Synergistic Regulation of Glutamatergic Transmission by Serotonin and Norepinephrine Reuptake Inhibitors in Prefrontal Cortical Neurons

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Running title: Modulation of AMPARs by 5-HT and NE Dual Reuptake Inhibitors

Background: Serotonin and norepinephrine reuptake inhibitors (SNRIs) produce better therapeutic effects than single selective reuptake inhibitors, while the underlying mechanisms are largely unknown.

Results: Low-dose SNRIs, by acting on 5-HT₁A and α₂-AR, synergistically reduced glutamatergic transmission in prefrontal cortex (PFC).

Conclusion: SNRIs exerts a powerful impact on PFC synaptic activity.

Significance: It provides a mechanism for the therapeutic effects of SNRIs in mental disorders related to PFC dysfunction.

Monoamine system in prefrontal cortex (PFC) has been implicated in various mental disorders and has been the major target of anxiolytics and antidepressants. Clinical studies show that serotonin and norepinephrine reuptake inhibitors (SNRIs) produce better therapeutic effects than single selective reuptake inhibitors, while the underlying mechanisms are largely unknown. Here, we found that low-dose SNRIs, by acting on 5-HT₁A and α₂-adrenergic receptors (α₂-AR) receptors, synergistically reduced AMPAR-mediated excitatory postsynaptic currents (EPSC) and AMPAR surface expression in PFC pyramidal neurons, via a mechanism involving Rab5/dynamin-mediated endocytosis of AMPARs. The synergistic effect of SNRIs on AMPARs was blocked by inhibition of Activator of G protein Signaling 3 (AGS3), a G-protein modulator that prevents re-association of Gᵢ protein α subunit and prolongs βγ-mediated signaling pathway.

Moreover, the depression of AMPAR-EPSC by SNRIs required p38 kinase activity, which was increased by 5-HT₁A and α₂-AR co-activation in an AGS3-dependent manner. These results have revealed a potential mechanism for the synergy between serotonin and norepinephrine systems on the regulation of glutamatergic transmission in cortical neurons.

Keywords: AMPA receptor; serotonin; norepinephrine; reuptake inhibitors; AGS3; Rab5; p38 MAPK

Monoamines, such as serotonin (5-HT) and norepinephrine (NE), play important roles in cognitive and emotional processes mediated by prefrontal cortex (PFC, 1,2). Changing 5-HT and NE transmission has been the core mechanism among antidepressants and anxiolytics (3-7). Since monoamines do not pass blood brain barrier, effective drugs are reuptake inhibitors to raise their synaptic concentrations. Clinical trials suggest that drugs increasing both 5-HT and NE levels at synapses give more robust therapeutic effects than those raising single neurotransmitter alone, including faster onset and lower relapse rate (4,8,9). However, little is known about the underlying mechanisms.

Both serotonin receptors and adrenergic receptors have multiple subtypes that can be grouped into different classes based on their pharmacological and signaling properties (10,11). Among these
receptors, 5-HT_{1A} and \( \alpha_2 \)-AR are G_{i}-coupled GPCRs enriched in dendritic spines (12,13), where glutamatergic transmission occurs. Abnormal expression and signaling mediated by 5-HT_{1A} and \( \alpha_2 \)-AR have been highly implicated in psychiatric disorders. For example, genetic knockout of 5-HT_{1A} or \( \alpha_2 \)-AR show elevated anxiety phenotype (14,15). Postmortem brains of depressed patients show altered receptor levels or ligand binding (16,17). The 5-HT_{1A} and \( \alpha_2 \)-AR gene polymorphisms or altered receptor functions correlate well with the prevalence of depression in humans (18), as well as the effectiveness of antidepressants (19,20).

In the present study, we have identified that AMPAR is the cellular target of monoamines in prefrontal cortex. Low-dose of serotonin and norepinephrine reuptake inhibitors (SNRIs), by simultaneously activating 5-HT_{1A} and \( \alpha_2 \)-AR, synergistically suppresses glutamatergic transmission in PFC pyramidal neurons through a mechanism involving AMPAR internalization. Given the significance of AMPAR-mediated excitation in PFC-dependent functions (21,22), our results provide a potential basis for the better effects of dual reuptake inhibitors in therapeutic intervention of mental disorders related to PFC dysfunction.

