Biochemical and Biophysical Research Communications 451 (2014) 461–466

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Tricyclic antidepressants exhibit variable pharmacological profiles at the α_{2A} adrenergic receptor

Christopher Cottingham [a,b], Stefanie Percival [a], Tana Birky [a], Qin Wang [a,*]

[a] Department of Cell, Molecular and Developmental Biology, University of Alabama at Birmingham, Birmingham, AL 35294, United States
[b] Department of Biology and Chemistry, Morehead State University, Morehead, KY 40351, United States

ARTICLE INFO

Article history:
Received 8 July 2014
Available online 12 August 2014

Keywords:
α_2 adrenergic receptor
Tricyclic antidepressants
Arrestin
Endocytosis

ABSTRACT

Antidepressant mechanisms of action remain shrouded in mystery, greatly hindering our ability to develop therapeutics which can fully treat patients suffering from depressive disorders. In an attempt to shed new light on this topic, we have undertaken a series of studies investigating actions of tricyclic antidepressant drugs (TCAs) at the α_{2A} adrenergic receptor (AR), a centrally important receptor, dysregulation of which has been linked to depression. Our previous work established a particular TCA, desipramine, as an arrestin-biased α_{2A}AR ligand driving receptor endocytosis and downregulation but not canonical heterotrimeric G protein-mediated signaling. The present work is aimed at broadening our understanding of how members of the TCA drug class act at the α_{2A}AR, as we have selected the closely related but subtly different TCAs imipramine and amitriptyline for evaluation. Our data demonstrate that these drugs do also function as direct arrestin-biased α_{2A}AR ligands. However, these data reveal differences in receptor affinity and in the extent/nature of arrestin recruitment to and endocytosis of α_{2A}ARs. Specifically, amitriptyline exhibits an approximately 14-fold stronger interaction with the receptor, is a weaker driver of arrestin recruitment, and preferentially recruits a different arrestin subtype. Extent of endocytosis is similar for all TCAs studied so far, and occurs in an arrestin-dependent manner, although imipramine uniquely retains a slight ability to drive α_{2A}AR endocytosis in arrestin-null cells. These findings signify an important expansion of our mechanistic understanding of antidepressant pharmacology, and provide useful insights for future medicinal chemistry efforts.

1. Introduction

In the field of antidepressant pharmacology, the need to better understand mechanisms of action remains high, as precious little progress has been made since the advent of the drug class some six decades ago. Available evidence indicates that as many as half of all patients suffering from depressive disorders are not adequately treated by current therapeutics [1–3]. Meanwhile, work such as the massive Sequenced Treatment Alternatives to Relieve Depression (STAR*D) clinical trial [4,5] and meta-analyses of the literature [6,7] has revealed that newer-generation drugs, such as the selective serotonin reuptake inhibitors (SSRIs), are not necessarily more efficacious than older-generation drugs, which include the tricyclic antidepressants (TCAs). Expansion of our present understanding of antidepressant drugs is therefore imperative, as it can contribute to much-needed improvements in therapeutic approaches for depression.

To that end, we have recently completed a series of *in vitro* and *in vivo* studies regarding the mechanism of action for a classic TCA, desipramine (DMI) [8–10]. More specifically, we have investigated the actions of this drug as a direct ligand at the α_{2A} adrenergic receptor (AR). The α_{2A}AR, predominant among α_2AR subtypes in the central nervous system [11–13], is a prototypical G protein-coupled receptor (GPCR) with well-established roles in noradrenergic neurotransmission [14–17]. Further, our own extensive review of the literature has revealed that increased expression and activity of α_2ARs is associated with the occurrence of depressive disorders in patients [18]. Our work on DMI has established that this TCA functions as a direct arrestin-biased ligand at the α_{2A}AR, selectively driving recruitment of the classic GPCR adaptor protein arrestin to the receptor while not activating any canonical

Abbreviations: AMI, amitriptyline; AR, adrenergic receptor; Arr, arrestin; CFP, cyan fluorescent protein; DMI, desipramine; ESC, escitalopram; FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; GPCR, G protein-coupled receptor; HA, hemagglutinin; IMI, imipramine; MEF, mouse embryonic fibroblast; NE, norepinephrine; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant; YFP, yellow fluorescent protein.

* Corresponding author. Address: 1918 University Boulevard, 986 MCLM, Birmingham, AL 35294, United States.
E-mail address: qinwang@uab.edu (Q. Wang).

http://dx.doi.org/10.1016/j.bbrc.2014.08.024
0006-291X/

heterotrimeric G protein-mediated signal transduction [8]. This arrestin recruitment leads to receptor endocytosis and, with chronic exposure, α_{2A}AR downregulation *in vitro* and *in vivo*.

While all TCAs share a common polycyclic chemical structure, namely a pair flanking benzene rings fused to a central heptagonal ring, there are a number of subtle variations between them, primarily in the form of substitutions to the central ring and the single long side chain arising from that ring. These drugs are therefore a useful set of ready-made tools for exploring antidepressant-target interactions. In the present study, we have expanded on our previous work with DMI by evaluating a pair of subtly different TCAs, imipramine (IMI) and amitriptyline (AMI), as direct α_{2A}AR ligands. Our findings demonstrate that, while also functioning as arrestin-biased α_{2A}AR ligands, the chemical differences in these compounds lead to functional differences in the degree of arrestin recruitment and characteristics of endocytosis stimulated. Consequently, these data represent an important contribution to our knowledge of antidepressant pharmacology.

