mTORC1-dependent protein synthesis underlying rapid antidepressant effect requires GABA$_B$R signaling

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**Abstract**

Administration of N-methyl-D-aspartate receptors (NMDAR) antagonists initiates a rapid anti-depressant response requiring mammalian Target of Rapamycin Complex 1 (mTORC1) kinase; however the molecular mechanism is unknown. We have determined that upon NMDAR blockade, dendritic γ-aminobutyric acid B receptors (GABA$_B$R) facilitate dendritic calcium entry. The GABA$_B$R-mediated increase in calcium signal requires the availability of dendritic L-type calcium channels. Moreover, GABA$_B$R can activate mTOR and increase mTOR dependent expression of BDNF under the same NMDAR blocked conditions. In vivo, blocking GABA$_B$R prevents the fast-acting, anti-depressant effect of the NR2B antagonist, Ro-25-6891, decreases active mTORC1 kinase, and reduces expression of BDNF and the AMPA receptor subunit GluA1. These findings propose a novel role for GABA$_B$Rs in the antidepressant action of NR2B antagonists and as an initiator/regulator of mTORC1-mediated translation.

**1. Introduction**

Major depressive disorder (MDD) is a chronic disease with low remission rates (~33%) and relies on lengthy use of antidepressants for therapy (Kessler et al., 2003; Murrough, 2012; Rush et al., 2011, 2006). Several well-characterized N-methyl-D-aspartate receptor (NMDAR) antagonists (AP-7, Ro-25-6981, MK-801, and ketamine) act as rapid onset antidepressants; however the mechanism by which this occurs is largely unknown (Autry et al., 2011; Trullas and Skolnick, 1990). A recent finding demonstrates that rapid antidepressants can increase the activity of the mammalian target of rapamycin (mTOR), a serine/threonine kinase critical for translation (Li et al., 2010). mTOR consists of two complexes mTORC1 and C2, with C1 mediating protein synthesis (Hay and Sonenberg, 2004; Hoeffer and Klann, 2010). Because activation of mTOR kinase is generally regarded as a consequence of NMDAR stimulation, it is unknown how NMDAR antagonists increase active mTOR (Gong et al., 2006; Raab-Graham et al., 2006). In this study we address the fundamental question of how mTOR kinase is activated during NMDAR blockade.

Many molecular changes occur when NMDAR activity is altered. For example, NMDAR activation negatively regulates the surface expression of γ-aminobutyric acid type B receptors (GABA$_B$Rs) (Chalifoux and Carter, 2011a; Guetg et al., 2010; Padgett and Slesinger, 2010). GABA$_B$Rs inhibit neuronal activity by reducing neurotransmitter release, via the inhibition of presynaptic calcium channels, and by mediating slow inhibitory postsynaptic potentials via the activation of postsynaptic potassium channels. Whether changes in GABA$_B$R signaling play a role in the molecular basis of rapid antidepressants is unknown.

Herein, we demonstrate that a functional shift of postsynaptic GABA$_B$Rs underlies the enhanced mTOR activity induced by NMDAR antagonists. GABA$_B$Rs shift from reducing to increasing resting dendritic calcium signal and require L-type calcium channels to do so. Consistent with the requirement for new protein synthesis of plasticity related proteins, blocking GABA$_B$R signaling in mice treated with the rapid antidepressant Ro-25-6891 reduces the protein levels of the brain-derived neurotropic factor (BDNF) and the synaptic protein GluR1/GluA1 in the prefrontal cortex. We extend these findings by demonstrating that block of GABA$_B$Rs in vivo prevents both the up regulation of mTOR and the expected behavioral phenotype arising from blocking NMDARs.
2. Material and methods

2.1. Cell culture and transfections

Primary neurons were prepared as previously described (Ma et al., 2002). For imaging experiments, hippocampal neurons were plated at 200,000 cells/12 mm coverslip and used at day in vitro (DIV) 14-18. For Western blots, cortical neurons were plated at 2 million cells/35 mm well and used at DIV14-18. DIV 12-14 hippocampal neurons were transfected using 0.4 μg DNA according to manufacturer's instructions (Invitrogen) with the exception that the transfection was done in Neurobasal media and neurons were returned to conditioned media 4–6 h post transfection. HEK293FT cells were transfected according to the manufacturer's instructions (Invitrogen) and fixed 24 h post-transfection. Constructs used were: GCaMP3 (Addgene), GABAAR1 and GABAAR2 (Margeta-Mitrovic et al., 2000).