EXPERIMENTAL PROCEDURES

**Electrophysiological Recordings in slices and cultures.** The whole-cell voltage clamp technique was used to record AMPAR-EPSC in PFC layer V pyramidal neurons from postnatal rats (3-4 weeks) as we described previously (23-25). Slices (300 \( \mu \)m) was perfused with ACSF bubbled with 95% O_{2} and 5% CO_{2} containing APV (25 \( \mu \)M) and bicuculline (10 \( \mu \)M). The internal solution contained (in mM): 130 Cs-methanesulfonate, 10 CsCl, 1 NaCl, 1 MgCl_{2}, 10 HEPES, 5 EGTA, 2.2 QX-314, 12 phosphocreatine, 5 MgATP, 0.5 Na_{2}GTP, pH 7.2-7.3, 265-270 mosM. Neurons were visualized with a 40 x water-immersion lens and illuminated with near infrared IR light. All recordings were performed using a Multiclamp 700A amplifier with neurons held at -70mV. Tight seals (2-10 GΩ) were generated by applying negative pressure, followed by additional suction to disrupt the membrane and obtain the whole-cell configuration. EPSCs were stimulated by exciting the neighboring cortical neurons with a bipolar tungsten electrode (FHC, Inc.) located at a few hundred micrometers away from the neuron under recording.

Rat cortical cultures were prepared by modified methods as described previously (24,26). After treatment or transfection, the mEPSC recordings in culture neurons (DIV 21-24) were performed in the presence of APV (25 \( \mu \)M), bicuculline (10 \( \mu \)M) and TTX (1 \( \mu \)M).

The agents we used include fluoxetine, 8-hydroxy-2(di-n-propylamino) tetralin (8-OH-DPAT), WAY-100635 (Sigma), desipramine, venlafaxine, clonidine hydrochloride, yohimbine hydrochloride, SB203480, PD98059 and dynamin inhibitory peptide (Tocris). They were made up as concentrated stocks in water or DMSO and stored at -20°C. Stocks were thawed and diluted immediately prior to experiments.

**Biochemical measurement of surface receptors.** The surface AMPA receptors were detected as described previously (23,24). After treatment, PFC slices were incubated in PBS containing 1 mg/ml Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) on ice for 40 minutes. The slices were then rinsed three times in TBS to quench the biotin reaction, followed by homogenization in 500 \( \mu \)l modified RIPA buffer containing 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 50 mM NaPO_{4}, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 mg/ml leupeptin. The homogenates were centrifuged at 16,000 x g for 30 min at 4°C. Supernatant was collected and incubated with Neutravidin Agarose (Pierce) for 2 hr at 4°C. Bound proteins were washed 3 times by RIPA buffer and subjected to SDS-PAGE. Western blots were performed on biotinylated (surface) and total proteins using antibodies against GluR1 or GluR2 (both 1:500; Chemicon).

**Immunocytochemistry.** After transfection and treatment, neurons were fixed in 4%
paraformaldehyde in PBS for 20 min at room temperature and incubated with 5% bovine serum albumin (BSA) for 1 hour to block nonspecific staining. Cells were then permeabilized with 0.2% triton for 20 min at room temperature, followed by incubation with the primary antibody at 4°C overnight. For measuring the level of total and active p38 MAPK, anti-p38 and anti-Thr180/Tyr182 phospho-p38 antibodies (1: 500, Cell Signaling) were used. After washing, neurons were incubated with Alex488 (green)- or Alex594 (red)-conjugated secondary antibodies (Molecular Probe, 1:500) for 2 hr at room temperature. After washing in PBS for three times, the coverslips were mounted on slides with VECTASHIELD mounting media (Vector Laboratories, Burlingame, CA). Images were captured with identical conditions and analyzed with identical parameters. The intensity of p38 signal was quantified using Image J software.