2. Materials and methods

2.1. Pharmacological agents

Drugs were purchased from Sigma–Aldrich, and stock solutions for all were prepared freshly prior to each experiment in distilled water, with the exception of escitalopram, which was dissolved in DMSO. Note that all treatments with norepinephrine (NE) were done in the presence of 1 μM prazosin (an α_1 and $\alpha_{2B/C}$ AR antagonist) and propranolol (a non-selective βAR antagonist), to pharmacologically isolate the α_{2A}AR.

2.2. Cell culture

All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) and 100 units/mL penicillin/streptomycin (Life Technologies) and maintained in a humidified 5% CO_2 incubator. Generation and characterization of the mouse embryonic fibroblast (MEF) [8,19] and HEK 293 [20] lines stably expressing N-terminal hemagglutinin (HA) epitope-tagged murine α_{2A}ARs have been previously described.

2.3. Radioassays

All radioassays were carried out in crude membrane preparations from the HEK 293 HA-α_{2A}AR stable cell line. Competition radioligand binding was carried out as previously described [8] in order to assess the affinity of α_{2A}ARs for antidepressants; the radioligand was a ^{3}H-labeled form of the α_2AR antagonist RX821002 (^{3}H-RX821002, PerkinElmer). All competition binding experiments were done in the presence of 5′-guanylimidodiphosphate (Sigma–Aldrich) to eliminate influence of heterotrimeric G proteins on binding affinity. Competition binding data was processed into dose-response curves and K_i values were calculated using GraphPad Prism software (GraphPad, Inc.). Ligand-stimulated coupling of heterotrimeric G proteins to the α_{2A}ARs was assessed by measuring the binding of a radiolabeled, non-hydrolyzable form of GTP ([^{35}S]GTPγS, PerkinElmer) to membrane preparations as previously described [8].

2.4. Assessment of ligand-stimulated α_{2A}AR endocytosis

Receptor endocytosis was assessed both qualitatively and quantitatively in MEF cells of the HA-α_{2A}AR stable lines; HA-α_{2A}ARs were detected using the HA.11 anti-HA primary antibody (mouse monoclonal, Covance). Qualitative assessment was done by immunofluorescent staining according to a primary antibody pre-labeling method for HA-α_{2A}ARs that has been previously described [8]. MEF cells were pre-labeled with HA.11 (1:125 dilution) prior to stimulation, followed by permeabilization and labeling with Alexa488-conjugated anti-mouse secondary antibody (1:1000 dilution, Molecular Probes). Stained cells were visualized by confocal microscopy using a Zeiss LSM 710 (Carl Zeiss) of the UAB High Resolution Imaging Facility.

Quantitative assessment of receptor endocytosis was done by an intact cell-surface ELISA technique which has been previously described [8]. Stimulated and intact (e.g. non-permeabilized) cells were labeled with HA.11 (1:3000 dilution) followed by HRP-conjugated anti-mouse secondary antibody (1:2000 dilution, Millipore). Endocytosis was measured as a percent decrease in cell-surface receptor density compared with control (no ligand) wells (set as 100% surface receptor).

2.5. Fluorescence lifetime imaging microscopy (FLIM)

Our previously established method for detecting α_{2A}ARs/arrestin interaction in live cells through FLIM-based FRET [8] was used to assess arrestin recruitment to the α_{2A}AR by antidepressants. Parental HEK 293 cells were transiently transfected (using Lipofectamine 2000, Sigma–Aldrich) with a plasmid encoding a CFP-tagged α_{2A}AR alone and together with a plasmid encoding either YFP-tagged Arr2 or YFP-tagged Arr3. Transfected, live cells were plated onto 8-well μ-slides (ibidi GmbH) and, following the appropriate treatment, were subjected to one-photon (confocal) FLIM imaging using a Becker and Hickl Simple Tau Time Correlated Single Photon Counting Module and pulsed diode 405 nm laser (Becker and Hickl GmbH) working in concert with the Zeiss LSM 710 confocal microscope. Analysis of FLIM images with the manufacturer's SPCImage software resulted in a CFP fluorescent lifetime value (in picoseconds) for each cell, obtained from a point at the cell surface. These raw lifetime values (t_{CFP} for CFP-only cells and t_{FRET} for CFP/YFP-expressing cells) were then used to calculate FLIM–FRET efficiency (E) values, according to the formula $E = 1 - (t_{FRET}/t_{CFP})$. At least 5–6 individual cells from 2 to 3 different transfections were subjected to FLIM for each treatment group.

3. Results and discussion

3.1. Basic pharmacological assessment of antidepressants as α_{2A}AR ligands

Following our extensive previous work on DMI, for the present study, we selected two members of the tricyclic antidepressant class for further study. These compounds, imipramine (IMI) and amitriptyline (AMI), have subtle chemical differences in comparison with each other and with DMI (Fig. 1A). To begin, we set out to probe the affinity of the α_{2A}AR for IMI and AMI. For a contrast, we also assessed α_{2A}AR affinity for the selective serotonin reuptake inhibitor (SSRI) escitalopram (ESC). As shown in Fig. 1B, the three antidepressant drugs returned three distinct competition binding curves. Analysis of the curves revealed K_i values of 5.07 μM for IMI, 330 nM for AMI, and 79.4 μM for ESC, while our previous study reported a K_i value of 4.62 μM for DMI at the α_{2A}AR [8]. These data show that the α_{2A}AR has nearly identical affinity for both IMI and DMI but approximately 14-fold stronger affinity for AMI than either IMI or DMI. In contrast, the α_{2A}AR/SSRI interaction occurs with approximately 15-fold lower affinity than even the lowest affinity α_{2A}AR/TCA interaction.

