D2 and alpha1-adrenergic receptors, as well as interacting with alpha2-adrenergic receptors via their main metabolite 1-PP (Eison and Temple, 1986). Another example of a commonly-used 5-HT_{1A} receptor agonist is 8-OH-DPAT, which is an important tool for preclinical research but which also acts as an agonist at other serotonin receptors, notably 5-HT₇, with clear physiological implications (Eriksson et al., 2008) and has stereoisomers with distinct receptor agonist efficacy and affinity profiles (Lejeune et al., 1997). Overall, there remains a clear need for selective and potent agonists to explore the mechanisms and therapeutic potential of serotonin receptors.

The trend in modern neuropharmacology has therefore been to enrich its therapeutic arsenal with increasingly specific agonists, e.g.,

with binding affinity for their target receptor that is ten or a hundred times greater than that for other receptors or off-targets. But even when using stringent selectivity criteria, agonist stimulation has its limits in terms of specificity of its therapeutic consequences. In fact, it is necessary to consider the multitude of molecular cascades that are activated following the binding of an exogenous agonist (or endogenous neurotransmitter) to its receptor target. Indeed, a specific agonist, (i.e., that binds to only one receptor target at a given concentration), can still activate several downstream signalling cascades. For example, the prototypical 5-HT_{1A} receptor agonist, 8-OH-DPAT, activates diverse G proteins (G_{αo}, G_{αi3}, G_{αi1} and G_{αz}) as well as the cAMP pathway, pERK, β-arrestin and internalisation processes (Albert and Vahid-Ansari, 2019; Raymond et al., 1999). However, not all these pathways are necessarily associated with the desired pharmacological and therapeutic effects. Moreover, some activated pathways may themselves be responsible for limiting the desired therapeutic activity or mediating side effects. It therefore appears that the target specificity of an agonist for an individual receptor target is a limited concept when the multiplicity of activated intracellular pathways is considered.

In this context, the concept of biased agonism has allowed the field to move forward by proposing not just increasingly potent and selective ‘super-agonists’ (Bollinger et al., 2010) that only activate a specific receptor subtype, but also innovative ‘biased agonists’ that preferentially engage well-defined transduction pathways (Newman-Tancredi et al., 2022). This concept has advanced our understanding of G protein-coupled receptors, which are not simply ‘toggle switches’ with binary conformations that are either inactive under the influence of an

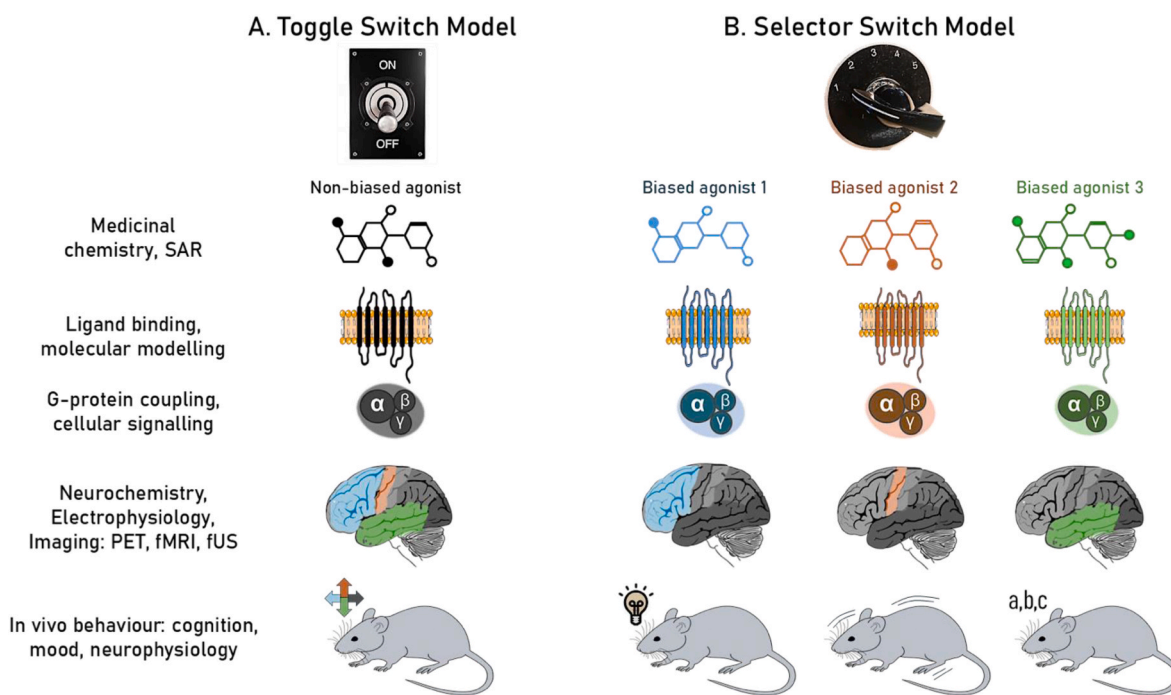


Fig. 1. The Toggle Switch and Selector Switch models of agonism at GPCR - the challenge of translating biased agonists from medicinal chemistry to therapeutic activity. A: The classical concept of receptor theory as a ‘toggle switch’ which is either ‘on’ or ‘off’ depending on the binding of a non-biased agonist to the receptor target. In this model, the agonist triggers the activation of all the associated G proteins and signalling cascades, thus influencing all the related brain regions and the (sometimes opposing) behavioural and physiological responses. B: The concept of biased agonism is more akin to a ‘selector switch’ model in which different biased agonists can select specific conformations of the receptor, and thereby trigger activation of different G proteins and/or other receptor signalling cascades. This can lead to activation of different brain regions and therefore different *in vivo* responses. In this example, biased agonists which are chemically closely related elicit distinct signalling, neurological and *in vivo* responses. Biased agonist-1 activates receptor-coupled cascades that elicit effects in frontal cortical regions associated with cognition and mood. In contrast, Biased agonist-2 elicits responses in motor cortex, which is associated with movement planning and coordination. Biased agonist-3 targets the temporal lobe, which is associated with auditory processing, language development and regulation of emotion. The same receptor can therefore be considered to constitute 3 different molecular ‘targets’ depending on its intracellular signalling and neurological effects. Importantly, these different targets can be distinguished pharmacologically. The key challenge is to show that biased agonists have effects which translate consistently through the multiple levels of investigation: from cellular signalling, through neurochemistry, brain imaging and behaviour. Non-invasive brain imaging techniques such as PET and fMRI are particularly powerful because they can be used across species and for clinical investigation.

antagonist or active under the influence of an agonist, but can be better compared to ‘selector switches’ or dials with multiple configurations and outputs (see Fig. 1).