2.2. Pharmacology

Drug treatment was done in media. All live imaging experiments were done in HEPES-based artificial cerebral spinal fluid (aCSF (in mM): 100 NaCl, 10 HEPES [pH 7.4], 3 KCl, 2 CaCl2, 1 MgCl2, 10 glucose) adjusted to match the osmolarity of cell culture media. For in vitro manipulations, neurons were pretreated with the NMDAR antagonist (p-(2-amino-5)-phosphonopentanoic acid (50 μM D-AP5; ACSF-AP5) for 5 min. For GABAAR activation, neurons were treated with baclofen (50 μM, 5–10 min). To block GABAAR, neurons were treated with GGP-35348 (100 μM). For calcium blocker experiments, the following blockers were used: nifedipine (1 μM, L-type), u-conotoxin GIVA (1 μM, N type), and u-agatoxin-IVA (1 μM, P type). For Western blots, rapamycin (200 nM, 30 min prior to harvesting and 60 min after AP5 application) was used to block mTORC1. To isolate the postsynaptic GABAAR component, the following blockers were added to ACSF just prior to imaging: NBOX (10 μM, AMPAR), pictoroxin (20 μM, GABAAR), bicuculline (20 μM, GABAAR), TTX (1 μM, Na+ channels), MPEP (10 μM, mGluR), LY367387 (100 μM, mGluR); and for conditions where NMDARs were blocked, APS (50 μM) was included. All drugs were purchased from Tocris.

2.3. Microscopy

Images were acquired with a Leica SP5 disk confocal microscope using an oil-immersion 63X lens or a 63X water immersion lens for live imaging. For immunostaining, a 100X oil immersion lens or a 63X water immersion lens was used. Alexa Fluor 488 or 555 labeled secondary antibodies) or 5% nonfat milk. Membranes were incubated O/N at 4°C and washed in TBST for 60 min. Membranes were then stripped by incubation in stripping buffer (2 M Tris, 40% SDS, 1% BME) for 1 h, washed in TBST for 60 min and probed for total ERK as above. For BDNF blots, membranes were probed by enhanced chemiluminescence. ImageJ was used for densitometry analysis. The antibodies used were as follows: phospho-mTOR (29715, Ser2448, Cell Signaling), mTOR (553144, Invitrogen), Arc (S17839, Santa Cruz for Fig. 7, or 156,002, Synaptic Systems for Fig. 5), GluR1/GluA1 (PC246, Millipore), pERK (4370, Cell Signaling), ERK (4372, Cell Signaling), BDNF (sc 20981, Santa Cruz) and approved by the UT-Austin Institutional Animal Care and Usage Committee (IACUC).

2.8. Isolation of synaptoneurosomes and neuronal lysates

Synaptoneurosomes (SN) were isolated from DIV14-18 cortical cultures or from mouse prefrontal cortex (PFC) by a modified method previously described (Quinlan et al., 1999). Briefly, neurons were harvested in buffer B (20 mM HEPES, pH 7.4; 5 mM EDTA, pH 8.0; protease inhibitor cocktail (Complete, Roche); phosphatase inhibitor) and homogenized. Homogenate was filtered first through a sterile 100 μm nylon filter followed by a 5 μm filter. SNs were pelleted at 14,000 × g for 20 min at 4°C. SN pellet was solubilized with RIPA buffer (150 mM NaCl; 10 mM Tris, pH 7.4; 0.1% SDS; 1% Triton X-100; 1% deoxycholate; 5 mM EDTA; protease inhibitor cocktail and phosphatase inhibitor) for 20 min on ice. The insoluble fraction was then pelleted at 14,000 × g for 20 min at 4°C and the supernatant was used for immunoblot analysis.

2.9. Immunoblotting

Equal amounts of SN (15–40 μg) sample in RIPA and SDS loading buffer were run on a 4–20% gradient (BioRad) or 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane for 1 h in 20% transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). For BDNF, samples in RIPA and SDS loading buffer were run on a 12% gel and transferred to a PVDF membrane for 30 min in 10% transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). Membranes were then blocked for 1 h at RT while shaking in TBST (0.1% Tween-20) with either 5% bovine serum albumin (BSA, for phospho-specific antibodies) or 5% nonfat milk. Membranes were incubated O/N at 4°C in primary antibody and for 45 min at RT in secondary antibody. After each incubation step, membranes were washed for 6 × 10 min in TBST. Membranes were imaged with the Odyssey immunoblot software. To probe for total ERK, membranes were then stripped at 55°C in stripping buffer (2 M Tris, 40% SDS, 1% BME) for 1 h, washed in TBST for 6 × 10 min and probed for total ERK as above. For BDNF blots, membranes were probed by enhanced chemiluminescence. ImageJ was used for densitometry analysis. The antibodies used were as follows: phospho-mTOR (29715, Ser2448, Cell Signaling), mTOR (553144, Invitrogen), Arc (S17839, Santa Cruz for Fig. 7, or 156,002, Synaptic Systems for Fig. 5), GluR1/GluA1 (PC246, Millipore), pERK (4370, Cell Signaling), ERK (4372, Cell Signaling), BDNF (sc 20981, Santa Cruz) and α-Tubulin (ab15246, Abcam).