By examining the TCA chemical structures, we can see that the only difference between DMI (K_i = 4.62 μM) and IMI (K_i = 5.07 μM)

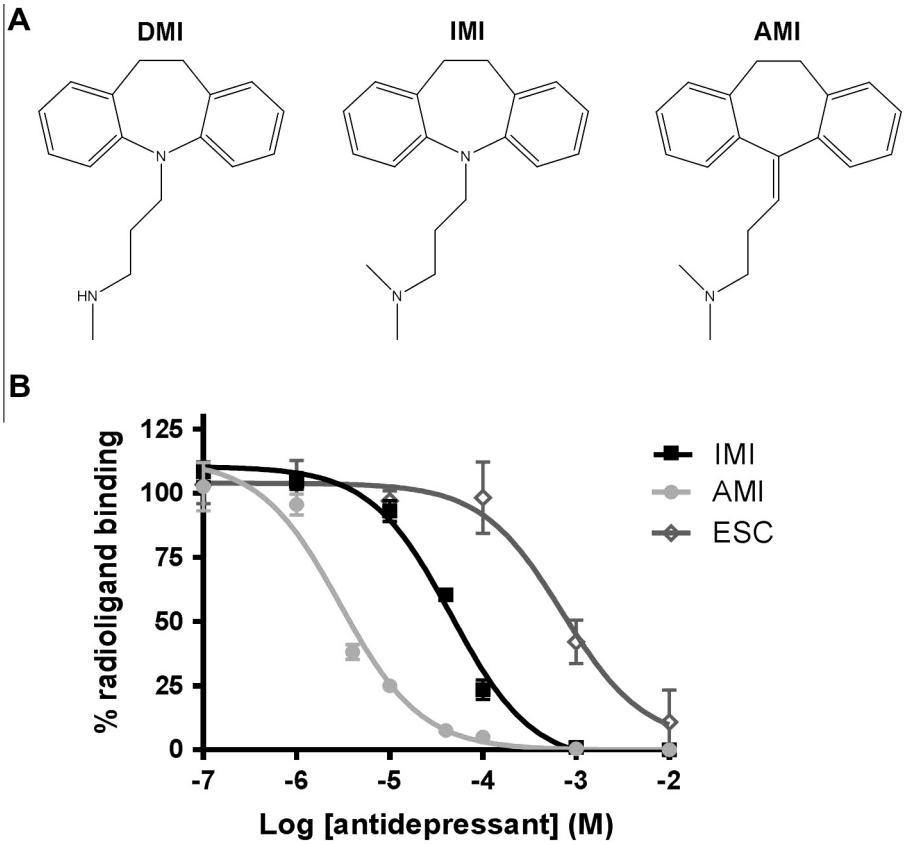

Fig. 1. The α_{2A}AR has variable affinity for different antidepressant drugs. (A) Chemical structures for the tricyclic antidepressants DMI, IMI, and AMI. (B) Receptor affinity for the antidepressants was assessed by competition with an α_2AR radioligand for binding to crude membrane preparations from the HEK 293 HA-α_{2A}AR stable cell line. Competition with the radioligand was measured at increasing concentrations of the tricyclics IMI and AMI or the SSRI escitalopram (ESC) as indicated. Each data point represents $n \geqslant 4$. Analysis of the curves returned K_i values as follows: IMI = 5.07 μM; AMI = 330 nM; ESC = 79.4 μM.

is the addition of a distal methyl group to the side chain, while the only difference between IMI (K_i = 5.07 μM) and AMI (K_i = 330 nM) is the substitution of carbon for nitrogen at the side chain attachment point on the middle ring, which also introduces a double bond to the proximal end of the chain. Our data therefore indicate that the proximal end of the side chain has a greater influence on the affinity of the α_{2A}AR/TCA interaction than the distal end. Additionally, it is important to note that the clinically-reported therapeutic ranges for antidepressant drugs are typically on the order of high nanomolar to low micromolar concentrations in patient serum samples [21]. Furthermore, K_i values for the α_{2A}AR/TCA interaction (330 nM–5.07 μM) are similar to the K_i value for α_{2A}AR interaction with its endogenous agonist NE (3.63 μM) [8]. These findings support the potential clinical relevance of α_{2A}AR/TCA interactions while also suggesting that α_{2A}AR/SSRI interactions are unlikely to occur at physiological concentrations.

Having assessed the affinity of the α_{2A}AR for our TCAs of interest, we next set out to determine whether IMI and AMI are capable of activating canonical signal transduction by the receptor. More specifically, we looked to establish whether these TCAs behave as α_2AR agonists or antagonists. To achieve this, we utilized the classic pharmacological technique of [^{35}S]GTPγS binding, which detects G protein coupling to and activation by a GPCR in the form of [^{35}S]GTPγS accumulation. Using crude membrane preparations from our HEK 293 HA-α_{2A}AR stable cell line, we assessed G protein coupling in response to the endogenous agonist NE, the classic competitive αAR antagonist phentolamine, and the TCAs. Our findings clearly demonstrate that the TCAs, even at the supra-physiological concentration of 1 mM, behave similarly to the antagonist phentolamine (Fig. 2). A clear and dramatic G protein coupling response by α_{2A}ARs is seen only in response the endogenous agonist NE, which drives a nearly twofold induction, consistent with our previous findings [8].