2. An overview of the genesis of the 5-HT_{1A} biased agonists concept

2.1. Biased agonists: an attractive concept offering targeted efficacy and limited side-effects

The concept of biased agonists, or functional agonists, developed gradually as knowledge of G protein-coupled receptors has grown, and according to different parallel histories that have intersected (Kenakin and Christopoulos, 2013). Firstly, impetus for research was provided by molecular pharmacologists such as Brian Kobilka and Robert Lefkowitz, who were jointly awarded the Nobel Prize for Chemistry in 2012 (Lin, 2013). They highlighted the complexity of the transduction pathways that occur when agonists dock into one of the molecular pockets of a receptor’s three-dimensional structure. Concurrently, pharmacologists made great strides in this field by synthesising increasingly specific G protein-coupled receptor agonists through chemical modulations validated by structure-activity relationships. Thus, in the area of serotonin receptors, potent and specific 5-HT_{1A} receptor agonists were developed in the late 1990s and early 2000s based on novel chemical pharmacophores (Lemoine et al., 2012; Maurel et al., 2007; Vacher et al., 1998, 1999). The lead compounds showed sub-nanomolar affinity and, above all, remarkable specificity for 5-HT_{1A} receptors compared with a wide palette of other receptors and brain-localised transporters. The receptor signalling story and the 5-HT_{1A} receptor selectivity story converged when it became clear that, among the series of agonists developed, some preferentially activated particular intracellular pathways (Newman-Tancredi et al., 2009, 2017). Of note, the differences in cellular signalling were associated with activation of distinct brain regions, since the expression of intracellular transduction pathways is modulated according to the expression distribution of 5-HT_{1A} receptors and of their coupled G-proteins (Llado-Pelfort et al., 2010; Mannoury la Cour et al., 2006; Newman-Tancredi et al., 2009). Thus, the definition of superagonists, possessing potent and specific activity for a given receptor, was complemented by the concept of biased agonists.

The new availability of molecules which, in addition to being highly receptor-specific, activate signalling pathways in a differential manner has opened up new perspectives in 5-HT_{1A} receptor neuropharmacology by, firstly, identifying compounds that activate the pathways necessary for a pharmacological, and therapeutic effects, without activating undesirable pathways responsible for side effects, and, secondly, by developing improved therapeutics that preferentially activate specific brain regions associated with pathologies of interest, due to regionally-accentuated expression of transduction pathways therein. The concept of such quasi-surgical precision neuropharmacology is highly innovative and allows researchers to envisage numerous therapeutic applications (Salaciak and Pytka, 2021; Sniecikowska et al., 2019b). A notable example of this is seen in recent medicinal chemistry efforts focused on identifying biased agonists for 5-HT_{2A} receptors (Kargbo, 2023; Marti-Solano et al., 2015; Pogorelov et al., 2023). This stems from the mounting interest in psychedelics and the desire to identify drug candidates that exhibit therapeutic properties but without the associated hallucinogenic and side-effect profiles. The hypothesis is therefore that some 5-HT_{2A} receptor biased agonists may retain beneficial effects on mood but not elicit a ‘psychedelic trip’. However, such investigation is still at a relatively early stage, whereas 5-HT_{1A} receptors have benefited more from biased agonist drug discovery than other serotonin receptor families. This is particularly due to a pharmaceutically rich chemical library of selective 5-HT_{1A} receptor agonists, mainly derivatives of 2-pyridinemethylamine (Vacher et al., 1998, 1999), synthesized by Pierre Fabre Laboratories and subsequently developed by the biopharma company, Neurolix. This portfolio of agonists, further expanded in

collaboration with a team at Jagiellonian University, Krakow (Sniecikowska et al., 2019a, 2020), constituted the basis for the emergence of an informal scientific consortium, led by the present authors, and bringing together multiple research teams focusing on serotonergic mechanisms and studying the properties of these molecules in *in vitro* and *in vivo* models.

