CHAPTER NINE

α_2 Adrenergic Receptor Trafficking as a Therapeutic Target in Antidepressant Drug Action

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Abstract

Antidepressant drugs remain poorly understood, especially with respect to pharmacological mechanisms of action. This lack of knowledge results from the extreme complexity inherent to psychopharmacology, as well as to a corresponding lack of knowledge regarding depressive disorder pathophysiology. While the final analysis is likely to be multifactorial and heterogeneous, compelling evidence exists for upregulation of brain α_2 adrenergic receptors (ARs) in depressed patients. This evidence has sparked a line of research into actions of a particular antidepressant drug class, the tricyclic antidepressants (TCAs), as direct ligands at α_{2A}ARs. Our findings, as outlined herein, demonstrate that TCAs function as arrestin-biased ligands at α_{2A}ARs. Importantly, TCA-induced α_{2A}AR/arrestin recruitment leads to receptor endocytosis and downregulation of α_{2A}AR expression with prolonged exposure. These findings represent a novel mechanism linking α_2AR trafficking with antidepressant pharmacology.

1. INTRODUCTION

Psychopharmacology is an exceedingly intricate discipline, aiming as it does to modulate human emotion, behavior, and other complex cognitive processes. Although our understanding of brain–behavior relationships has

Progress in Molecular Biology and Translational Science, Volume 132
ISSN 1877-1173
http://dx.doi.org/10.1016/bs.pmbts.2015.03.007

improved dramatically in recent decades, modern neuroscience still struggles to provide coherent pathophysiological mechanisms for psychiatric disorders. Further complicating matters, at a molecular level, the psychopharmacologist must contend with an almost bewildering array of neurotransmitters, ionotropic and metabotropic receptors, transporters, and enzymes both catabolic and anabolic. These myriad considerations help to explain why our understanding of the therapeutic actions underlying psychoactive agents remains frustratingly limited decades after the psychopharmacology revolution of the 1950s.

Antidepressant drugs (ADs) are among the most widely used psychopharmacological agents. These therapeutics have application not just in managing the depressive disorders but also in treating conditions such as migraine headaches[1–3] and chronic neuropathic pain.[1,3–5] All currently available ADs function by affecting, in some fashion, monoamine neurotransmitter systems in the brain.[6] The central monoamine neurotransmitters, which include serotonin (5-HT), dopamine (DA), and norepinephrine (NE), come complete with a corresponding set of enzymes, transporters, and primarily metabotropic receptors. All of these proteins can and have served as molecular antidepressant targets. Indeed, the earliest ADs were the monoamine oxidase inhibitors, which inhibit the activity of the monoamine oxidase enzyme responsible for catabolic inactivation of released monoamines. While these drugs still enjoy some limited therapeutic use, the vast majority of contemporary ADs function primarily as transporter blockers, a therapeutic mechanism known as reuptake inhibition. Transporter blockers include the selective serotonin reuptake inhibitors (SSRIs), the serotonin-norepinephrine reuptake inhibitors (SNRIs), and the tricyclic antidepressants (TCAs).

Regardless of what enzyme or transporter is inhibited, one can expect a resulting increase in synaptic availability of these monoamine neurotransmitters. Beyond this statement of fact, the mechanisms of action remain largely mysterious, as one must then consider which of the fourteen 5-HT receptors,[7] five DA receptors,[7] or nine adrenergic receptors[7] are being affected by the increased neurotransmitter level. In addition, some of these drugs, particularly the TCAs, are classic examples of pharmacologically "dirty" drugs, having multiple direct ligand interactions with these receptors alongside their reuptake inhibition.[6]

Given the extreme complexity inherent to antidepressant pharmacology outlined above, it seems prudent to apply a measured, systematic approach to understanding how these therapeutic agents affect their various molecular

targets. To that end, we have expended considerable energy in recent years to characterize how TCAs function as direct ligands at the α_{2A} adrenergic receptor (AR), a G protein-coupled receptor (GPCR) with numerous physiological roles throughout the central nervous system (CNS) and which has itself been implicated in the neurobiology of depressive disorders. Our research efforts have uncovered extensive novel information, especially regarding the induction of arrestin recruitment to and subsequent arrestin-mediated trafficking of α_{2A}ARs by TCAs. This chapter will therefore be aimed at summarizing our findings on the arrestin-biased behavior of TCAs at the α_{2A}AR, and placing these findings in their proper physiological and pharmacological context.