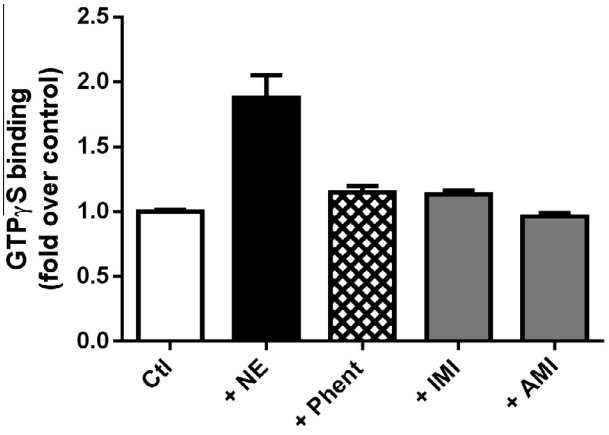

Fig. 2. Tricyclic antidepressants do not stimulate α_{2A}AR/heterotrimeric G protein coupling. Binding of [^{35}S]GTPγS to crude membrane preparations from the HEK 293 HA-α_{2A}AR stable cell line was assessed in response to the endogenous adrenergic agonist NE (10 μM), the classic non-selective αAR antagonist phentolamine (Phent, 1 μM), and the tricyclic antidepressants IMI and AMI (each at 1 mM). Data are expressed as a fold increase in [^{35}S]GTPγS binding over non-stimulated control (set as 1.0-fold); data are shown as mean ± SEM.

3.2. Tricyclic antidepressants drive α_{2A}AR endocytosis in an arrestin-mediated fashion

Although the above data would seem to indicate that IMI and AMI can be pharmacologically classified as α_{2A}AR antagonists, our previous work with DMI suggests another possibility. We know DMI to in fact be a biased ligand at the α_{2A}AR, silent with respect to canonical signal transduction but active with respect to the separate response of ligand-induced receptor endocytosis. To determine whether IMI and AMI share this property, we first qualitatively assessed α_{2A}AR endocytosis via immunofluorescent staining. Specifically, the use of a primary antibody pre-labeling technique allows us to observe only cell-surface receptors and receptors which have undergone endocytosis from the cell surface. As shown in Fig. 3A, stimulation of ArrWT MEF cells with either IMI or AMI alone induced clear receptor endocytosis, indicated by the appearance of perinuclear punctate containing internalized receptors which are absent in no-ligand control cells. Further quantitative assessment of α_{2A}AR endocytosis via intact cell-surface ELISA confirmed the occurrence of significant IMI- and AMI-stimulated receptor internalization in ArrWT MEF cells (Fig. 3B, black bars). These results demonstrate that IMI and AMI, acting alone as direct α_{2A}AR ligands, are capable of driving α_{2A}AR endocytosis. Importantly, this characteristic is not shared by the classic competitive antagonist phentolamine [8]. Note that immunofluorescent staining was performed following 30 min of stimulation to ensure accumulation of visible punctate; ELISA in ArrWT cells revealed no significant difference between 10 and 30 min time points (data not shown).

It is well-appreciated that many GPCRs undergo ligand-stimulated endocytosis through a mechanism mediated by the ubiquitously expressed arrestins, arrestin2 and arrestin3 (sometimes referred to as β-arrestin1 and β-arrestin2) [22–24]. Indeed, we have previously established that α_{2A}AR endocytosis stimulated by both NE and DMI is fully dependent on arrestins [8]. To make the same determination for IMI and AMI, we repeated the intact cell-surface ELISA technique following stimulation of arrestin-null (Arr2,3$^{-/-}$) MEF cells [25]. This experiment demonstrated that α_{2A}AR endocytosis stimulated by AMI is fully lost in the arrestin-null cells, with no reduction in surface receptor versus control (Fig. 3B, gray bars). IMI-stimulated α_{2A}AR endocytosis was fully lost at the 5 min time point but only significantly attenuated at the 10 min time point, with a very slight but significant response retained in the arrestin-null cells (Fig. 3B, gray bars). This response to IMI, though, is still dramatically lower than the corresponding response in ArrWT cells (~10% reduction in surface receptor versus ~37% reduction in ArrWT). Taken together with our previous findings, these data indicate that TCAs drive α_{2A}AR endocytosis via a typical arrestin-mediated mechanism for GPCRs. These data also highlight the potential complexity of addressing whether a particular ligand is an agonist or an antagonist at a given GPCR. In the case of TCAs at the α_{2A}AR, the choice depends on the specific ligand-stimulated response under study, be it signal transduction or endocytosis.