2.2. Preclinical evidence

A large body of preclinical data has been generated focusing particularly on a selection of biased agonists targeting 5-HT_{1A} receptors. Some of their nomenclature has changed over the years since their initial discovery. Befradol was originally known as F13640 and is now referred to as NLX-112; F15599 (Newman-Tancredi et al., 2009) is now known as NLX-101. This should not obscure the fact that three molecules have been the most studied to date: NLX-101, NLX-112 and F13714. These biased agonists show different patterns of action in *in vitro*, electrophysiological, neurochemical and behavioural models (see (Newman-Tancredi et al., 2022) for a comprehensive review). For example, NLX-101 preferentially activates G α i G proteins (Alabdali et al., 2023), and shows pronounced activity in cortical areas expressing postsynaptic 5-HT_{1A} receptors, which are associated with antidepressant and procognitive activity (Cabanu et al., 2022; R. Depoortere et al., 2021; Gluch-Lutwin et al., 2021; Papp et al., 2023). It also shows robust activity on respiratory function controlled by brainstem regions (Levitt et al., 1985). In contrast, F13714 is highly active at 5-HT_{1A} autoreceptors in the raphe nuclei, with possible implications for anxiolytic activity and control of aggressive behaviours, and at postsynaptic basal ganglia 5-HT_{1A} receptors associated with motor control (de Boer and Newman-Tancredi, 2016; Depoortere et al., 2019; Jastrzebska-Wiesek et al., 2018). NLX-112 preferentially activates G α o G proteins and shows activity in raphe nuclei, cortical areas and striatal regions that control motor function, as well as in the spinal cord, where it has analgesic activity, and in the cerebellum, where it is associated with anti-ataxic properties. In contrast to NLX-101, NLX-112 and F13714 show clear effects on body temperature and corticosterone release, which are under the control of hypothalamic 5-HT_{1A} receptors (Assie et al., 2010; Newman-Tancredi et al., 2018b). Importantly, the 5-HT_{1A} specificity of the biased agonists has been demonstrated by blocking their activity with selective antagonists. This indicates that their differential profiles *in vivo* are not due to interactions at cross-reacting sites but due to differential activity at the target sites. More recently, novel and selective 5-HT_{1A} receptor biased agonists have been identified that preferentially target beta-arrestin signalling (Sniecikowska et al., 2020). The functional profiles of these compounds is under early exploration but could lead to additional opportunities for therapeutic development.

2.3. Towards clinical applications

From a therapeutic point of view, a major challenge is to match compounds that selectively activate 5-HT_{1A} receptors in specific brain regions with the most relevant target psychiatric or neurological disease, while avoiding potential side effects due to 5-HT_{1A} activation in other brain regions.

2.3.1. Potential in depression and anxiety

The 5-HT_{1A} receptor biased agonist chemical series was initially developed for psychiatric indications, notably depression (Maurel et al., 2007). In the 1990s and 2000s, evidence supporting agonist stimulation of 5-HT_{1A} receptors as an adjuvant and accelerator of antidepressant effects emerged strongly (Smith et al., 2023). Consequently, several molecules with a 5-HT_{1A} receptor partial agonist component, such as buspirone and related azapirones, have been investigated in preclinical studies and tested in humans. However, clinical trials failed to show compelling outcomes (Kishi et al., 2013; Shin et al., 2020), leading to the gradual de-emphasis of this therapeutic avenue, although gepirone was

eventually approved for treatment of depression by the FDA (Kaur Gill et al., 2019). Explanations for these failures included the lack of specificity of the agonists and/or their partial agonist nature. Nevertheless, this large body of work further buttressed the important role of 5-HT_{1A} receptors in antidepressant responses, particularly showing that autoreceptors located in the raphe slow down the onset of therapeutic efficacy and pointing to 5-HT_{1A} heteroreceptors located in cortical areas as mediating antidepressant effects (Salaciak and Pytka, 2021). Further support for the importance of the latter subpopulation of 5-HT_{1A} receptors came from studies using ketamine, an NMDA receptor antagonist that exhibits rapid-acting clinical antidepressant activity and which indirectly activates cortical 5-HT_{1A} receptors (Fukumoto et al., 2018, 2020). The emergence of 5-HT_{1A} receptor biased agonists therefore provided a new strategy to target the therapeutically relevant brain areas, with the aim of specifically activating 5-HT_{1A} heteroreceptors in cortical regions. Several biased agonists (F13714, NLX-112, NLX-101, NLX-204) have been tested for their preclinical antidepressant properties using a variety of behavioural tests in rodents (FST, sucrose consumption, etc.) and in models of depression (e.g., chronic mild stress, chronic corticosterone) and in models of anxiety behaviour (e.g., marble burying test, social interaction under high light levels, elevated plus maze, Vogel test, etc.) (Newman-Tancredi et al., 2022; Powell et al., 2021). Notably, NLX-101 and NLX-204 exhibit very robust and rapid antidepressant-like responses in rodent models which closely resemble those of ketamine, suggesting that direct agonist activation of cortical 5-HT_{1A} heteroreceptors is sufficient to elicit a therapeutic benefit on mood (Papp et al., 2023, 2024a). This is supported by data from transgenic mice which over-express 5-HT_{1A} autoreceptors: in these mice, NLX-101 rapidly remodels serotonergic neurotransmission and Raphe-cortical projections, consistent with a powerful antidepressant profile (Vahid-Ansari et al., 2024).

2.3.2. Potential in cognition

Another area of therapeutic interest at the interface between psychiatry and neurology is that of procognitive molecules, particularly in the context of cognitive decline associated with neurodegenerative pathologies. In this case, the target is the frontal region, more specifically the 5-HT_{1A} receptors located in the prefrontal cortex, which are known to play a role in cognitive processes, notably in relation to neurotrophic factors and neuroplasticity (Homborg et al., 2014).

5-HT_{1A} receptors have been shown to play a key role in cognition and memory, leading to interest in the use of 5-HT_{1A} agonists to alleviate cognitive impairment (Schreiber and Newman-Tancredi, 2014). The biased agonists NLX-101 and F13714 have been tested in a number of behavioural models assessing different aspects of cognition and memory, such as the delayed non-matching to position model, the hole-board model, and have demonstrated improvements in working memory and attention and pattern separation in rodents upon both acute and chronic administration (Depoortere et al., 2010; van Goethem et al., 2015; van Hagen, 2020; van Hagen et al., 2022).