2. PHYSIOLOGICAL BASIS FOR STUDYING α_{2A}AR TRAFFICKING IN DEPRESSION

A careful review of the basic and clinical literature reveals broad support for the study of α_{2A}AR trafficking in the context of antidepressant psychopharmacology. While it is important to note that the neurobiology of depressive disorders is likely to be multifactorial and heterogeneous, currently available evidence demonstrates that dysregulation of α_2AR expression and/or function level is involved in at least some cases of depression. More specifically, the evidence supports a link between upregulation of α_2AR expression and/or function and major depressive disorder (MDD). For a more detailed explanation and discussion of this evidence, the reader is referred to our recent review article on this subject.[8] Here, we will focus on a concise overview of the findings.

Many studies in this area have been unable to look specifically at the α_{2A}AR, meaning that some clarification on α_2ARs is required before proceeding. The α_{2A}AR is one of three subtypes (α_{2A}AR, α_{2B}AR, and α_{2C}AR) comprising the α_2AR subfamily,[9,10] itself one of three AR subfamilies (α_1ARs, α_2ARs, and βARs).[7] Our ability to investigate with subtype specificity is hindered by a lack of subtype-selective ligands for the different α_2ARs. This is significant given that most published studies have relied on radioligand binding to determine α_2AR levels. However, it is well established that the α_{2A}AR is the most predominantly expressed α_2AR subtype within the CNS,[11–13] and is largely responsible for mediating classic α_2AR functions such as the regulation of neurotransmitter release from presynaptic terminals[14,15] and the centrally mediated sedative, hypotensive, and analgesic effects of α_{2A}AR agonists.[16–20]

Clinical studies on α_2AR levels in depression have largely taken one of two major approaches, assaying receptor density through radioligand binding in platelet samples obtained from living patients[21–30] or in postmortem brain tissue obtained mainly from depressed suicide completers. Regardless of approach, these studies as a whole support the existence of α_2AR upregulation in cases of MDD, with the upregulation being observed in brain regions including the frontal and prefrontal cortex,[31–37] hippocampus,[31] and locus coeruleus.[38–40] One study specifically found an upregulation of α_{2A}AR mRNA in the prefrontal cortex of depressed suicide completers.[41]

The neurobiological consequences of α_2AR upregulation are likely to be far-reaching and complex, given the broad reach of noradrenergic projections from the locus coeruleus throughout the brain. Furthermore, while the presynaptic role of α_{2A}AR autoreceptors in regulating NE release from and firing activity of locus coeruleus neurons has long been appreciated, more recent evidence has revealed significant heteroreceptor and postsynaptic roles for α_{2A}ARs in the CNS.[20,42–45] As a general rule, α_2ARs have an inhibitory effect on CNS function, as they classically couple to $G\alpha_{i/o}$-containing heterotrimeric G proteins, thereby linking to inhibition of adenylyl cyclase.[46,47] More specifically, at the cellular level, α_2AR activation links to inhibition of neurotransmitter release via inhibition of voltage-gated Ca^{2+} channels and neuronal hyperpolarization via activation of G protein-coupled inwardly rectifying K^+ channels.[44,46,47] On a larger physiological scale, α_2ARs mediate sedative, hypotensive, and anti-epileptogenic[48] effects. It can therefore be reasonably assumed that α_{2A}AR upregulation will have a generally inhibitory effect on central neurotransmission.