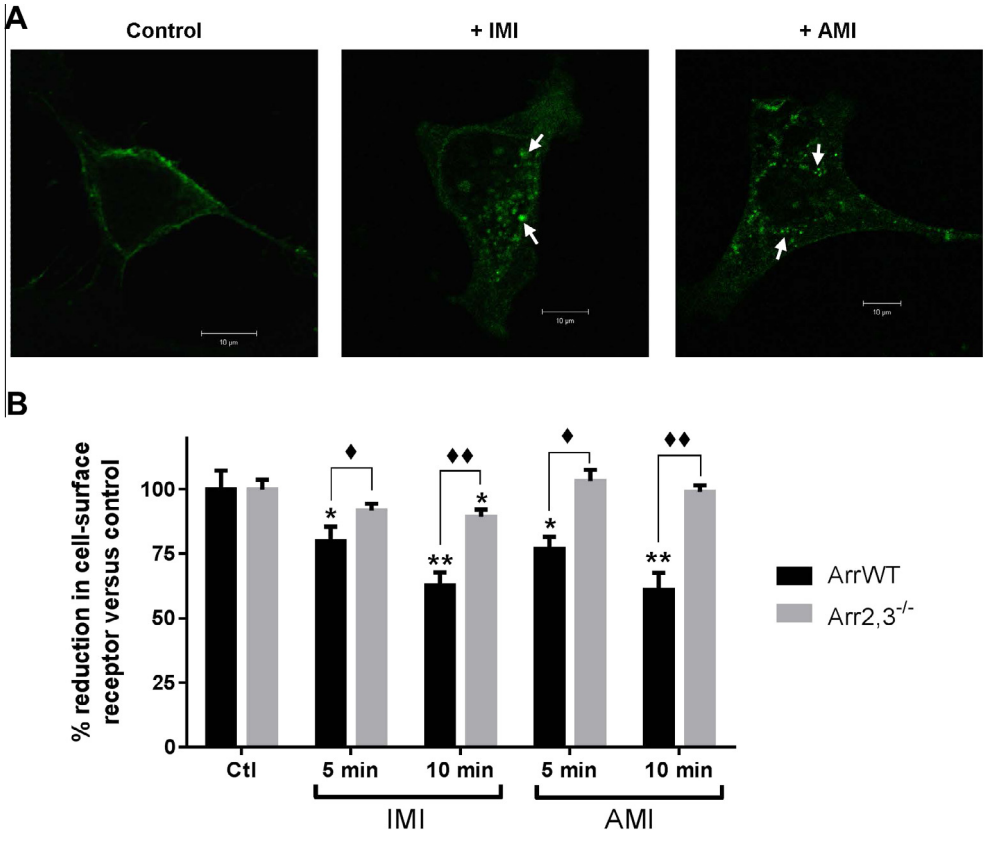

Fig. 3. Tricyclic antidepressants drive α_{2A}AR endocytosis in an arrestin-mediated fashion. (A) Immunofluorescent staining of ArrWT MEF cells reveals that both IMI (10 μM) and AMI (1 μM) drives endocytosis of α_{2A}ARs following 30 min stimulation. Arrows indicate perinuclear punctate staining, characteristic of endocytosed receptors contained in late endosomes. Confocal images were obtained using a 63× objective and are representative of three independent samples. (B) α_{2A}AR endocytosis was assessed quantitatively using intact cell-surface ELISA in both ArrWT and Arr2,3$^{-/-}$ MEF cells following 5 and 10 min stimulate with either IMI (10 μM) or AMI (1 μM) as indicated. Data shown are mean ± SEM and represent $n \geqslant 7$ replicates for each group. $^{*}p < 0.05$; $^{**}p < 0.01$ by unpaired Student's *t*-test, stimulated group versus control group. $^{\blacklozenge}p < 0.05$; $^{\blacklozenge\blacklozenge}p < 0.01$ by unpaired Student's *t*-test, ArrWT versus Arr2,3$^{-/-}$.

3.3. Variable recruitment of arrestins to the α_{2A}AR by tricyclic antidepressants

In addition to demonstrating that DMI is a functionally biased ligand at the α_{2A}AR favoring endocytosis but not signal transduction, our previous work established that DMI functions as an arrestin-biased ligand [8]. Arrestin-biased agonism, wherein a ligand stimulates arrestin recruitment to a GPCR but not G protein coupling, is becoming an increasingly appreciated phenomenon [26,27]. Such biased agonism contrasts with classical agonism, wherein both G protein coupling and arrestin recruitment occur. Our report on DMI provided the first documented evidence of arrestin-biased agonism at the α_{2A}AR specifically, and in the present study, we can confirm other TCAs share this characteristic.

Through the use of FLIM–FRET, we assessed the ability of IMI and AMI to drive arrestin recruitment to the α_{2A}AR in live cells. Fig. 4A shows our successful expression of CFP-tagged α_{2A}ARs and YFP-tagged arrestins in HEK 293 cells. Following 10 min stimulation of such cells, specifically expression YFP-arrestin3, we found that IMI (10 μM) drives a clear and significant increase in FLIM–FRET efficiency, indicating α_{2A}AR/arrestin3 interaction at the cell surface (Fig. 4B). The degree of interaction stimulated by IMI (~25% FLIM–FRET *E*) is essentially identical to that stimulated by DMI [8]. Further, a 10-fold lower concentration of IMI was equally effective at stimulating the α_{2A}AR/arrestin3 interaction (data not shown). In contrast, AMI (1 μM) drove α_{2A}AR/arrestin3 recruitment to a much lesser, though still significant, degree (Fig. 4B). Given the complete arrestin-dependence of AMI-induced α_{2A}AR endocytosis, we additionally assessed the ability of AMI to drive recruitment of arrestin2 to the receptor, and found that AMI (1 μM) does in fact stimulate a significantly stronger α_{2A}AR/arrestin2 interaction (Fig. 4B). It seems likely then that AMI-stimulated α_{2A}AR endocytosis is mediated by a combination of arrestin3 and arrestin2. These data also suggest that the carbon-for-nitrogen substitution at the side chain attachment point in AMI not only affects the affinity of its α_{2A}AR interaction but also impacts which of the two arrestins is recruited.