Other models have also investigated episodic memory in rats or mimicked cognition/memory deficits induced by glutamatergic NMDA receptor antagonism using phencyclidine (Depoortere et al., 2010; Horiguchi and Meltzer, 2012), by the muscarinic antagonist scopolamine (Depoortere et al., 2021) or by chronic mild stress (Papp et al., 2024b). These cognition and memory tests indicate that NLX-101, which preferentially targets postsynaptic cortical 5-HT_{1A} receptors, consistently and robustly improves cognitive performance with effects that are superior to those of F13714 or 8-OH-DPAT, 5-HT_{1A} receptor agonists known to target 5-HT_{1A} receptors at the presynaptic level. Taken together, this body of data strongly supports developing post-synaptic cortical 5-HT_{1A} receptor biased agonists for treatment of conditions involving deficits in cognitive function. At a clinical level, this is of interest for treatment of neuropsychiatric disorders such as schizophrenia or bipolar disorder. Indeed, these conditions are characterized by clear cognitive impairments involving cortical neurotransmission,

particularly involving imbalance of glutamatergic signalling (Ibrahim and Tammimga, 2012; McCutcheon et al., 2023). It is notable that antipsychotic drugs that have been approved over the last decade or two, notably aripiprazole, perospirone, lurasidone, cariprazine and brexpiprazole, have pronounced 5-HT_{1A} receptor agonist properties and this may contribute to improved treatment of cognitive symptoms (Newman-Tancredi and Kleven, 2011; Razakarivony et al., 2021). Moreover, some studies have shown that adjunctive treatment with (non-biased) 5-HT_{1A} receptor agonists can improve the cognitive status of schizophrenia patients (Meltzer and Sumiyoshi, 2008; Sumiyoshi et al., 2007). Taken together these observations suggest that a cortical 5-HT_{1A} receptor biased agonist, such as NLX-101, could be of therapeutic benefit for alleviating cognitive impairment in psychotic disorders.

An additional therapeutic area of relevance is the treatment of rare autism spectrum disorders, such as Fragile X syndrome and Rett syndrome, both of which are characterized by intellectual disability and for which there is a large unmet medical need (NLX-101 has been granted Orphan Drug designation for both of these indications). In the case of Fragile X syndrome, NLX-101 shows robust effects in transgenic mice, reducing their sensory hypersensitivity (Tao et al., 2023) and rescuing their EEG profile (Tao et al., 2023). In the case of Rett syndrome, NLX-101 also shows a potentially beneficial targeting of the brainstem. This is of interest because Rett syndrome is characterised by respiratory irregularity of central origin, probably involving serotonergic neurotransmission (Abdala et al., 2014; Azmitia, 2001). This led to the study of the effects of NLX-101 in mouse models of Rett syndrome, which revealed proventilatory properties, presumably via preferential activation of 5-HT_{1A} heteroreceptors in the brainstem (Abdala et al., 2014; Levitt et al., 2013).

2.3.3. Potential in Parkinson's disease

Parkinson's disease (PD) is another area of application for 5-HT_{1A} receptor agonists. While the dopaminergic system is the primary target for the vast majority of current antiparkinsonian therapeutic strategies, the serotonergic system has increasingly become a focus of interest in this pathology, particularly in the context of levodopa-induced dyskinesia (Al-Kassmy et al., 2023; Buchanan et al., 2024; Carta et al., 2010; Corsi et al., 2021). Parkinson's disease is also the indication where the clearest translational validation of the therapeutic potential of selective 5-HT_{1A} receptor biased agonists is currently available.

In early PD, levodopa is converted to dopamine (DA) in DAergic neurons by levodopa decarboxylase, thus replenishing depleted DA levels. However, as PD progresses, DA neurons are lost and levodopa is taken up by serotonergic neurons which also possess levodopa decarboxylase and hence produce and release DA as a 'false neurotransmitter' (Carta et al., 2010; Politis et al., 2014; Carta et al., 2008). However, 5-HT neurons lack control mechanisms to regulate synaptic DA levels, resulting in physiologically inappropriate DA release and pulsatile stimulation of post-synaptic DA receptors, generating levodopa-induced dyskinesias (LID). One mechanism to counter this LID is therefore to activate 5-HT_{1A} autoreceptors on 5-HT neurons, thus inhibiting the latter's activity and dampening false neurotransmitter DA release.

As mentioned above, NLX-112 preferentially activates Gao G proteins *in vitro*, targets 5-HT_{1A} autoreceptors in ERK phosphorylation measures *ex vivo*, and potentially inhibits the electrical activity of 5-HT neurons and their release of 5-HT (Llado-Pelfort et al., 2012). Correspondingly, NLX-112 robustly inhibits levodopa-evoked DA release in parkinsonian (i.e., 6-OH-DA lesioned) rats (Inderberg et al., 2015) and, at a behavioural level, decreases their dyskinesia. The changes in central 5-HT_{1A} signalling can be clearly shown at an electrophysiological and brain imaging level, using tools that can be also employed in higher species (Brys et al., 2018; Chaib et al., 2023). Notably, these rodent data translated well to non-human primates (i.e., MPTP treated marmosets and macaques (Depoortere et al., 2020; Fisher et al., 2020) and, importantly, also to a proof-of-concept, randomized, double-blind,

placebo-controlled clinical trial in Parkinson's disease patients with troublesome levodopa-induced dyskinesia (Svenningsson et al., 2023). In that study, NLX-112 significantly reduced levodopa-induced dyskinesia from basal levels, whereas placebo-treated patients did not show significant change from baseline. Interestingly, the patients in the NLX-112 group also exhibited lower parkinsonian symptoms, suggesting that NLX-112 may have antiparkinsonian properties of its own. This observation echoed earlier observations in rat and marmosets where improved motor activity was also observed (Fisher et al., 2020; Iderberg et al., 2015).

Although further clinical studies are necessary to confirm these findings, the strong anti-LID activity exhibited by NLX-112 in rodent and non-human primate models of PD did translate successfully to initial clinical investigation.