Small interference RNA. To knock down the endogenous AGS3 expression, we used the small interfering RNA (siRNA) specifically targeting AGS3 (Santa Cruz, CA): 5’- CGAGAGCACUCUACAACAU, 5’- GCUGAACAUUAACAAGGA-3 and 5’- CCAAGCAUAGGGUCUUGUA. AGS3 siRNA oligos were co-transfected with EGFP into cultured PFC neurons (DIV21) using the Lipofectamine 2000 method. A scrambled siRNA was used as control. Western blot analysis of AGS3 expression was performed to validate the gene silencing efficiency of AGS3 siRNA using anti-AGS3 antibody (1:500, Santa Cruz). Biochemical, immunocytochemical, or electrophysiological experiments were performed after 2-3 days of transfection.

Cloning, Expression, and Purification of Proteins. Purified Rab5 protein was generated with the similar method as described previously (27). Using mouse brain total RNA, wild-type Rab5 cDNA was cloned by RT-PCR, followed by sequence verification. The cDNA was then subcloned into the bacterial expression vector pQE-80 (QIAGEN, Valencia, CA), which added a His6 tag at the N terminus of the protein. Rab5 mutants (S34N and Q79L) were generated by site-directed mutagenesis using the QuickChange Kit from Stratagene (La Jolla, CA). Rab5 expression in the M15 strain of E. Coli (QIAGEN) was induced by adding isopropyl β-D-thiogalactoside to 1 mM final concentration for 4-5 hr at 25°C (to minimize the formation of inclusion bodies). Rab5 in cleared E. coli lysate was purified by affinity chromatography using the His Gravitrap column (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) according to the manufacturer’s protocol. His6-tagged Rab5 proteins were eluted from the column in a buffer containing 50 mM Tris, 500 mM NaCl, and 300 mM imidazole, pH 7.4. Fractions of eluate were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining to identify the peak fractions containing Rab5 proteins. Western blotting with the polyclonal anti-Rab5 (Santa Cruz Biotechnology) was also performed to verify the expression of purified Rab5 protein. The most pure 1-2 fractions (shown as a single band by Coomassie blue staining) were dialyzed against phosphate-buffered saline before being used in electrophysiological experiments.

Statistics
Data analyses were performed with AxoGraph (Axon Instruments), Kaleidagraph (Albeck Software, Reading, PA), Origin 6 (Microcal Software, Northampton, MA), and Statview (Abacus Concepts, Calabasas, CA). All data are expressed as the mean ± SEM. Experiments with more than two groups were subjected to one-way ANOVA or two-way ANOVA, followed by post hoc Tukey tests. Experiments with two groups were analyzed statistically using unpaired Student’s t-tests.

Results
Serotonin and norepinephrine reuptake inhibitors (SNRIs) induce a synergistic reduction of AMPAR-mediated synaptic transmission in PFC pyramidal neurons.

To understand the interactive role of serotonin (5-HT) and norepinephrine (NE) systems in glutamatergic transmission, we recorded AMPAR-mediated excitatory postsynaptic currents
(EPSC) in PFC slices. Reuptake inhibitors, fluoxetine and desipramine, were used to elevate synaptic concentration of endogenous 5-HT and NE, respectively. As shown in Fig. 1A and 1B, bath application of fluoxetine (10 μM) or desipramine (10 μM) alone did not significantly alter AMPAR-EPSC (fluox: 7.2 ± 1.7%, n = 5; des: 9.8 ± 1.6%, n = 7, p>0.05, ANOVA). However, co-application of both inhibitors induced a substantial reduction of AMPAR-EPSC amplitude by 48.8 ± 4.9% (n = 10, p<0.001, ANOVA). Dose-dependent experiments using fluoxetine and/or desipramine were shown in Fig. 1C. AMPAR-EPSC was not sensitive to either inhibitor at the dosage of less than 10 μM, but was significantly reduced by both inhibitors at low-doses (5 μM: 23.5 ± 1.8% and 10 μM: 49.0 ± 3.9%, p<0.001, ANOVA). Since the dual action of fluoxetine and desipramine at 10 μM gave a saturating effect on AMPAR currents, we selected this concentration for the remaining experiments.