These data represent variability in the pharmacological relationship between the α_{2A}AR and TCAs. In addition to the variable affinity for the ligands described earlier, there is variable arrestin recruitment to the receptor by these ligands. Among the three TCAs studied previously and presently, DMI and IMI appear largely identical while AMI stands apart. AMI binds the α_{2A}AR with mid-nanomolar affinity, but is the weakest driver of arrestin recruitment. Meanwhile, DMI and IMI bind the α_{2A}AR with low micromolar affinity, but drive arrestin recruitment more strongly. Furthermore, AMI alone appears to preferentially drive arrestin2 recruitment, as our previous work established that DMI preferentially drives arrestin3 recruitment [8]. The present data do indicate one difference functional difference between DMI and IMI though, as IMI retains a slight but significant ability to drive α_{2A}AR endocytosis in arrestin-null cells (Fig. 3B). This suggests that IMI, when binding the α_{2A}AR, can at least partially engage some arrestin-independent endocytic mechanism, but further study will be

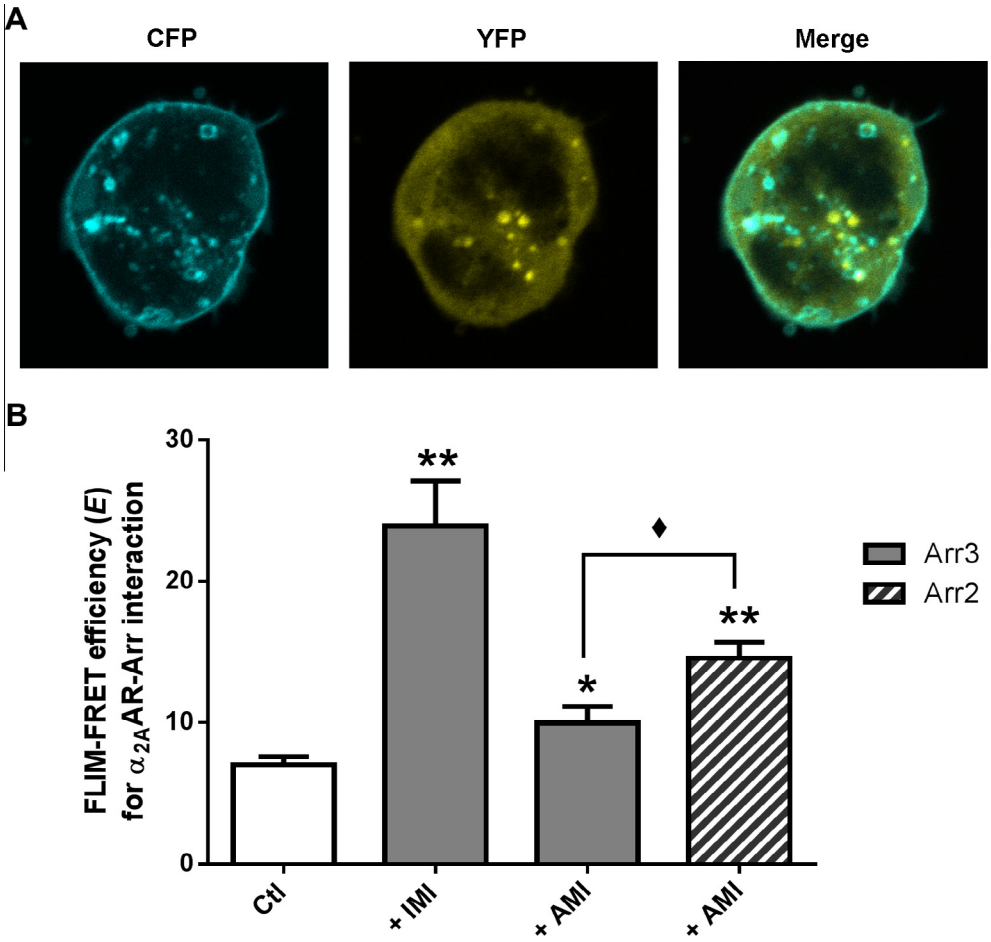

Fig. 4. Arrestin recruitment to the α_{2A}AR by tricyclic antidepressants is variable. (A) Representative confocal images showing the expression of CFP-α_{2A}AR and YFP-Arr in live transfected HEK 293 cells. (B) FLIM–FRET efficiency (*E*) values (%) for α_{2A}AR/Arr3 interaction (solid gray bars) and α_{2A}AR/Arr2 interaction (hashed bar). Data were collected following 10 min stimulation with either IMI (10 μM) or AMI (1 μM); data shown are mean ± SEM and represent $n = 5–6$ for each group. $^{*}p < 0.05$; $^{**}p < 0.0001$ by unpaired Student's *t*-test, stimulated versus control. $^{\blacklozenge}p < 0.05$ by unpaired Student's *t*-test, Arr3 versus Arr2.

necessary to elucidate an explanation. Overall, these data demonstrate that it is difficult to draw a clear correlation between receptor affinity and strength of arrestin recruitment or between strength of arrestin recruitment and degree of receptor endocytosis induced.