2.10. Statistical analysis

All statistical analyses were performed using Graphpad Prism software. For all experiments: significance was calculated at α = 0.05 level. For both 1-way and 2-way ANOVA, significance is represented by: *p < 0.05, **p < 0.01, ***p < 0.001; n.s. indicates no significant difference. For Fig. 2B, significance was calculated with a one sample t-test where μ = 0. Error bars represent SEM.
3. Results and discussion

3.1. NMDAR inhibition promotes GABA\(_B\)R surface expression and shifts its function to increase resting calcium

To establish an in vitro model that visualizes site-specific molecular changes associated with rapid antidepressants, we blocked NMDARs in cultured cortical or hippocampal neurons by applying D-\((2R)\)amino-5-phosphovaleric acid (AP5) for 90 min. Because NMDAR activation reduces the dendritic surface expression of GABA\(_B\)Rs by endocytosis (Guert et al., 2010; Terunuma et al., 2010), we predicted that NMDAR blockade would increase GABA\(_B\)Rs on the dendritic membrane. To label surface GABA\(_B\)Rs, we used an antibody against the extracellular N-terminus and compared that to the total amount of GABA\(_B\)Rs labeled with an antibody against the C-terminal, cytoplasmic tail of GABA\(_B\)R1 (Fig. S1A). To verify our surface staining protocol, human embryonic kidney (HEK) 293 cells were transfected with cDNAs coding for GABA\(_B\)R1a alone or with GABA\(_B\)R1a and GABA\(_B\)R2. Plasma membrane expression of the functional dimeric GABA\(_B\) receptor requires both GABA\(_B\)R1 and R2. In HEK cells that only express GABA\(_B\)R1a, GABA\(_B\)Rs will not be expressed on the surface as GABA\(_B\)R1a will be retained intracellularly. This serves as a negative control for surface expression (Margeta-Mitrovic et al., 2000). Indeed, in non-permeabilized HEK cells transfected with GABA\(_B\)R1a, the extracellular antibody did not detect any surface GABA\(_B\)Rs. However, permeabilized cells displayed strong intracellular staining, consistent with intracellular retention of GABA\(_B\)R1a in the absence of GABA\(_B\)R2 (Margeta-Mitrovic et al., 2000). In contrast, co-expression of GABA\(_B\)R1 and R2 resulted in abundant surface GABA\(_B\)R expression which was detected by both the extracellular and cytoplasmic antibodies (Fig. S1B).

Using these antibodies, we quantitated the surface expression of GABA\(_B\)R1 in hippocampal neurons treated with vehicle and AP5. At the completion of the treatment, neurons were fixed and surface GABA\(_B\)R1s were immunostained with the extracellular antibody. Afterwards, neurons were permeabilized and total GABA\(_B\)R1 expression was determined using the C-terminal antibody. Consistent with our prediction, we found that blocking NMDARs for 90 min increased the ratio of surface to total GABA\(_B\)Rs by 36 ± 0.07% relative to control (Fig. 1A and B).

We next sought the functional significance of increased GABA\(_B\)R surface expression during NMDAR blockade by examining dendritic calcium. GABA\(_B\)R activation can affect calcium signaling through 1) blocking presynaptic release of GABA leading to reduced inhibition and increased dendritic calcium, 2) blocking presynaptic glutamate release which will likely reduce membrane depolarization and decrease dendritic calcium, or 3) opening postsynaptic potassium channels, thereby preventing calcium entry. To assess how NMDAR blockade changes the GABA\(_B\)R-induced calcium signals, we used hippocampal and cortical cultured neurons filled with the calcium indicator OGB-488AM to determine the change in calcium signal due to baclofen, a GABA\(_B\) agonist. Neurons under both control (vehicle, H\(_2\)O) and AP5 conditions had comparable dendritic calcium (Fig. S2A). As an additional control, neurons treated with AP5 were imaged over 10 min to ensure that the baseline calcium levels remained steady for the duration of the imaging protocol (Fig. S2B). Consistent with GABA\(_B\)R activation decreasing glutamate release or opening postsynaptic potassium channels, baclofen significantly decreased dendritic calcium compared to vehicle (Fig. 2A, hippocampal: −15 ± 4%, left and Fig. 2B–C; Fig. S2C, cortical: −8 ±4%). In contrast, GABA\(_B\)R activation in AP5-treated neurons significantly increased the resting calcium signal (Fig. 2A hippocampal: 13 ± 4%, right and Fig. 2B–C; Fig. S2C, cortical: 43 ±15%). We measured the time course of dendritic calcium changes of AP5-treated neurons in response baclofen. Of note, we observed a three to four minute delay before the onset of calcium increase (Fig. S2E–F). The effects of baclofen were completely blocked by inclusion of the GABA\(_B\) antagonist CGP-35348 (100 \(\mu\)M, Fig. 2B and C). These results demonstrate that after blockade of NMDARs, GABA\(_B\)R activation increases dendritic resting calcium.