Next, we tested whether the synergistic reduction of AMPAR-EPSC by SNRIs also occurred in vivo. Animals were intraperitoneally injected with fluoxetine (5 mg/kg, 1hr) and/or desipramine (1 mg/kg, 1hr) before slicing and recording. As shown in Fig. 1D and 1E, AMPAR-EPSC was not altered in animals injected with fluoxetine or desipramine alone (saline: n=12; fluox: n=11; des: n=10), but was significantly reduced in those co-injected with desipramine and fluoxetine (des+fluox: n=10, F_{4,192}=13.8, p<0.001, ANOVA). These data suggest that endogenous serotonin and norepinephrine induce a synergistic down-regulation of glutamatergic transmission in vivo.

To test whether a bona fide SNRI have the same effect as fluoxetine plus desipramine, we examined venlafaxine, a SNRI used for treating certain anxiety disorders with depression. As shown in Fig. 1F, bath application of venlafaxine to cortical slices reduced AMPAR-EPSC in a dose-dependent manner with a significant effect at 50 μM (25.5 ± 4.2%, n=5, F_{3,16}=11.2, p<0.001, ANOVA). PFC neurons from animals with an i.p. injection of venlafaxine (8 mg/kg, 1 hr) also showed a significant reduction of AMPAR-EPSC (saline: n=10; venlafaxine n=10, F_{4,90}=7.6, p<0.001, two-way ANOVA).

Co-activation of 5-HT_{1A} and α_2-AR receptors mediates the synergistic reduction of AMPAR currents and surface expression by SNRIs.

To determine which receptors underlie the synergistic regulation of AMPARs by SNRIs, we used specific receptor antagonists. As shown in Fig. 2A-C, 5-HT_{1A} antagonist WAY-100635 (200 nM) or α_2-AR antagonist yohimbine (40 μM) alone completely blocked the synergistic reduction of AMPAR-EPSC by desipramine and fluoxetine (WAY: 3.1 ± 2.8%, n = 5, yohi: 7.0 ± 1.5%, n = 6), suggesting that simultaneous activation of both receptors is required for the effect of SNRIs. However, dopamine D4 receptor antagonist L741,742 (10 μM) failed to alter the synergistic reduction of AMPAR-EPSC by desipramine and fluoxetine (WAY: 3.1 ± 2.8%, n = 5, yohi: 7.0 ± 1.5%, n = 6), excluding the potential involvement of D4 receptors. Moreover, co-application of 5-HT_{1A} agonist 8-OH-DPAT (5 μM) and α_2-AR agonist clonidine (5 μM) significantly reduced AMPAR-EPSC (40.6 ± 6.3%, n = 5), while neither of the single agonist was effective (8-OH-DPAT: 7.0 ± 1.9%, n = 8, clonidine: 3.6 ± 1.9%, n = 5, p>0.05, ANOVA). Taken together, these results suggest that co-activation of 5-HT_{1A} and α_2-AR underlies the synergistic reduction of AMPAR currents by dual reuptake inhibitors.

Stimulation of serotonin and norepinephrine receptors may alter presynaptic glutamate release, which can contribute to the change in AMPAR-EPSC. To examine this possibility, we recorded miniature EPSC (mEPSC), which results from the random release of single transmitter vesicles. Significant changes in mEPSC amplitude reflect modifications of post-synaptic glutamate receptors. As shown in Fig. 2D-F, co-application of 8-OH-DPAT (5 μM) and clonidine (5 μM) primarily reduced mEPSC amplitude (26.3 ± 1.6%, n=6, p<0.001, ANOVA), but not frequency (12.5 ± 3.2%, n=6, p>0.05, ANOVA). No significant reduction of mEPSC was observed by 8-OH-DPAT alone (amp: 7.8 ± 1.9%; freq: 11.4 ± 2.4%, n=7, p>0.05, ANOVA).
ANOVA) or clonidine alone (amp: 3.3 ± 1.2%; freq: 9.8 ± 2.0%, n=6, p>0.05, ANOVA). Moreover, the paired-pulse (interval: 0.1 sec) ratio of AMPAR-EPSC (an index of presynaptic glutamate release) was unchanged in PFC slices treated with fluoxetine and desipramine (control: 1.37 ± 0.05; fluox+des: 1.4 ± 0.04, n=4, p>0.05, ANOVA). These data suggests that the reduction of AMPAR-EPSC by 5-HT1A and α2-AR co-activation does not result from presynaptic changes.