3.4. Implications

The present findings are an important expansion of our previous work and provide novel information regarding both antidepressant pharmacology and ligand-selective regulation of the α_{2A}AR. We have now demonstrated that multiple members of the tricyclic antidepressant drug class function as arrestin-biased ligands at the α_{2A}AR, a property which precludes the classification of these drugs as simply receptor antagonists. Importantly, the present work further demonstrates that the particular strength of interaction with and recruitment of arrestin to the α_{2A}AR varies among the different TCAs. In addition, there seems to be some variability in whether arrestin2 or arrestin3 is preferentially recruited. Given the evidence for differential distribution of these arrestin subtypes in the brain [28], this variability raises the possibility that different TCAs cause distinct patterns of central α_{2A}AR regulation.

The clinical significance of our findings arises from evidence linking upregulation of α_2AR expression and activity with depression in human patients [18]. Although we have not directly observed downregulation in response to IMI and AMI, the collective efforts of our previous and present work strongly suggest that TCAs, by acting as direct arrestin-biased ligands, are able to drive a reduction in α_{2A}AR levels. We believe that this effect represents an important component of the therapeutic mechanism of action for these TCAs, but not drugs such as SSRIs, which essentially have no physiologically relevant receptor interaction. Moving forward, studies such as our own, together with increasing structural knowledge of GPCR ligand binding and activation, should contribute to the development of more selective therapeutics.

Acknowledgments

Our thanks to Dr. Robert J. Lefkowitz (Duke University) for generously providing the Arr2,3$^{-/-}$ MEF cell line, to Drs. Kai Jiao (UAB) and Kurt Gibbs (Morehead State) for helpful discussions, and to Bethanny Glahn (Morehead State) for routine maintenance of cell lines. This work was supported by a National Institute of Mental Health Grant (MH081917), NARSAD Independent Investigator Award (to QW) and a National Institute of General Medical Sciences Grant (P20GM103436).

References

[1] M.H. Trivedi, A.J. Rush, S.R. Wisniewski, A.A. Nierenberg, D. Warden, L. Ritz, et al., Evaluation of outcomes with citalopram for depression using measurement-based care in STAR*D: implications for clinical practice, Am. J. Psychiatry 163 (2006) 28–40, http://dx.doi.org/10.1176/appi.ajp.163.1.28.

[2] E.H. Turner, A.M. Matthews, E. Linardatos, R.A. Tell, R. Rosenthal, Selective publication of antidepressant trials and its influence on apparent efficacy, N. Engl. J. Med. 358 (2008) 252–260, http://dx.doi.org/10.1056/NEJMsa065779.

[3] J.C. Fournier, R.J. DeRubeis, S.D. Hollon, et al., Antidepressant drug effects and depression severity: a patient-level meta-analysis, JAMA 303 (2010) 47–53, http://dx.doi.org/10.1001/jama.2009.1943.

[4] M. Fava, A.J. Rush, S.R. Wisniewski, A.A. Nierenberg, J.E. Alpert, P.J. McGrath, et al., A comparison of mirtazapine and nortriptyline following two consecutive failed medication treatments for depressed outpatients: a STAR*D report, Am. J. Psychiatry 163 (2006) 1161–1172, http://dx.doi.org/10.1176/appi.ajp.163.7.1161.

[5] P.J. McGrath, J.W. Stewart, M. Fava, M.H. Trivedi, S.R. Wisniewski, A.A. Nierenberg, et al., Tranylcypromine versus venlafaxine plus mirtazapine following three failed antidepressant medication trials for depression: a STAR*D report, Am. J. Psychiatry 163 (2006) 1531–1541, http://dx.doi.org/10.1176/appi.ajp.163.9.1531 (quiz 1666).

[6] I.M. Anderson, Selective serotonin reuptake inhibitors versus tricyclic antidepressants: a meta-analysis of efficacy and tolerability, J. Affect. Disord. 58 (2000) 19–36.

[7] B. Arroll, S. Macgillivray, S. Ogston, I. Reid, F. Sullivan, B. Williams, et al., Efficacy and tolerability of tricyclic antidepressants and SSRIs compared with placebo for treatment of depression in primary care: a meta-analysis, Ann. Fam. Med. 3 (2005) 449–456, http://dx.doi.org/10.1370/afm.349.

[8] C. Cottingham, Y. Chen, K. Jiao, Q. Wang, The antidepressant desipramine is an arrestin-biased ligand at the alpha(2A)-adrenergic receptor driving receptor down-regulation in vitro and in vivo, J. Biol. Chem. 286 (2011) 36063–36075, http://dx.doi.org/10.1074/jbc.M111.261578.

[9] C. Cottingham, X. Li, Q. Wang, Noradrenergic antidepressant responses to desipramine in vivo are reciprocally regulated by arrestin3 and spinophilin, Neuropharmacology 62 (2012) 2354–2362, http://dx.doi.org/10.1016/j.neuropharm.2012.02.011.