3.2. \(L\)-type calcium channels mediate GABA\(_B\)R-induced increase in dendritic calcium signal when NMDARs are blocked

To characterize the baclofen-mediated increase in dendritic calcium when NMDARs are blocked, we performed live calcium imaging of AP5-treated neurons in the presence of N-type (\(\omega\)-conotoxin GVIA), P-type (\(\omega\)-agatoxin), or \(L\)-type (nifedipine), calcium channel blockers before and after baclofen application. Blocking N-type channels had no effect on either basal calcium levels or the baclofen-mediated increase in dendritic calcium (Fig. 3A–B). Although \(\omega\)-agatoxin increased the basal calcium signal in AP5-treated neurons (Fig. 3A and Fig. S3A; 18 ±7%), blocking P-type channels did not abrogate the GABA\(_B\)R-mediated increase in dendritic calcium (Fig. 3B and Fig. S3A). Nifedipine, which blocks \(L\)-type channels, was the lone blocker to decrease the resting dendritic calcium signal (−18 ±6%, Fig. 3A) and to abolish the baclofen-induced increase in calcium signal (−24 ± 8%, Fig. 3B–C) of AP5-treated neurons. Indeed, inclusion of nifedipine mimicked the

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**Fig. 1.** NMDAR blockade increases GABA\(_B\)R surface expression on dendritic membrane (A) Representative images of GABA\(_B\)R surface expression in control or AP5 neurons (Scale = 15 \(\mu\)m). (B) Summary graph shows baclofen significantly increases surface expression of GABA\(_B\)R in dendrites (right, \(N = 15 − 25\), df = 35, \(t = 2.22\)) but not cell body (left, \(N = 9\) neurons, df = 16, \(t = 0.98\)). Significance calculated with Student’s T-test at \(\alpha = 0.05\) level. Error bars represent SEM.
baclofen-induced decrease in resting dendritic calcium seen in control neurons (−24 ± 8%). In the absence of AP5, nifedipine did not alter the baclofen mediated decrease in resting calcium signal (Fig. S3B–C). These results indicate that L-type calcium channels are required for the GABAergic-mediated increase in calcium signal.

3.3. L-type channel activity requirement in the AP5-induced GABAergic shift in function persists when dendritic GABAergic component is isolated

Since L-type calcium channels are reported to be predominantly postsynaptic in hippocampal neurons, these data led us to ask the question if the shift in GABAergic function occurs postsynaptically (Catterall et al., 2005; Magee et al., 1998; Wheeler et al., 2012). We reasoned that if the baclofen-induced calcium signal persisted when we isolated the postsynaptic component of GABAergic by using blockers that prevented neurotransmitter input, then we would be able to assess whether presynaptic and/or postsynaptic GABAergicRs triggered the increase in calcium signal. To isolate the dendritic component of the GABAergic-mediated increase in calcium signal, we performed live calcium imaging in a cocktail of glutamate receptor (MEP (10 μM, mGlu1), LY 367385 (100 μM, mGlu1), NBQX (20 μM, AMPAR)), sodium channel (TTX (1 μM, Na+ channel)), and GABAergic (picotrin (20 μM, GABAergic), bicuculline (20 μM, GABAergic)) blockers. These blockers were washed onto the cell's primary dendrites when treated with baclofen as compared to vehicle treatment (−8 ± 2%; Fig. 4D–E). In contrast, baclofen significantly increased dendritic fluorescence in neurons exposed to NMDAR blockade (19 ± 3%; Fig. 4D–E). These results indicate that postsynaptic GABAergicRs can mediate the increase in dendritic calcium signal as measured by two different calcium sensors. To confirm the requirement for L-type calcium channels we repeated the experiment in the presence of nifedipine. Indeed, blocking L-type channels prevented the baclofen-mediated increase in calcium signal (Fig. 4D–E) in AP5-treated neurons. This finding provides additional evidence that postsynaptic L-type calcium channels participate in GABAergic-mediated elevation of dendritic resting calcium when NMDAR signaling is abated.