2.3.4. Potential in pain management

The serotonergic system is involved in pain mechanisms: a descending serotonergic pathway emanating from the raphe nuclei projects to regions of the spinal cord acts as a relay for pain messages, and SERT or SERT/NET class antidepressants have been shown to be effective in alleviating various painful conditions. More specifically, the 5-HT_{1A} receptor is involved in analgesic activity and NLX-112 has been extensively tested in various rodent models of pain, demonstrating robust antinociceptive/analgesic activity. For example, in rats, NLX-112 produces intra- and post-operative analgesia in several animal models, including a surgical pain model, a spinal cord injury model, a trigeminal neuropathic pain model and an arthritic pain model (Newman-Tancredi et al., 2022; Salat et al., 2017). Moreover, intrathecal administration of NLX-112 elicits clear analgesic effects in the formalin test, suggesting that a biased agonist that specifically targets the spinal cord sub-population of 5-HT_{1A} receptors could constitute a promising analgesic agent (Newman-Tancredi et al., 2018a). In addition, those data suggest that, as well as treating levodopa induced dyskinesia, NLX-112 could also alleviate some non-motor symptoms of PD, i.e., chronic pain, which is highly co-morbid in PD patients.

2.3.5. Potential in neuroprotection & neuroplasticity

An intriguing additional area of intervention with 5-HT_{1A} receptor biased agonists is for treatment of neurodegenerative disorders and/or brain injury. Considerable *in vitro* and *in vivo* evidence indicates that 5-HT_{1A} receptors mediate neuroprotective and neuroplastic effects, as observed in models of ischemia using 8-OH-DPAT and another 5-HT_{1A} receptor agonist, repinotan (Aguilar et al., 2020; Berends et al., 2005; Cheng et al., 2008; Mauler and Horvath, 2005; Olsen et al., 2012). Evidence has also accumulated supporting targeting of 5-HT_{1A} receptors in neurodegenerative disorders, notably Parkinson's disease. Indeed, repinotan slowed the manifestation of motor abnormalities in parkinsonian (MPTP lesioned) mice and macaques via protection of nigrostriatal dopamine neurons (Bezard et al., 2006), effects that may be mediated via 5-HT_{1A} receptors expressed on astrocytes (Miyazaki and Asanuma, 2016). Other studies used non-selective compounds, mirtazapine and rotigotine, that possess some 5-HT_{1A} receptor agonism: in both cases their neuroprotective activity was prevented by blocking 5-HT_{1A} receptors (Isooka et al., 2020; Kikuoka et al., 2020).

In the case of the recent selective biased agonists, NLX-101 showed robust capacity to induce biomarkers of neuroplasticity, including BDNF and PSD95, as well as promoting functional recovery, neuronal sprouting and dendrite remodeling in cortical and hippocampal brain regions in a mouse model of ischemia (Aguilar et al., 2019; Cabanu et al., 2022; van Hagen et al., 2022). NLX-101 also elicited neurogenesis and improved cognitive performance in aged rats, increasing scores in a pattern separation task (Aguilar et al., 2020). Importantly, this study found that the pre-synaptic biased agonist, F13714, failed to improve cognitive performance, suggesting that the neuroplastic/neurorestorative properties of NLX-101 are specifically mediated by post-synaptic 5-HT_{1A} receptor activation. Other studies add

further support to this assertion: in rats rendered 'depressive' by chronic mild stress, NLX-101 and a related 5-HT_{1A} biased agonist, NLX-204, exerted antianhedonic and procognitive effects that lasted for several weeks after treatment cessation, suggesting that they had modified the cortical neuronal networks regulating mood and cognition (Papp et al., 2024a). This is consistent with the observation that, in 'depressed' mice which overexpress 5-HT_{1A} autoreceptors, NLX-101 extensively remodeled serotonergic projections in a brain region-specific manner (Vahid-Ansari et al., 2024).

In studies carried out in the context of Parkinson's disease, the pre/post-synaptic agonist, NLX-112, was investigated in MPTP-treated mice and showed robust protection of dopaminergic neurons in substantia nigra and striatum, *anti*-neuroinflammatory activity and upregulation of GDNF, a protective neurotrophic factor that promotes neuronal survival and differentiation (Powell et al., 2023). In a study of transgenic mice modelling spinocerebellar ataxia3 (a.k.a. Machado Joseph's disease), NLX-112 again protected dopaminergic neurons in the Substantia nigra and attenuated the progression of motor impairment (Ferreira-Lomba et al., 2022).

Overall, there is considerable support for the use of 5-HT_{1A} receptor biased agonists as neuroprotective agents. However, this has not yet been therapeutically tested and it remains to be determined whether they can bring clinically measurable advances as disease modifying agents for treatment of neurodegenerative diseases and brain injury disorders.

3. Challenges and prospects

Although the volume of validating information supporting the development of 5-HT_{1A} receptor biased agonists is compelling, the dissemination and understanding of the concept of biased agonists still suffers from conceptual and strategic limitations, due to its poor integration and consideration in the neuropharmacological approach by pharmacologists, therapists and drug manufacturers alike. One reason for this is the, as yet, unclear understanding of the molecular mechanisms underlying the pharmacological phenomenon of biased agonism. Elucidating this would also allow better definition of the concept, whose terminology is still heterogeneous. In particular, this will require more predictive experimental models, which will ultimately facilitate translation to humans.

3.1. Better understand the mechanism of biased agonists

As mentioned before, the literature is now rich in preclinical molecular, functional and neuroimaging data demonstrating the existence of highly precise pharmacological targeting by 5-HT_{1A} receptor biased agonists. However, there is still insufficient understanding of molecular-level interactions to explain this specific targeting. In other words, by what receptor/signalling mechanism(s) does a biased agonist specifically activate a transduction pathway associated with its target receptor?