We next examined the effect of SNRIs on AMPAR surface expression. As shown in Fig. 2G and 2H, the level of surface GluR1 and GluR2 subunits were significantly decreased in PFC slices co-treated with fluoxetine and desipramine (GluR1: 0.53±0.08 of control; GluR2: 0.62 ± 0.04 of control, n=4, p<0.001, ANOVA), but not with the application of fluoxetine alone (GluR1: 0.98 ± 0.15 of control; GluR2: 0.95 ± 0.11 of control, n=4, p>0.05, ANOVA) or desipramine alone (GluR1: 0.85 ± 0.01 of control; GluR2: 1.04 ± 0.16, n=4, p>0.05, ANOVA). It suggests that SNRIs might alter AMPAR-mediated synaptic responses by changing the surface expression of AMPARs.

The regulation of AMPARs by SNRIs involves dynamin/Rab5-dependent AMPAR endocytosis.

Since SNRIs reduce surface AMPARs, we tested whether it results from increased internalization of surface AMPARs or from decreased AMPAR transport along cytoskeletons. To do so, we dialyzed neurons with a dynamin inhibitory peptide (50 μM), which disrupts the amphiphysin-dynamin binding and thus prevents endocytosis through clathrin-coated pits (28). As shown in Fig. 3A and 3B, the dynamin inhibitory peptide prevented the reduction of AMPAR-EPSCs by fluoxetine and desipramine (5.8 ± 1.6%, n = 7), while a scrambled control peptide was ineffective (46.0 ± 3.3%, n = 6). Dialysis of reagents that perturb microtubule or actin stability failed to alter the effect of SNRIs on AMPAR-EPSC (colchicine: 30 μM; 43.8 ± 2.2%, n = 6; latrunculin: 5 μM; 41.0 ± 3.2%, n = 6, Fig. 3B), which ruled out the involvement of cytoskeleton-dependent transport of AMPARs. These data suggest that the regulation of AMPARs by SNRIs relies on dynamin-dependent AMPAR endocytosis.

AGS3, a G protein modulator, is involved in the synergistic regulation of AMPARs by SNRIs.

We next sought to examine the molecular mechanisms underlying the synergistic regulation of AMPARs by SNRIs. Both α2-AR and 5-HT1A are G-i-coupled receptors. G-protein signaling initiates with the dissociation of βγ and α subunits, and is terminated by re-association of the two. Activator of G protein signaling 3 (AGS3) binds to βγ subunit and prevents it from re-associating with G i α subunit, resulting in persistent activation of βγ-mediated signaling (32). To test whether AGS3 could be the key synergistic modulator in the regulation of AMPARs by SNRIs, we dialyzed neurons with a peptide derived from AGS3 (GRKRRQRRRPTMGEDFDDLLAKSQSKRM DDQRVDLAK), which interrupts the AGS3-βγ binding (33). As shown in Fig. 4A-C, AGS3 peptide abolished the reduction of AMPAR-EPSC by co-
application of fluoxetine and desipramine (AGS3 pep: 12.2 ± 1.6%, n = 5; control pep: 40.2 ± 3.5%, n = 5). Moreover, inhibiting intracellular AGS3 function by infusing an AGS3 antibody gave similar blockade (AGS3 ab: 10.8 ± 1.8%, n = 6; heat-inactivated ab: 49.3 ± 1.9%, n = 5).

To further test the involvement of AGS3, we utilized the siRNA approach to knockdown AGS3. As shown in Fig. 4D-F, AGS3 siRNA induced a significant suppression of AGS expression in cortical cultures. 8-OH-DPAT plus Clonidine failed to reduce mEPSC in AGS3 siRNA-transfected neurons (amp: 8.0 ± 3.4%, freq: 5.6 ± 2.6%, n = 7), but not in scrambled siRNA-transfected cells (amp: 26.1 ± 2.5%, freq: 9.3 ± 1.6%, n = 6). Taken together, these lines of evidence indicate that 5-HT1A and α2-AR synergistically down-regulates AMPAR-EPSC through a mechanism involving AGS3-facilitated G protein signaling.