3.4. GABAergic activation increases dendritic mTOR1 activity and protein synthesis with NMDAR blockade

Others have reported that injection of rapid antidepressants into rodents increases mTOR and extracellular signal-regulated kinase

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**Fig. 2.** GABAergic activation increases dendritic calcium signal in AP5-treated neurons. (A) Representative images of calcium signal change pre- (top) and post-baclofen (bottom) treatment. Neurons are pseudo-colored to indicate signal intensity with 0 being the lowest and 255 being the highest intensity (Scale −25 μm). (B) Hippocampal neurons were preincubated in vehicle (control) or AP5 for 90 min prior to imaging. Averaged fluorescence signals, normalized by baseline, over 1 min before and after the addition of vehicle or baclofen in vehicle (Veh) or AP5-treated neurons either with or without GABAergic blockers included in bath solution. (C) Summary graph shows significant increase in dendritic calcium signal (ΔF/F) by Tukey's 1-way ANOVA in hippocampal neurons treated with AP5. Inclusion of GABAergic blocker CGP-35348 prevents baclofen-mediated increase in calcium signal. (N = 8–12 neurons, F1,35 = 14.19). * indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001, and n.s. indicates not significant. Error bars represent SEM.
(ERK) signaling (Duman et al., 2012). Several cell types use calcium entry through L-type calcium channels as a second messenger to activate these pathways (Hegner et al., 2009; Wu et al., 2005). Although our results demonstrate that GABA<sub>B</sub>Rs increase calcium, it is unknown whether endogenous GABA is sufficient to trigger mTOR and ERK activity in vivo. We have previously shown that AP5 treatment reduces mTOR activity in vitro (Raab-Graham et al., 2006). Thus, we expect that our in vitro model would require GABA<sub>B</sub>R activation with baclofen to turn on these signaling pathways via calcium.

To test this hypothesis we used Western blot analysis of synaptoneurosomes isolated from untreated control- or AP5-treated cultured neurons. In control neurons, baclofen did not increase ERK or mTOR activity (Fig S4A and B). Unlike the reported in vivo findings, AP5 alone did not change ERK activity; however as expected, AP5 treatment reduced mTOR activity (Fig. 5A and B). Furthermore, the addition of baclofen to AP5-treated neurons increased the activity of mTOR back to control levels with no significant effect on ERK activity. The effect of GABA<sub>B</sub>R activation on mTOR activity was reduced by pretreatment with rapamycin (1.03 ± 0.02 AP5+baclofen vs. 0.46 ± 0.03 AP5+baclofen + rapamycin, Fig. SB).

Through immunocytochemistry we sought the subcellular localization of the increased mTOR activity during NMDAR blockade. We used an antibody against phospho-S6 (pS6), since increased pS6 signal reflects heightened mTORC1 activity (Cammalleri et al., 2003). In untreated, control neurons pS6 activity was found in dendrites as distinct puncta or hotspots (Fig. 5C). pS6 expression in control and baclofen-treated neurons had similar number of hotspots in dendrites (0.88 ± 0.21 as fold of control, Fig. 5C and D). To confirm that the increased pS6 was due to mTORC1 activation, neurons exposed to AP5 and baclofen were treated with the mTORC1-specific inhibitor rapamycin. The addition of rapamycin prevented the activation of mTORC1 kinase by baclofen as indicated by the absence of pS6 hotspots in dendrites compared to control neurons (0.07 ± 0.02 as fold of control, Fig. 5C and D). However, activation of GABA<sub>B</sub>Rs in AP5-treated cells restored the number of pS6 hotspots to control levels (0.69 ± 0.30 as fold of control, Fig. 5C and D). To confirm that the increased pS6 was due to mTORC1 activation, neurons exposed to AP5 and baclofen were treated with the mTORC1-specific inhibitor rapamycin. The addition of rapamycin prevented the activation of mTORC1 kinase by baclofen as indicated by the absence of pS6 hotspots in dendrites (Fig. 5C and D). These results demonstrate that GABA<sub>B</sub>R signaling activates mTORC1 kinase in vitro when NMDARs are blocked.

To assess if GABA<sub>B</sub>R-mediated activation of mTORC1 kinase increases protein synthesis of plasticity-related mRNAs, we performed Western blot analysis on synaptoneurosomes. Changes in protein expression were determined for the brain-derived neurotrophic factor (BDNF), a protein reported to be synthesized by the mTOR pathway (Henry et al., 2012), and activity regulated cytoskeleton-associated protein (Arc), whose synthesis has been reported to be regulated by both ERK and mTOR (Panja et al., 2009;
We reasoned that mRNAs whose translation is regulated by mTOR and not ERK would be sensitive to rapamycin. Arc protein levels did not change significantly under any condition (Fig. 5E). In contrast, NMDAR blockade alone reduced the levels of BDNF protein by \( \approx 33\% \) (0.67/0.02, Fig. 5F). Furthermore, the addition of baclofen to AP5-treated neurons increased BDNF expression above control levels (1.51/0.10). Preincubation with rapamycin prevented the baclofen-mediated increase in BDNF, consistent with mTORC1-mediated protein synthesis (Fig. 5F). These results show that in AP5-treated neurons, GABABR activation of the mTOR pathway mediates synthesis of BDNF but not Arc.