If α_{2A}AR upregulation represents at least a component of MDD pathophysiology, it stands to reason that receptor downregulation represents a valid AD therapeutic strategy. Indeed, additional evidence indicates that the chronic exposure to ADs necessary for symptom relief in MDD is associated with decreases in α_2AR expression and/or activity level. This evidence comes from both clinical studies[21,22,26,29,33,38,49–52] and preclinical modeling[53–60] of AD pharmacological activity and is especially associated with chronic exposure to TCAs.

It is here that we find the potential link between MDD and GPCR trafficking, as it has long been appreciated that downregulation of receptor expression can result from prolonged exposure to ligands and ligand-induced receptor endocytosis. Although it would be logical to assume that downregulation of neurotransmitter receptor expression could result from

the elevated neurotransmitter levels caused by AD-mediated reuptake inhibition, there is a dearth of evidence to support that assumption. In fact, with respect to ARs, chronic exposure to NE reuptake inhibitors has been shown to sustain only a mild increase in brain NE levels to 10 nM from a typical baseline near 1 nM,[57,61,62] suggesting that an additional mechanism is necessary to explain TCA-induced α_2AR downregulation. As mentioned previously, TCAs exhibit a large degree of molecular promiscuity, with nontransporter targets that include α_2ARs,[6,63–65] providing an attractive potential additional mechanism.

3. ARRESTIN-BIASED REGULATION OF THE α_{2A}AR BY THE TCA DRUG CLASS

The need for a mechanistic explanation regarding AD-induced α_2AR downregulation, coupled with the knowledge that TCAs can bind directly to these highly relevant GPCRs, has sparked a productive line of research in our laboratories. Our initial work sought to characterize the immediate molecular consequences of TCA binding to α_{2A}ARs. Although we began with a focus on a specific TCA, desipramine (DMI),[60] we have since expanded our work to include two additional representative members of the TCA drug class, imipramine (IMI) and amitriptyline (AMI).[66] TCAs share a common basic chemical structure which has been slightly modified to generate the various specific drugs (Fig. 1), and each of these TCAs exhibits a slightly different molecular profile in terms of their relative affinities for the α_{2A}AR (both murine, as assessed experimentally, and human, as reported in the literature), the NE transporter (NET), and the 5-HT transporter (SERT), data which are summarized in Table 1. DMI and IMI share a similar α_{2A}AR affinity in the low micromolar range, similar to α_{2A}AR affinity for the endogenous ligand NE, while AMI has an approximately 14-fold stronger α_{2A}AR affinity, in the nanomolar range. It is interesting to note that using transporter affinities results in different groupings, as DMI exhibits a strong selectivity for NET over SERT, while IMI and AMI exhibit a strong selectivity for SERT over NET. This finding suggests that different chemical determinants are relevant for receptor interactions versus transporter interactions.

Beyond their molecular profile, our work has sought to investigate what, if any, actions these TCAs might have when acting as direct α_{2A}AR ligands. With respect to signaling activity, the TCAs appear to behave similarly to classic neutral antagonists, as they drive no appreciable coupling of

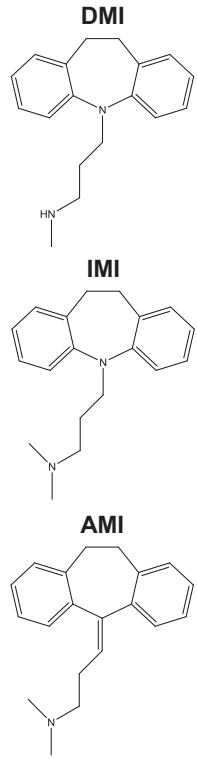

Figure 1 Chemical structures of the three TCAs.

heterotrimeric G proteins to the α_{2A}AR (Table 1). Furthermore, we have been unable to observe any activation of typical α_{2A}AR downstream signaling targets such as MAP kinases[69,70] or Akt[60] in response to TCA stimulation of α_{2A}ARs,[60] although we certainly cannot and should not rule out the possibility of novel signal transduction at this time. As well, it is important to note that TCAs do not appear to function simply as classic neutral antagonists. While attempting to determine whether DMI could block NE-induced α_{2A}AR signaling, as would be expected for an antagonist, we found that DMI actually potentiates NE-induced MAP kinase activation by α_{2A}ARs,[71] indicating a more complex pharmacological interaction.