**5-HT1A and α2-AR receptors synergistically activate p38 kinase in an AGS3-dependent manner.**

We further investigated the mechanism downstream of AGS3/G protein signaling that leads to the reduction of AMPAR-EPSC by SNRIs. A potential candidate is p38 MAPK, whose activation can induce the removal of synaptic AMPARs (34). To test this, we dialyzed neurons with p38 inhibitor SB2303480 (20 µM). The p44/42 MAPK inhibitor PD98059 (40 µM) was used as a control. As shown in Fig. 5A and 5B, fluoxetine and desipramine lost the capability to reduce AMPAR-EPSC significantly in the presence of SB2303480 (11.0 ± 1.7%, n = 6), but not PD98059 (45.6 ± 3.5%, n = 5).

To examine whether co-stimulation of 5-HT1A and α2-AR receptors activates p38 MAPK via AGS3, we transfected neurons with AGS3 siRNA, followed by measuring the level of active (Thr180/Tyr182 phosphorylated) p38 MAPK (35). As shown in Fig. 5C and 5D, co-treatment of 8-OH-DPAT and clonidine increased p38 activity in neurons transfected with a control siRNA (2.1 ± 0.15 of control, n = 4), and this effect was abolished in cells transfected with AGS3 siRNA (1.25 ± 0.1 of control, n = 4). Application of 8-OH-DPAT or clonidine alone did not affect p38 activity. The activity of p44/42 was not altered by co-treatment of 8-OH-DPAT and clonidine. Consistent with the Western blotting results, immunocytochemical data (Fig. 5E and 5F) showed that 8-OH-DPAT plus clonidine treatment increased the level of active p38 in cultured neurons transfected with a control siRNA (control: 1 ± 0.05, DPAT+Clon: 1.87 ± 0.05, n=19-22, p<0.001, ANOVA), but not AGS3 siRNA (control: 0.93 ± 0.11, DPAT+Clon: 0.83 ± 0.05, n=19-21). Taken together, these results suggest that 5-HT1A and α2-AR receptor co-activation synergistically reduces AMPARs via AGS3-dependent activation of p38 MAPK.

**Discussion**

Monoamine hypofunction has long been proposed to underlie the pathophysiology of depression and anxiety disorders (6,36). Data suggest that the interplay of NE with 5-HT may exert neurobiological normalization in mental illnesses (37). Dual enhancement of NE and 5-HT systems produces better therapeutic outcome than targeting only one monoamine system (4,8). The class of serotonin and norepinephrine reuptake inhibitors (SNRIs) now comprises three medications: venlafaxine, milnacipran, and duloxetine, all of which are efficacious in treating a variety of anxiety disorders and are helpful in relieving chronic pain associated with and independent of depression (38). Evidence from meta-analysis of clinical trials supports the greater efficacy of venlafaxine and duloxetine in moderate to severe depression compared to selective serotonin reuptake inhibitors (9).

Using combined electrophysiological, biochemical and immunocytochemical approaches, we have found that application of low-dose SNRI synergistically decreases AMPAR surface expression and function in PFC pyramidal neurons. Since high concentrations of single reuptake inhibitors are toxic and even lethal (39), our results suggest that using low-dose SNRI may provide more effective regulation of synaptic transmission in prefrontal cortex, which is important for cognitive and emotional processes. Moreover, the SNRI-
induced synergistic activation of signaling cascades to alter synaptic function may accelerate the onset of therapeutic effects.

Pharmacological evidence suggests that the synergistic regulation of AMPARs by SNRIs is mediated by 5-HT1A and α2-AR receptors, both of which are enriched at glutamatergic synapses (12,13). An important player involved in the synergistic actions of α2-AR and 5-HT1A receptors is identified as Activator of G protein Signaling 3 (AGS3), which prolongs βγ-mediated signaling by preventing the re-association of Gi α subunit (32). Consistently, AGS3 also mediates the synergy between mu-opiate and cannabinoid CB1 receptors (40).