[10] C. Cottingham, A. Jones, Q. Wang, Desipramine selectively potentiates norepinephrine-elicited ERK1/2 activation through the alpha2A adrenergic receptor, Biochem. Biophys. Res. Commun. 420 (2012) 161–165, http://dx.doi.org/10.1016/j.bbrc.2012.02.135.

[11] H. De Vos, G. Vauquelin, K.J. De, J.P. De Backer, L. Van I, Regional distribution of alpha 2A- and alpha 2B-adrenoceptor subtypes in postmortem human brain, J. Neurochem. 58 (1992) 1555–1560.

[12] M. Sastre, J.A. García-Sevilla, Alpha 2-adrenoceptor subtypes identified by [3H]RX821002 binding in the human brain: the agonist guanoxabenz does not discriminate different forms of the predominant alpha 2A subtype, J. Neurochem. 63 (1994) 1077–1085.

[13] R. Wang, L.B. Macmillan, R.T. Fremeau Jr., M.A. Magnuson, J. Lindner, L.E. Limbird, Expression of alpha 2-adrenergic receptor subtypes in the mouse brain: evaluation of spatial and temporal information imparted by 3 kb of 5′ regulatory sequence for the alpha 2A AR-receptor gene in transgenic animals, Neuroscience 74 (1996) 199–218.

[14] L. Hein, J.D. Altman, B.K. Kobilka, Two functionally distinct alpha2-adrenergic receptors regulate sympathetic neurotransmission, Nature 402 (1999) 181–184.

[15] A.E. Knaus, V. Muthig, S. Schickinger, E. Moura, N. Beetz, R. Gilsbach, et al., Alpha2-adrenoceptor subtypes – unexpected functions for receptors and ligands derived from gene-targeted mouse models, Neurochem. Int. 51 (2007) 277–281, http://dx.doi.org/10.1016/j.neuint.2007.06.036.

[16] R. Gilsbach, C. Roser, N. Beetz, M. Brede, K. Hadamek, M. Haubold, et al., Genetic dissection of alpha2-adrenoceptor functions in adrenergic versus nonadrenergic cells, Mol. Pharmacol. 75 (2009) 1160–1170.

[17] A.D. Shields, Q. Wang, D.G. Winder, Alpha2A-adrenergic receptors heterosynaptically regulate glutamatergic transmission in the bed nucleus of the stria terminalis, Neuroscience 163 (2009) 339–351.

[18] C. Cottingham, Q. Wang, α2 adrenergic receptor dysregulation in depressive disorders: Implications for the neurobiology of depression and antidepressant therapy, Neurosci. Biobehav. Rev. 36 (2012) 2214–2225, http://dx.doi.org/10.1016/j.neubiorev.2012.07.011.

[19] A.E. Brady, Q. Wang, P.B. Allen, M. Rizzo, P. Greengard, L.E. Limbird, Alpha 2-adrenergic agonist enrichment of spinophilin at the cell surface involves beta gamma subunits of Gi proteins and is preferentially induced by the alpha 2A-subtype, Mol. Pharmacol. 67 (2005) 1690–1696, http://dx.doi.org/10.1124/mol.104.005215.

[20] N.L. Schramm, L.E. Limbird, Stimulation of mitogen-activated protein kinase by G protein-coupled alpha(2)-adrenergic receptors does not require agonist-elicited endocytosis, J. Biol. Chem. 274 (1999) 24935–24940.

[21] R.J. Baldessarini, Drug therapy of depression and anxiety disorders, in: L. Brunton, J.S. Lazo, K.L. Parker (Eds.), Goodman Gilmans Pharmacol. Basis Ther., eleventh ed., McGraw-Hill Inc., New York, 2006, pp. 429–460.

[22] S.K. Shenoy, R.J. Lefkowitz, Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling, Biochem. J. 375 (2003) 503–515, http://dx.doi.org/10.1042/BJ20031076.

[23] C.M. Tan, A.E. Brady, H.H. Nickols, Q. Wang, L.E. Limbird, Membrane trafficking of G protein-coupled receptors, Annu. Rev. Pharmacol. Toxicol. 44 (2004) 559–609, http://dx.doi.org/10.1146/annurev.pharmtox.44.101802.121558.

[24] A.C. Hanyaloglu, M. von Zastrow, Regulation of GPCRs by endocytic membrane trafficking and its potential implications, Annu. Rev. Pharmacol. Toxicol. 48 (2008) 537–568, http://dx.doi.org/10.1146/annurev.pharmtox.48.113006.094830.

[25] T.A. Kohout, F.S. Lin, S.J. Perry, D.A. Conner, R.J. Lefkowitz, Beta-arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 1601–1606, http://dx.doi.org/10.1073/pnas.041608198.

[26] J.D. Violin, R.J. Lefkowitz, Beta-arrestin-biased ligands at seven-transmembrane receptors, Trends Pharmacol. Sci. 28 (2007) 416–422, http://dx.doi.org/10.1016/j.tips.2007.06.006.

[27] S. Rajagopal, K. Rajagopal, R.J. Lefkowitz, Teaching old receptors new tricks: biasing seven-transmembrane receptors, Nat. Rev. Drug Discov. 9 (2010) 373–386, http://dx.doi.org/10.1038/nrd3024.

[28] E.V. Gurevich, J.L. Benovic, V.V. Gurevich, Arrestin2 and arrestin3 are differentially expressed in the rat brain during postnatal development, Neuroscience 109 (2002) 421–436.