One hypothesis is related to differential biodistribution of the biased agonists, with regio-specific targeting leading to preferential activation of 5-HT_{1A} receptors in each brain region, which in turn are linked to different intracellular signalling pathways. Indeed, 5-HT_{1A} receptors in dorsal raphe nucleus and cortex are reported to couple to G_{αi3} G-proteins, whereas hippocampal 5-HT_{1A} receptors are described as preferentially coupled to G_{αo} G-proteins, and are activated in an agonist-dependent manner (Mannoury la Cour et al., 2006; Valdizan et al., 2010). However, if there exists a differential biodistribution of the biased agonists, this would be expected to be accompanied by differences in the cerebral distribution of the fluorine-18 radiolabelled agonists observed by PET imaging *in vivo*. But this physico-chemical hypothesis lacks confirmation. In fact, different 5-HT_{1A} receptor biased agonists have very similar lipophilicity properties and the corresponding radiolabelled molecules, in which fluorine-19 is replaced by fluorine-18

(i.e., ^{18}F -NLX-101, ^{18}F -NLX-112 and ^{18}F -F13714), show no significant differences in cerebral biodistribution by PET imaging in rodents (Lemoine et al., 2010, 2012; Vidal et al., 2018a) and see discussion below).

A second hypothesis is that the conformation of the agonist binding sites within the receptor itself differ between brain regions and are themselves associated with specific transduction pathways. Indeed, 5-HT_{1A} receptor biased agonists have small chemical variations corresponding to differences in chemical substituents. This results in different three-dimensional properties, both in terms of spatial conformation and electronic environment. Thus, a biased agonist would bind specifically to a particular 3D conformation of the agonist (orthosteric) binding pocket, resulting in the activation of transduction pathways that are preferentially expressed in specific brain regions.

A third mechanistic hypothesis assumes that 5-HT_{1A} receptors have the same initial orthosteric binding niche configuration. However, this niche would adopt a different conformation according to the three-dimensional structure of the agonist, which would then favour subsequent coupling to a specific transduction pathway. Thus, the specific conformation of the receptor, and hence its preferential coupling, would be determined by the three-dimensional shape induced by the biased agonist-receptor interaction, orienting and stabilising distinct conformations of receptors that are biased towards specific intracellular transducers and distinct signalling cascades.

Fourthly, a number of 5-HT_{1A} heteroreceptor complexes, or heterodimers, have recently been found to be associated with ascending serotonin pathways, which are known to play important roles in pathophysiological processes (Borroto-Escuela et al., 2018; Mitroshina et al., 2023). These 5-HT_{1A} complexes differ depending on the brain area, e.g. 5-HT_{1A}/FGFR1 (fibroblast growth factor receptor 1 subtype) in the hippocampus and dorsal raphe, 5-HT_{1A}/5-HT_{2A} isoreceptor complexes in the hippocampus and limbic cortex (Borroto-Escuela et al., 2017). These heteroreceptor complexes may be involved in the possible mechanism of biased agonism by constituting additional (quaternary) levels of complexity for regulation of signalling.

Overall, at present the precise molecular mechanisms underlying the targeted actions of biased agonists remain poorly understood, partly because of a lack of appropriate tools. Significant advances have been made in structural biology, with the ability to purify proteins combined with advances in X-ray crystallography and cryo-electron microscopy. These techniques have revealed high-resolution structures of different CGPR states, including transducer-coupled forms (Calebiro et al., 2021). However, these studies often required modifications to the proteins involved, such as truncations, fusions or mutations of their amino acid sequences. Moreover, molecular modelling tools, which have made spectacular progress for cell surface receptors in recent years, do not yet include interacting intracellular proteins in their models, although efforts are made to progress in this direction (Castleman et al., 2019; Kogut-Gunthel, 2024). Overall, there remains a clear need for improved understanding of the mechanisms underlying biased agonism at a molecular level.

A related, and equally fundamental, question concerns the identification of the specific receptor signalling pathways that differentiate the effects of 5-HT_{1A} receptor biased agonists in specific brain regions and mediate the behavioural/physiological effects mentioned above. 5-HT_{1A} receptors are coupled to several G-proteins of the Gi/o family and to a variety of signalling responses. Investigations of 5-HT_{1A} biased agonist signaling have mainly focused on various G-proteins, cAMP inhibition, pERK, calcium mobilisation, β -arrestin recruitment and receptor internalisation. However, it remains unclear which of these measures best predict the differential properties of compounds in whole animal assays. Indeed, it's worth noting that although NLX-101 and (+)8-OH-DPAT have different profiles in G-protein activation assays, they both show accentuated activation of pERK *in vitro* (Newman-Tancredi et al., 2009; Sniecikowska et al., 2020). Therefore, focusing on this *in vitro* functional measure alone does not fully capture the differential profiles of the

compounds *in vivo*. Indeed, the biased agonists show diverging profiles of pERK activation in *ex vivo* assays (Buritova et al., 2009; Newman-Tancredi et al., 2009), emphasizing the need for whole animal experimentation. Future studies should therefore determine whether 5-HT_{1A} receptor-biased agonists can be distinguished on the basis of combinations of signalling profiles, or on other signalling assays, that can be correlated with *in vivo* or *ex vivo* effects.

3.2. Clarifying the different terminologies to clarify the concept of biased agonism

As indicated above, the concept of biased agonists is very attractive because it offers the possibility of selective intervention in intracellular signalling pathways. Thus, biased agonists could be used therapeutically in contexts where specific intracellular pathways need to be activated and undesirable pathways need to be silenced. In practice, however, the activation of intracellular pathways is not entirely specific. In other words, each biased agonist has its own signalling profile that activates pathways at different levels of intensity. For 5-HT_{1A} receptors, several dozen biased agonists have been characterized *in vitro*, some of which are broad and others more specific in their spectra of activated intracellular pathways (Sniecikowska et al., 2019b). The concept of specific targeting by a biased agonist must therefore account for the multidimensional signalling pharmacology of these agonists, as they exhibit a bias between different G protein subtypes and signalling cascades. Bias expression is therefore a relative measure that compares signalling properties to a reference ligand, which is usually the endogenous agonist. Bias is easily determined when a ligand exclusively activates a particular signalling pathway, known as 'modality bias'. In cases where a ligand is less effective or potent for a particular pathway, its preference for other pathways can be quantified using a metric known as the 'bias factor'. This factor compares the signalling activity of the ligand with that of an unbiased reference agonist, and provides a measure of bias for different signalling pathways (Toth et al., 2024). Functionally selective effects can result from different mechanisms, often referred to as 'types' or 'dimensions' of bias.