Co-stimulation of 5-HT1A and α2-AR receptors induces AGS3-dependent activation of p38 MAPK, probably via G protein βγ subunit, small GTPase Rap1, and MAPK kinase 3/6, similar to metabotropic glutamate receptors (mGluRs, 41). Activation of p38 MAPK is found to be required for the removal of synaptic AMPARs and synaptic depression (34). Our previous study has also shown that G protein-coupled receptors, such as mGluRs, could induce the activation of p38 MAPK, leading to Rab5-mediated AMPAR endocytosis and long-term depression in PFC neurons (42). Here we demonstrate that p38 MAPK is the key molecule responsible for SNRI reduction of AMPAR-EPSC. It has been found that p38 MAPK modulates endocytic trafficking by regulating the functional cycle of Rab5 between membrane and cytosol, which is controlled by guanyl nucleotide dissociation inhibitor (GDI, 43). Thus, SNRI activation of p38 MAPK may accelerate AMPAR endocytosis by stimulating the formation of Rab5:GDI complex. Another possibility is that SNRI alters AMPAR recycling, since some AMPA receptors undergo constitutive endocytic recycling, and dynamin and rab5 blockade could impact receptor recycling directly.

Taken together, our study has revealed the synergistic regulation of PFC glutamatergic transmission by co-activation of serotonin and norepinephrine systems, which provides a potential basis for the better therapeutic effects of dual reuptake inhibitors in the treatment of mental disorders.
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Footnotes
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Figure Legends

Fig 1. Serotonin and Norepinephrine Reuptake Inhibitors (SNRIs) produce a synergistic reduction of AMPAR-EPSC in PFC pyramidal neurons. **A**, Plot of normalized peak AMPAR-EPSC recorded in PFC slices treated with the selective 5-HT reuptake inhibitor fluoxetine alone (10 µM), the selective NE reuptake inhibitor desipramine alone (10 µM) or both. **B**, Representative current traces taken at time point denoted by # in A. Scale bar: 50 pA, 10 ms. **C**, Dose-response plot showing the percentage reduction of EPSC by single or dual application of fluoxetine and desipramine at various concentrations. *: p < 0.01, **: p <0.05, two-way ANOVA. **D**, Input-output curve of AMPAR-EPSC amplitude evoked by different stimulation intensities in PFC slices taken from animals injected (i.p.) without or with fluoxetine (5 mg/kg), desipramine (1 mg/kg) or both. *: p < 0.01, **: p <0.05, two-way ANOVA. **E**, Representative EPSC traces in animals injected with different inhibitors. Scale bar: 100 pA, 20 ms. **F**, Dose-response plot showing the percentage reduction of AMPAR-EPSC by application of venlafaxine at various concentrations. *: p < 0.01, **: p <0.05, one-way ANOVA. **G**, Input-output curve of AMPAR-EPSC amplitude evoked by different stimulation intensities in PFC slices taken from animals injected with saline or venlafaxine (8 mg/kg, i.p.). *: p < 0.01, **: p <0.05, two-way ANOVA.

Fig 2. 5-HT$_{1A}$ and α$_2$-AR co-activation mediates the regulation of AMPAR-EPSC and AMPAR surface expression by SNRIs. **A, B**, Plot of normalized AMPAR-EPSC showing the effect of SNRIs in the presence of 5-HT$_{1A}$ antagonist WAY-100635 (200 nM, A) or α$_2$-AR antagonist yohimbine (40 µM, B). Inset: Representative current traces taken from time point denoted by #. Scale bars: 50 pA, 10 ms. **C**, Bar graph (mean ± SEM) showing the percentage reduction of AMPAR-EPSCs by SNRIs in the presence of various pharmacological reagents affecting 5-HT, NE or DA systems. *: p < 0.01, one-way ANOVA. **D, E**, Cumulative distribution of mEPSC amplitudes (D) or inter-event intervals (E) in cultured PFC neurons co-treated with 8-OH-DPAT (5 µM, 10 min) and clonidine (5 µM, 10 min). Scale bar: 100 pA, 10 ms. **F**, Cumulative data (mean ± SEM) summarizing the percentage reduction of mEPSC amplitude and frequency by 5-HT$_{1A}$ and α$_2$-AR agonists. *: p < 0.01, one-way ANOVA. **G, H**, Biotinylation assay showing the levels of surface GluR1 and GluR2 in PFC slices treated with fluoxetine, desipramine or both (10 µM, 10 min). *: p < 0.01, one-way ANOVA.