Further complicating the attempt to pharmacologically classify TCAs as direct α_{2A}AR ligands, we discovered that these agents exhibit agonist-like properties with respect to receptor-arrestin recruitment. Through a combination of coimmunoprecipitation and fluorescence lifetime imaging (FLIM)-based FRET approaches, we have shown that TCAs have a variable

Table 1 Summary of TCA Pharmacological Properties as Direct α_{2A}AR Ligands

Parameter	DMI[60]	IMI[66]	AMI[66]
K_i (murine α_{2A}AR)	4.62 μM	5.07 μM	330 nM
K_i (human α_2AR)	3.44 μM[67,68]	3.10 μM[67]	402 nM[67,68]
Transporter binding[6]			
NET K_i (nM)	0.8	37	34.5
SERT K_i (nM)	17.5	1.4	4.3
G protein coupling	No	No	No
Endocytosis			
Peak internalization (%)	35	40	40
Arrestin dependence	Yes	Yes	Yes
Arrestin recruitment (by FLIM-FRET)			
Arrestin2	+	ND	++
Arrestin3	+++	+++	+

Data for murine K_i, G protein coupling, endocytosis, and arrestin recruitment come from Cottingham *et al.*[60] or Cottingham *et al.*,[66] as indicated. Human K_i values are as reported in Cusack *et al.*[67] and/or Owens *et al.*[68]; values for DMI and AMI are an average of values reported in both publications. Transporter binding values are as reported in Baldessarini.[6]

capacity to drive recruitment of arrestins to the α_{2A}AR.[60,66] The nonvisual arrestins, arrestin2 and arrestin3, also sometimes referred to as β-arrestin1 and β-arrestin-2, are well appreciated as key regulators of GPCR function. These adaptor or scaffolding-type proteins are often essential to classical GPCR endocytosis via clathrin-coated pits,[72–75] a process which leads to receptor desensitization,[76,77] and have more recently been appreciated as multi-functional proteins capable of transducing intracellular signaling independent of heterotrimeric G proteins.[78–81] Additionally, expression of the nonvisual arrestins has been reported to be dysregulated in cases of MDD[82,83] and to be affected by chronic exposure to ADs.[82–84] Coupled with the evidence on α_2AR dysregulation described earlier, such findings highlight the potential importance of α_{2A}AR arrestin-mediated trafficking to MDD and its psychopharmacological management.

As mentioned above, and summarized in Table 1, our research has revealed that TCAs are able to drive α_{2A}AR-arrestin recruitment at concentrations near or below their α_{2A}AR affinity values. Both DMI (at concentrations of 1 and 10 μM) and IMI (10 μM) drive a similar degree

of robust arrestin3 recruitment to the receptor, when the process is observed via FLIM-FRET in live cells. AMI (1 μM) drives arrestin3 recruitment less strongly than the others, though still significantly. Furthermore, we have found that certain TCAs preferentially recruit one arrestin over the other. Specifically, we have uncovered clear preferential arrestin3 recruitment to the α_{2A}AR by DMI, with much weaker arrestin2 recruitment by this TCA.[60] Meanwhile, AMI drives a more robust recruitment of arrestin2 to the α_{2A}AR compared with arrestin3.[66] This finding provides yet another example of the subtle biomolecular differences between these chemically similar pharmacological agents and has additional significance in light of evidence supporting differential expression patterns for arrestin2 versus arrestin3 in the brain.[85]