### 3.5. Selective inhibition of NR2B/GluN2B activity further stimulates mTORC1 signaling through GABA\(_B\)R activation

NMDA receptors are tetramers often composed of two subunits: NR1 (or GluN1) and two NR2 (or GluN2) subunits (Paoletti and Neyton, 2007). Recent clinical trials for major depressive disorder showed that NR2B/GluN2B antagonists have a greater antidepressant effect than general NMDAR antagonists. Furthermore, NR2B/GluN2B antagonists lack the psychotomimetic effects and abuse potential of some of the more general NMDAR blockers (e.g. ketamine) (Ibrahim et al., 2011; Mathews et al., 2012; Preskorn et al., 2008; Skolnick et al., 2009). In a more recent study, the NR2B/GluN2B antagonist Ro-25-6891 acts as a rapid antidepressant and involves mTORC1–dependent protein synthesis (Li et al., 2010). We hypothesized that specifically blocking NR2B/GluN2B-containing channels would induce the GABA\(_B\)R-mediated increase in active mTOR. Unlike AP5, treating cultured neurons with Ro-25-6891 for 90 min did not alter the number of pS6 hotspots relative to control. However, activation of GABA\(_B\)Rs in Ro-25-6891-treated neurons further enhanced the number of pS6 hotspots (3.32 ± 0.72; Fig. 6A and B). Similar to AP5-treated neurons, rapamycin prevented the baclofen-mediated increase in active mTOR (Fig. 6C). These results...
demonstrate that blocking NR2B/GluN2B-containing NMDARs is sufficient to induce GABABR-mediated activation of mTOR. Unlike the general NMDAR antagonist AP5, NR2B/GluN2B-specific inhibition maintains baseline mTOR activity, thus allowing further activation of mTOR with GABABR stimulation.

3.6. GABABRs are required for mTORC1-dependent protein synthesis in vivo with NR2B/GluN2B antagonist

Not surprisingly, application of NMDAR antagonists alone in vitro is not sufficient to increase mTOR kinase activity, as it does in vivo (Li et al., 2010), and requires GABABR activation. One possible explanation for the discrepancy is that culture systems do not preserve neuronal connections and perhaps do not recapitulate the in vivo ratio between glutamate and GABA as implied by increased miniature excitatory postsynaptic synaptic currents (mEPSCs) in vitro (Chang et al., 2010; De Simoni et al., 2003; Ivenshitz and Segal, 2010). Is endogenous GABA in vivo therefore sufficient to trigger mTOR-dependent protein synthesis through GABABR when NMDAR is blocked? To test this, we injected mice with either saline or Ro-25-6891. Forty-five minutes post-injection we prepared synaptoneurosomes from prefrontal cortex (PFC), since this region

![Fig. 5. GABABR stimulation increases active mTORC1 pathway but not ERK while NMDARs are blocked. (A) NMDAR blockade, GABABR activation, nor rapamycin significantly changes levels of active ERK (N = 4, F3,11 = 0.45). (B) GABABR activation rescues decrease in active mTORC1 as a result of NMDAR blockade. Rescue is prevented when neurons are pretreated with mTORC1 inhibitor rapamycin (N = 4–7, F3,20 = 23.87). (C) Representative images of neuronal pS6 signal or hotspot (Scale = 15 μm). Arrows indicate hotspots of pS6 activity. (D) Summary graph shows decreased total pS6 hotspots in AP5-treated neurons is rescued by baclofen treatment and prevented by pretreatment with rapamycin (Bonferroni’s 2-way-ANOVA). (N = 18–22 neurons, F11,112 = 7.413). Hotspots are reported for the first 50 μm of the dendrite from the soma. (E) Neither GABABR activation nor NMDAR blockade significantly changes levels of the ERK-dependent protein, Arc (N = 4, F3,10 = 2.65). (F) GABABR activation while NMDAR are blocked increases BDNF expression by 50% compared to control and rescues reduced BDNF expression due to NMDAR blockade (N = 4, F3,10 = 14.86). Increase in BDNF levels is prevented in neurons pretreated with mTORC1 inhibitor rapamycin. N = 4 independent cultures. Significance was assessed by Tukey’s 1-way ANOVA. * indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001. Error bars represent SEM.](image-url)
has been used to demonstrate mTOR activity underlying rapid antidepressants (Li et al., 2010). Consistent with previous observations, mTOR kinase activity was significantly elevated in the PFC of Ro-25-6891-injected mice compared to saline-injected controls (Fig. 7A) (Li et al., 2010). The elevated mTOR activity was absent when the GABABR antagonist CGP-35348 was co-injected with Ro-25-6891 (Fig. 7A). Again, NMDAR blockade with Ro-25-6891 had no effect on ERK activity at 45 min post-injection; however its activity was reduced with co-injection of GABABR antagonists (Fig. S5A).