However, the terminology used to describe biased signalling and its different forms is not consistent in the literature. In the case of 5-HT_{1A} biased agonists, we refer to 'ligand bias' (Newman-Tancredi et al., 2022; Sniecikowska et al., 2019b), which refers to the ability of these agonists to activate coupling pathways, as recommended by the Nomenclature and Standards Committee of the International Union of Basic and Clinical Pharmacology (Kolb et al., 2022).

This mechanism of biased agonism must be distinguished from other forms of bias described by IUPHAR, such as temporal bias, which occurs as a result of the different signalling kinetics of different agonists; spatial or localisation bias, which can occur as a result of differences in the ability of different ligands to access intracellular receptors; geographical bias, which results from differences in the lifetime of internalised agonist-receptor transducer complexes; and systemic bias, which results from differences in the abundance of the signalling proteins involved. It cannot be ruled out that different forms of bias may coexist, and again the multidimensional aspect must be considered when observing the effect of a drug involving a biased mechanism. Recently, an atlas (The Biased Signaling Atlas; <https://biasedsignalingatlas.org/>) has been made available, providing resources for retrieval of biased ligand and pathway effect data, analysis of ligand-bias relationships, study design, bias calculation and data deposit (Caroli et al., 2023).

3.3. More predictive experimental models

The majority of small molecule agonists, including those targeting the 5-HT_{1A} receptor, are identified and characterised by initial studies focusing solely on *in vitro* signalling responses in various cell systems, usually recombinant. The first step is to determine the specificity of the candidate molecules towards the target of interest, in our case the 5-

HT_{1A} receptor. Receptor affinity (expressed as pKi) is determined in competition binding experiments at 5-HT_{1A} and other receptors, using membranes prepared from rat brain tissue or cell lines expressing recombinant human receptors. The compounds are also compared for their ability to activate different signalling cascades, such as G-protein-mediated signalling or β -arrestin recruitment. The different profiles of these compounds for each cascade are considered to provide a biased agonist 'signature'. However, in most cases, it has not been demonstrated whether these signalling profiles are found at the tissue level or in whole animals and, ultimately, to what extent the concept of biased agonism can actually be translated into a clinical setting and produce superior efficacy in the treatment of disease states. *In vitro* assays, which are informative at the level of cell signalling and G protein activation, therefore need to be complemented by *ex vivo* and *in vivo* experiments. In the case of NLX-101, NLX-112 and related compounds, two functional responses were studied under *ex vivo* conditions in rats, notably ERK phosphorylation (pERK) in 3 different brain regions (hippocampus, frontal cortex and hypothalamus) and c-Fos gene expression in 8 different brain regions. The biased agonist activity in different brain regions was also studied by measuring neuronal electrical activity by electrophysiology and neurotransmitter release by microdialysis. By determining the discharge rate of serotonergic neurons in the dorsal raphe nucleus (DRN) and cortical pyramidal neurons, the preferential biased agonist properties of NLX-112 and NLX-101 on 5-HT_{1A} autoreceptors and postsynaptic receptors, respectively, were characterized (Llado-Pelfort et al., 2010, 2012).

However, while these different preclinical approaches are rich in molecular and functional information, and provide a bridge from *in vitro* to *in vivo* systems, they are only partially translational because they have no direct equivalent in humans. In other words, the concept of biased agonism at the level of receptor signalling has not yet been directly demonstrated in humans due to a lack of appropriate tools.

Nevertheless, biomedical imaging approaches (notably PET, MRI, fUS) offer unique translational opportunities as these non-invasive technologies can be used in small animals (i.e., rodents), large animals (notably non-human primates) and ultimately in humans, including both healthy volunteers and patients. Among the biased agonists developed for neuropharmacology, 5-HT_{1A} receptor agonists have benefited most from these neuroimaging techniques to demonstrate their brain region-specific properties (Newman-Tancredi et al., 2022). *In vivo* molecular and functional PET and MRI neuroimaging provide complementary information in terms of biased agonist bioavailability, drug occupancy and consequent functional activations. Thus, the key contribution of PET and MRI imaging to the study of biased agonism is their use for both pharmacological investigation and clinical translatability (Charveriat, 2020; Colom et al., 2019; Razakarivony et al., 2021; Zimmer, 2024).

The compound which has been most studied so far in this context is NLX-112 (i.e. befiradol, F13640). In microdosing PET imaging, this 5-HT_{1A} agonist was radiolabelled by replacing one of its (non-radioactive) fluorine-19s with a radioactive fluorine-18 to generate ¹⁸F-F13640 (i.e., ¹⁸F-NLX-112), the first full agonist PET radiopharmaceutical for 5-HT_{1A} receptors. This compound, whose biological behaviour remains unchanged, was visualised by PET imaging, providing valuable information on its cerebral bioavailability, distribution and pharmacokinetics in both animal models and humans (Colom et al., 2021; Courault et al., 2023; Vidal et al., 2018a). Importantly, PET imaging using ¹⁸F-F13640 allows investigation of the occupancy rate of 5-HT_{1A} receptors to be measured during the administration of other agonist drug candidates. This has long been problematic because of a lack of agonist PET tracers for 5-HT_{1A} receptors. Indeed, whereas antagonist PET radiotracers label both active and inactive-state GPCRs, agonist PET radiotracers preferentially label active-state receptors providing more therapeutically relevant measures of receptor interaction by agonist drug candidates (Bantick et al., 2004; Colom et al., 2019).