Importantly, we have been able to establish that TCA-dependent arrestin recruitment to the α_{2A}AR has a functional consequence, namely to induce receptor endocytosis. By stimulating both heterologous cells stably expressing α_{2A}ARs and primarily cultured cortical neurons endogenously expressing α_{2A}ARs, we demonstrated that TCA stimulation drives α_{2A}AR endocytosis in an arrestin-dependent fashion.[60,66] Each of the TCAs assayed was able to drive a similar degree of receptor internalization, peaking at approximately 35–40% loss of cell surface receptor density. Further, we have demonstrated that TCA-induced α_{2A}AR endocytosis occurs in a clathrin-dependent fashion,[60] confirming the involvement of the classical arrestin- and clathrin-mediated pathway for GPCR endocytosis.

Collectively, the data summarized in the preceding paragraphs establish the TCAs as arrestin-biased ligands at the α_{2A}AR, meaning that they are able to selectively drive arrestin recruitment to the receptor while stimulating no detectable heterotrimeric G protein coupling activity. Arrestin bias is a more recent phenomenon in the GPCR field,[80,81] and one that had not previously been observed at the α_{2A}AR specifically. As well, this particular form of arrestin bias selects for a trafficking response but not for any readily apparent arrestin-mediated signal transduction. Most previously reported arrestin-biased ligands acted as agonists, simply initiating signal transduction via arrestin rather than heterotrimeric G proteins. Nonetheless, as stated above, we cannot rule out the possibility of novel TCA-induced arrestin-mediated signaling by α_{2A}ARs.

Given the evidence for α_2AR upregulation in MDD outlined earlier, the potential clinical significance of our findings to this point should be readily apparent. It has long been appreciated that prolonged or chronic exposure, on the order of several hours to days, to agonists can drive a downregulation

of receptor expression,[72,74] an effect which has clear therapeutic relevance in the context of MDD. Indeed, we have shown that prolonged exposure, on the order of 4–24 h, to a TCA can drive downregulation of overall α_{2A}AR expression in cultured heterologous cells and primary cortical neurons.[60] This downregulation effect reaches a peak of approximately 50% reduction in α_{2A}AR expression at the 24-h time point, a reduction which is sufficient to attenuate NE-stimulated α_{2A}AR-mediated MAP kinase activation in the treated cells. To be clear, this downregulation effect occurs in response to TCA treatment alone in non-noradrenergic cells, which would not be releasing NE into the culture environment. Additionally, prolonged treatment of cultured cells with NE at 10 nM, the concentration reported to result from chronic NE reuptake inhibition,[57,61] is not sufficient to drive α_{2A}AR downregulation.

A final set of *in vivo* experiments underscores the physiological relevance of TCA-induced α_{2A}AR downregulation. Through the use of subcutaneous osmotic mini-pumps, we exposed mice to a 2-week course of chronic TCA treatment. After the 2-week period, mice were sacrificed and their cerebral cortices were used to generate a crude synaptosomal membrane preparation. In these preparations, we found a significant decrease in α_{2A}AR expression via radioligand binding, indicating receptor downregulation.[60] Most importantly, the effect on receptor expression was completely lost in arrestin3-null mice, suggesting the *in vivo* occurrence of arrestin-mediated α_{2A}AR trafficking and downregulation. This last piece of evidence also supports the *in vivo* relevance of the preferential recruitment of arrestin3 over arrestin2 to α_{2A}ARs by DMI observed *in vitro*.

Although additional study is necessary to directly observe this phenomenon in the brains of depressed human patients, our data strongly implicate arrestin-biased regulation of α_{2A}ARs by TCAs leading to receptor downregulation in the therapeutic mechanism of action of these ADs. It is important to note that our experiments have largely utilized physiologically relevant drug concentrations, a fact which is apparent when reported clinical therapeutic levels for these drugs (summarized in Table 2) are considered.