To determine if blocking GABABR activation affects levels of known proteins whose translation is mediated by mTOR activity, we assayed the levels of BDNF and GluR1/GluA1 at 45 min and 24 h post-injection, respectively (Henry et al., 2012; Li et al., 2010). Consistent with mTOR kinase-dependent translation, CGP-35348 reduced the Ro-25-6891-mediated increases in BDNF and GluR1/GluA1 to control levels (0.87 ± 0.13, Figs. 7B and 0.63 ± 0.08, Fig. 7C). As we observed in vitro, Arc protein levels did not increase with Ro-25-6891 injection. Of note, its expression was reduced

![Graph](image-url)
with GABABRs antagonists, similar to ERK activity (Fig. S5B). These results suggest that block of NR2B/GluN2B-containing NMDARs in vivo increases mTOR kinase activity through GABABRs, promoting translation of BDNF and GluR1.

3.7. GABABR is necessary for the antidepressant effects of NR2B antagonist, Ro-25-6891

The forced swim test (FST; also known as the behavioral despair test) is a widely-used assay for depression-like behaviors in rodents (Duman, 2010; Yan et al., 2010). Although depression is a complex disease with many phenotypes, the forced swim test, whereby rodents are forced to swim without escape, elicits a response of helplessness or despair by becoming immobile (Roubicek et al., 1975). This behavior is reminiscent of other animal behaviors associated with clinically depressed patients (Harlow and Suomi, 1974; Matthews et al., 2005; Miller et al., 1975). Moreover, immobility induced by the FST has been shown to have good predictive ability and sensitivity to antidepressant treatment as the majority of antidepressants used

Fig. 8. GABABR is necessary for antidepressant behavioral effect of NR2B antagonists. (A) Co-injection of CGP-35348, a GABABR antagonist, blocks the antidepressant behavior (decrease in immobility) of mice when injected with Ro-25-6891, a NR2B/GluN2B antagonist. (N = 4–8 animals per condition, F1,20 = 10.64). Statistical difference assessed by Bonferroni’s 2-way ANOVA. (B) Co-injection of the GABABR agonist, baclofen, has no added effect on immobility of mice injected with Ro-25-6891. Rapamycin blocks Ro-25-6891-induced decreases in immobility. (N = 3–4 animals per condition, F1,11 = 6.47), Statistical difference assessed by 1-way-ANOVA with Neuman-Keuls Post Test. * indicates p < 0.05, *** indicates p < 0.001, and. Error bars represent SEM.

Fig. 9. Working model of GABABR-mediated activation of mTOR-dependent translation. Under control conditions (left), basal NMDAR activity allows calcium to enter the dendrite and activate the mTOR signaling cascade via Ca2⁺–dependent activation of P13K (Hoeffer and Klann, 2009) leading to basal levels of mRNA translation. With rapid antidepressants (right), GABABR surface expression increases and its function shifts to increase resting L-type calcium channel activity, which in turn activates the mTOR signaling leading to activity-dependent mRNA translation.
medically can decrease the length of immobility (Castagne et al., 2011). Hence, the forced swim test can serve as a test for potential antidepressant drugs. To test if the antidepressant action of Ro-25-6891 is mediated by activation of GABA_BRs endogenously, we measured antidepressant behavior using the FST in mice injected with a GABA_BR antagonist or GABA_BR antagonist + Ro-25-6891. Consistent with antidepressant behavior, Ro-25-6891-injected mice displayed a significant reduction in immobility (Li et al., 2010). In contrast, mice injected with both Ro-25-6891 and CGP-35348 had immobility scores that were relative to control mice. Importantly, injection of CGP-35348 alone had no significant effect on immobility time (Fig. 8A). These results suggest that the antidepressant effect produced by NR2B/GluN2B antagonists requires GABA_BR activity.

Next, we examined whether further activation of GABA_BRs with baclofen enhanced the antidepressant efficacy of Ro-25-6891. Forty-five minutes post-injection, mice were scored for mobility. As previously observed, injection of Ro-25-6891 alone produced a marked decrease in immobility time (Fig. 8) (Li et al., 2010). Mice injected with baclofen alone were extremely lethargic and could not be tested in the forced swim test. Surprisingly, mice co-injected with baclofen and Ro-25-6891 had reduced immobility times relative to saline control and similar to Ro-25-6891 alone, thus blocking the lethargic affect produced by baclofen alone (Fig. 8B). Moreover, exposure to rapamycin abrogated the effect of the NR2B antagonist co-injected with baclofen, similar to previous observations (Li et al., 2010). These results suggest that baclofen does not provide additional benefit in mitigating depressive behavior; however Ro-25-6891 can reverse the negative effects of baclofen.