Fig 3.Dynamin/Rab5-dependent AMPAR endocytosis underlies the effect of SNRIs on AMPAR-EPSC. **A**, Plot of normalized AMPAR-EPSC showing the effect of SNRIs in neurons injected with the dynamin inhibitory peptide (50 µM) vs. a control peptide. **B**, Cumulative data (mean ± SEM) showing the effect of SNRIs on AMPAR-EPSC in neurons dialyzed with various reagents to perturb endocytosis or cytoskeleton. *: p < 0.01, one-way ANOVA. **C-E**, Plot of normalized AMPAR-EPSC showing the effect of SNRIs in neurons injected with Rab5WT, Rab5DN vs. Rab5CA proteins (all 4 µg/ml, C, D), or a Rab5 antibody vs. heat-inactivated antibody (2 µg/ml, E). Inset: Representative current traces taken from time point denoted by #. Scale bars: 50 pA, 10 ms. **F**, Cumulative data (mean ± SEM) showing the effect of SNRIs on EPSC in neurons dialyzed with various reagents affecting Rab5 activity. *: p < 0.01, one-way ANOVA.

Fig 4. AGS3, a G protein modulator, is involved in the synergistic regulation of AMPARs by SNRIs. **A, B**, Plot of normalized peak AMPAR-EPSC showing the effect of SNRIs in neurons dialyzed with AGS3 inhibitory peptide (50 µM) vs. a scrambled control peptide (A), or with anti-AGS3 (4 µg/ml) vs. the heat-inactivated antibody (B). **C**, Cumulative data demonstrating the percentage reduction of EPSC by SNRIs in the presence of various AGS3 inhibitors. *: p < 0.01, t-test. **D-E**, Cumulative distribution of mEPSC amplitudes in the absence and presence of clonidine plus 8-OH-DPAT in cultured PFC neurons transfected with AGS3 siRNA (D) vs. a scrambled control siRNA (E). Inset in D, Western blotting showing the knockdown of AGS3 expression in AGS3 siRNA-transfected cortical cultures. **F**, Summarized data showing the percentage reduction of mEPSC amplitude and frequency by 8-OH-DPAT plus clonidine in neurons transfected with different siRNAs. *: p < 0.01, t-test.
Fig 5. The p38 MAPK is important for SNRI regulation of AMPARs, and it is synergistically activated by 5-HT_{1A} and α2-AR receptors in an AGS3-dependent manner. **A**, Plot of normalized AMPAR-EPSCs showing the effect of SNRIs in neurons dialyzed with inhibitors for p38 (SB203480, 40 μM) or p44/42 (PD98059, 40 μM) MAPKs. **B**, Bar graphs summarizing the percentage reduction of EPSCs by SNRIs in the presence of different MAPK inhibitors. *: p < 0.01, one-way ANOVA. **C, D**, Western blots and quantifications showing the effect of 8-OH-DPAT and clonidine (both 5 μM, 10 min) on phos-p38 or phos-p42/44 MAPKs (active) in cultured neurons transfected with AGS3 siRNA or a scrambled control siRNA. *: p < 0.01, t-test. **E, F**, Immunocytochemical images and quantifications showing the effect of 8-OH-DPAT and clonidine on total p38 and phos-p38 levels in cultured neurons transfected with AGS3 siRNA or a scrambled control siRNA. *: p < 0.01, t-test.
Fig 2

A

B

C

D

E

F

G

H

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Fig 4

A. 

B. 

C. 

D. 

E. 

F. 

AGS3 siRNA

scrambled siRNA

control

DPAT+Clon

% reduction of mEPSC

amplitude

frequency

mEPSC amplitude (pA)

mEPSC amplitude (pA)
Fig 5

(A) Norm. peak of AMPAR-EPSC

(B) % reduc. of AMPAR-EPSC by Fluox+Des

(C) DPAT+Clon: scrm. siRNA, AGS3 siRNA

(D) DPAT+Clon: scrm. siRNA, AGS3 siRNA

(E) DPAT+Clon: scrm. siRNA, AGS3 siRNA

(F) DPAT+Clon: scrm. siRNA, AGS3 siRNA
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Eunice Y. Yuen, Luye Qin, Jing Wei, Wenhua Liu, Aiyi Liu and Zhen Yan

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