Table 2 Typical Therapeutic Ranges for TCAs

Clinical Therapeutic Range	DMI	IMI	AMI
ng/mL	125–600	175–300	100–250
μM	0.5–2.3	0.4–1.1	0.4–0.9

Values are clinically determined plasma concentrations as reported in Baldessarini.[6]

It is likely, then, that α_{2A}AR upregulation associated with MDD can be normalized, at least in part, due to the arrestin-biased interaction of a TCA with the receptor directly, leading to receptor trafficking and, ultimately, stable downregulation of receptor expression. Nevertheless, given the extreme complexity inherent to psychopharmacology, this putative mechanism is almost certainly just one piece of a massive puzzle that has yet to be fully solved.

As an addendum to the preceding evidence directly regarding receptor trafficking and downregulation, we have also demonstrated *in vivo* relevance for an α_{2A}AR- and arrestin3-mediated TCA mechanism through the use of behavioral pharmacology.[86] Porsolt's forced swim test[87,88] is a gold-standard preclinical model for assaying antidepressant effects on rodent behavior in which effective ADs show activity by reducing immobility, a form of learned helplessness behavior. Our work has shown that DMI induces an antidepressant effect on mouse behavior (i.e., reduces immobility) in an α_{2A}AR-dependent fashion, with the antidepressant effect lost in α_{2A}AR-null mice. Furthermore, we have shown that antidepressant responsiveness to DMI is significantly attenuated in arrestin3-null mice. While it is difficult to draw a clear mechanistic link to the trafficking line of evidence, these behavioral pharmacology findings nevertheless provide *in vivo* support for the importance of α_{2A}ARs and arrestin to TCA drug actions.

4. THERAPEUTIC IMPLICATIONS

The accumulated evidence presented thus far, including both clinical data and preclinical data from the literature and extensive work from our own laboratories, makes a compelling case for an α_{2A}AR-dependent AD therapeutic mechanism of action relying on physiologically normalizing arrestin-mediated downregulation of receptor expression. This putative mechanism is summarized in Fig. 2. In short, the arrestin-biased nature of TCAs at the α_{2A}AR means that these drugs do not stimulate heterotrimeric G protein coupling and would therefore be unable to contribute to the generally inhibitory neuronal effects classically driven by α_{2A}AR signaling. These TCAs are, however, capable of driving arrestin recruitment to the receptor, an effect which leads to receptor endocytosis and ultimately to α_{2A}AR downregulation following prolonged exposure. It is important to remember that this downregulation would likely represent a neuroadaptive effect, normalizing or correcting a pathophysiological α_{2A}AR upregulation underlying the occurrence of MDD. This is a particularly attractive

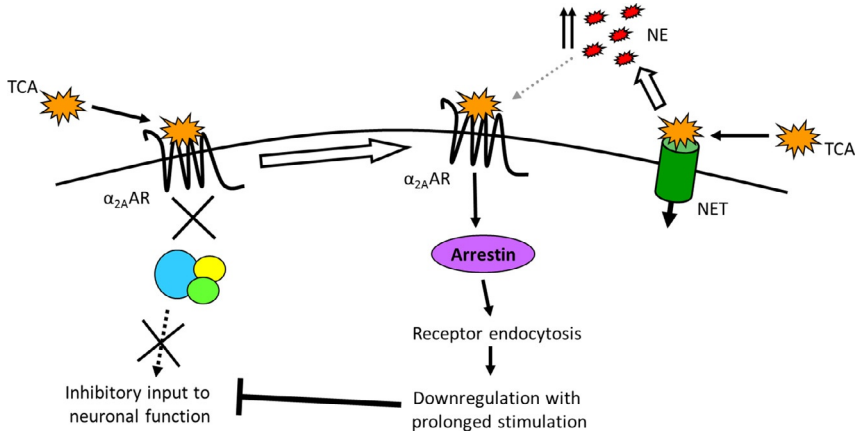

Figure 2 Working model for a TCA-induced therapeutic mechanism relying on α_{2A}AR downregulation. Exposure to a TCA leads to acute α_{2A}AR endocytosis in an arrestin-dependent fashion. With chronic exposure, this endocytic response transitions into a stable downregulation of receptor expression. This direct TCA-induced mechanism is necessary given that NE levels achieved by reuptake inhibition are insufficient to drive receptor downregulation. Downregulation of α_{2A}ARs would be expected to reduce overall signaling activity by this receptor, activity which represents generally inhibitory input to neuronal function.