4. Conclusions

Although it is well established that mTOR-dependent protein synthesis requires NMDAR activation during neuronal activity, little is known how mTOR is activated during NMDAR inhibition (Gong et al., 2006; Hoeffer and Klann, 2010; Raab-Graham et al., 2006). Using an in vitro model, we provide insight into the molecular changes underlying the activation of mTOR kinase in the presence of NMDAR antagonists. We show that blocking NMDARs (1) increases GABAAergic on the surface of the dendritic membrane and (2) shifts postsynaptic GABAAergic function from reducing to increasing dendritic calcium levels, requiring L-type calcium channels in normal and reduced states of synaptic activity (Fig. 9). While the signal transduction pathway through which GABA_BR increases dendritic calcium signal is yet undetermined, we have shown its requirement for the L-type calcium channel. Interestingly, this finding is opposite to recent reports suggesting that in dendrites, not exposed to NMDAR antagonists, GABA_BR activation inhibits calcium channels (Breton and Stuart, 2012; Chalifoux and Carter, 2011b; Perez-Garcia et al., 2013). However, it is consistent with work by other groups indicating that GABA_BR can increase L-type channel conductance during development through a Gq pathway that activates both PKA and PKC (Bray and Mynlieff, 2011). Collectively, our results indicate that neurons may utilize developmental pathways as a mode of inducing plasticity, consistent with what others have observed for the GABA_Aergic (Lee et al., 2011).

We show in vivo that GABA_BR activation is required to stimulate mTOR kinase activity and to promote the synthesis of plasticity-related proteins when NMDARs are blocked with rapid antidepressants (Fig. 9). These findings are surprising since GABA_BR signaling in central neurons mediates inhibition. One feasible explanation for a GABA_BR-facilitated increase in dendritic calcium is through the inhibition of presynaptic calcium channels in GABAergic interneurons, thus preventing GABA release (Farber et al., 1998). However, our results demonstrate that blocking presynaptic calcium channels, action potentials, and postsynaptic activity still permits the GABA_BR-mediated increase in dendritic calcium with NMDAR-blockade. In support of our findings, the recent report by Autry et al. demonstrates that inhibiting GABA_BR, a target of presynaptic GABA release, does not block the rapid antidepressant effect of NMDAR antagonists (2011). Together, these results argue for a role of postsynaptic GABA_BRs in mediating the rapid antidepressant effects.

Recent work into the mechanism of rapid antidepressants suggests that mTOR is necessary for the sustainment of rapid antidepressant effect of NMDAR antagonists. One model posits that rapid antidepressants promote secretion of the brain-derived neurotrophic factor (BDNF), which binds to postsynaptic tropomyosin-related kinase receptor B (TrkB) thus activating the mTOR kinase pathway (Duman et al., 2012). However, this model does not address how antidepressants that block NMDAR activity modulate resting dendritic calcium levels. Our findings have identified GABA_BR as an essential player in achieving relevant calcium signals during NMDAR inhibition by shifting its function from reducing to increasing calcium in the dendrites through L-type calcium channels (Chalifoux and Carter, 2010). This novel pathway may be critical for (1) BDNF synthesis, secretion, and activation of mTOR kinase, (2) mTOR-dependent translation of BDNF, GluR1/GluA1 and perhaps other synaptic proteins (Henry et al., 2012), and (3) facilitation of antidepressant effects of NMDAR antagonists.

The role of GABA_BRs receptors in depression is controversial. Both agonists and antagonists exhibit antidepressant effects (Ghose et al., 2011). NMDAR antagonists show promise as they can be effective in patients resistant to traditional medications (Murrough, 2012). Our results may help explain why both GABA_BR agonists and antagonists can relieve depression symptoms. Activation of GABA_BRs with NMDAR antagonists may decrease depressive symptoms through the activation of the mTOR kinase, a pathway that others have previously reported to be necessary to reverse depression (Li et al., 2010). On the other hand, GABA_BR antagonists alone may act over a longer time scale to decrease depressive symptoms by acting in concert with the serotonergic system (Slattery et al., 2005).

As protein synthesis is crucial for the induction of lasting changes in synaptic efficacy, GABA_BR signaling may play a role in activating mTOR kinase in response to reduced NMDAR function as induced by rapid antidepressants or perhaps in other diseases such as bipolar disorder (Diazgranados et al., 2010).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2013.05.037.


Perez-Garcí, E., Larkum, M.E., Nevian, T., 2013. Inhibition of dendritic Ca2+ spikes by GAB(A) receptors in cortical pyramidal neurons is mediated by a direct G(0/β-subunit interaction with Cav1 channels. The Journal of Physiology 591, 1599–1612.