Other brain imaging approaches, such as functional MRI or FDG-PET

imaging, make it possible to measure cerebral vascular or metabolic activation following administration of 5-HT_{1A} receptor biased agonists at pharmacological doses (Becker et al., 2016; Levigoureux et al., 2019; Vidal et al., 2020). Finally, the simultaneous combination of the two approaches using hybrid PET/MRI provides a complete picture of the cerebral PK/PD effects of 5-HT_{1A} receptor biased agonists, at least in rodents (Chaib et al., 2023; Vidal et al., 2018b). Specifically, the studies carried out using these techniques provided compelling demonstration of the differential brain region activation and regional connectivity of the biased agonists. In essence, NLX-101 shows robust activation of cortical brain regions, consistent with its prominent antidepressant and procognitive behavioural activity, whereas NLX-112 has a broader regional profile with notable activity in the raphe and thalamus, consistent with its strong therapeutic-like properties in models of movement disorders.

As well as generating abundant whole brain information on the activity of biased agonists, a major advantage of these techniques is that they can be translated from animals to humans. Any PET or MRI study performed in these animal models can be reproduced in humans, as has already been initiated for NLX-112 in a human PET imaging using ¹⁸F-F13640 (Colom et al., 2020; Courault et al., 2023). There are no scientific or technological limits at this stage preventing these techniques from being applied to the study of biased agonism in humans, and, indeed, it would be highly desirable for biased agonist drug candidates at other 5-HT receptors (such as 5-HT_{2A} and 5-HT₇) to also be investigated using these translatable techniques.

3.4. Less time in animal models and a faster passage to humans

Drug discovery priorities in neurology and psychiatry can differ significantly depending on whether programs are carried out in an academic or in an industrial pharmaceutical environment (Zimmer, 2024). Academic researchers are primarily concerned with elucidating pharmacological mechanisms. Animal models, despite their limitations in neurology and psychiatry, retain their value in this context. *In vitro* models can also provide valuable data, although, as mentioned above, they do not yet model the intracellular coupling associated with G protein-coupled receptors. In contrast, the priority of industrial researchers and drug regulatory authorities is not necessarily to understand the mechanism of action of a drug candidate. Their primary concern is to demonstrate a significant therapeutic effect that is superior to placebo and, ideally, comparable or superior to a reference molecule in the target clinical indication. They also seek to ensure the favourable safety and tolerability of the molecule when administered to humans as part of a safety pharmacology approach. In this context, and with a view to positioning 5-HT_{1A} receptor biased agonists in their rightful place in therapeutics, it is necessary to prioritise studies in humans. Ideally, this should include initial studies in healthy volunteers to establish pharmacokinetic constants using microdosing tools (Phase 0). In particular, PET imaging is a translational tool that has provided insights into the intracerebral pharmacokinetic constants of biased agonists. As mentioned above, NLX-112 was radiolabelled with fluorine-18 (as ¹⁸F-F13640) and administered to healthy volunteers, providing access to cerebral kinetic and biodistribution constants. Moreover, PET imaging of coadministration of ¹⁸F-F13640 with novel 5-HT_{1A} receptor biased agonists can provide valuable occupancy information, potentially demonstrating differential profiles of receptor engagement. A first step in this direction was recently described in a rat study (Depoortere et al., 2024) and could be translated to human. Specifically, the 5-HT_{1A} receptor occupancy profile of NLX-112 was compared to its dose-response for reducing abnormal involuntary movements (AIMs, a measure of dyskinesia in rats). A low level of occupancy (about 15%) was sufficient to reduce AIMs by half, and they were completely eliminated at 60% occupancy. These observations suggest that even modest doses of NLX-112 could provide substantial therapeutic benefit, an assertion which is supported by the recent proof-of-concept Parkinson's

dyskinesia trial, in which patients received a maximum of just 2 mg/day of NLX-112. Clinical occupancy data for 5-HT_{1A} receptors using ¹⁸F-F13640 are not yet available but there are no technical obstacles for studies to be carried out. Indeed, these clinical approaches, even if carried out only in healthy volunteers, would provide a real boost to future clinical trials by derisking some of the early development decision-making by industrial partners that is inherent in all drug discovery.

4. Conclusions

Biased agonism at serotonin receptors, a mechanism that enables new drug discovery strategies, is still in its infancy. For many receptors, studies have only been able to identify compounds that show distinct intracellular signalling in *in vitro* assays, leaving open the question of distinct properties in whole organs or animal models, let alone in clinical patient populations. However, in the case of 5-HT_{1A} receptors, a large body of work has been built over more than 15 years. *In vitro* investigation on 5-HT_{1A} receptor agonists includes receptor modelling, structural-functional activity, binding affinity and signalling studies. *In vivo* preclinical work ranges from electrophysiology to microdialysis, behavioural studies and brain imaging in several species including rodents, cats and non-human primates. In addition, two lead compounds, NLX-112 and NLX-101, are currently in clinical development for the treatment of movement disorders and autism spectrum disorders, respectively. Finally, new biased agonists (e.g. NLX-204) may address other therapeutic indications such as depression and/or pain.

Although clinically superior therapeutic efficacy by the novel biased agonists has yet to be formally demonstrated, NLX-112 has achieved a successful proof of concept in an early (Phase 2A) clinical trial assessing its safety, tolerability and efficacy for reduction of dyskinesia in Parkinson's disease, suggesting that the effects of NLX-112 seen in pre-clinical models translated well to the patient population. Nevertheless, important questions remain to be answered for the successful future identification and development of biased agonists. One of the challenges is to achieve sufficiently impressive clinical results to encourage larger clinical trials, paving the way for new drugs to be approved. This challenge must be accompanied by basic pharmacological work to elucidate the molecular mechanisms and thus guide the synthesis and design of novel drug candidates.

CRedit authorship contribution statement

Luc Zimmer: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Adrian Newman-Tancredi:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Adrian Newman-Tancredi is an employee and stockholder of Neurolix, Inc.

Data availability

No data was used for the research described in the article.

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