mechanism given the well-appreciated clinical lag time of 3–6 weeks between the start of antidepressant therapy and the onset of symptom relief.[6] Our findings are essential to progress in the field of AD psychopharmacology, given the lack of clear mechanistic models for AD therapeutics and the significant limitations of contemporary ADs.

All presently available ADs represent massive and broad biochemical alterations to the brain, making the typical AD a very blunt pharmacological instrument. This characteristic is clear for the TCAs and their molecular promiscuity, interacting with numerous receptor and transporter targets. However, even a clean drug such as an SSRI is not really clean at all, as the resulting increase in 5-HT levels will be global, affecting not only the serotonergic raphe nuclei but also any brain region receiving serotonergic inputs, and has the potential to increase activity of fourteen different 5-HT receptors. We believe that it is essential for the next generation of AD therapeutics to be more pharmacologically precise and finely tuned, thereby affecting a drastically narrower range of molecular targets.

Pharmacological imprecision is only one limitation of currently available AD therapeutics. While these drugs can certainly be quite effective at managing MDD symptoms with a minimum of undesirable side effects, far too

many patients are still unable to achieve symptom relief. Available evidence suggests that approximately half of all clinically depressed patients either have incomplete symptom relief or fail to respond at all to AD therapy.[89–91] Furthermore, the decades since the psychopharmacology revolution have seen precious little improvement in overall AD efficacy. Recent findings suggest that newer generation drugs such as the SSRIs and SNRIs are not significantly better at achieving symptom relief than older therapeutics such as the TCAs.[92–95] To be sure, the SSRIs and SNRIs do exhibit better tolerability and smaller side effect profiles when compared with the TCAs. Nevertheless, our apparent inability to improve antidepressant efficacy underscores the extreme difficulty inherent to understanding this complex pharmacological problem.

All of these limitations have understandably led to doubts regarding the value of monoamine-based therapeutic strategies. Much attention has been given of late to the application of the glutamatergic compound ketamine, which antagonizes NMDA-type ionotropic glutamate receptors, as a rapid-acting AD.[96–98] As well, the mixed MT_1/MT_2 melatonin receptor agonist-5-HT_{2C} receptor antagonist agomelatine has shown promise as an AD, with an efficacy that approximates traditional ADs,[99] and has been approved for use in Europe, although not yet in the United States. While there is significant evidence to support these therapeutic applications, it is important to caution that there is just as little information supporting an etiological role for glutamatergic or melatonergic systems in MDD as there is for monoaminergic systems. Therefore, the basis for ketamine and agomelatine as ADs comes primarily from the symptomatic side, as was true for the first monoaminergic drugs. Furthermore, it seems unwise to completely discount monoaminergic mechanisms, which have been the foundation of AD psychopharmacology for decades. Continuing to improve our knowledge base on existing therapeutics, as we have done in our line of research, should allow for both a better understanding of the neurobiology of MDD and improved design of future therapeutics.

Our research has demonstrated that slight chemical variations on the common base tricyclic structure leads to differing molecular pharmacological profiles, both in terms of relative α_{2A}AR versus transporter affinity and ability to drive arrestin recruitment. This evidence raises the possibility that, with further chemical tweaking, a more selective arrestin-biased α_{2A}AR ligand can be developed. Such a hypothetical drug would meet our desired characteristic of greater pharmacological precision and would be able to more cleanly target and normalize upregulated α_2AR expression without

the broad-spectrum biomolecular effects of the original TCAs. Given the current and ever-expanding GPCR structural revolution,[100,101] which is now capable of probing the receptor/arrestin interaction itself,[102] the time is ripe for just such a drug design effort.

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