Metabotropic, but not allosteric, effects of neurosteroids on GABAergic inhibition depend on the phosphorylation of GABA\(_A\) receptors

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Neuroactive steroids (NASs) are synthesized within the brain and exert profound effects on behavior. These effects are primarily believed to arise from the activities of NASs as positive allosteric modulators (PAMs) of the GABA-type A receptor (GABA\(_A\)R). NASs also activate a family of G protein–coupled receptors known as membrane progesterone receptors (mPRs). Here, using surface-biotinylation assays and electrophysiology techniques, we examined mPRs’ role in mediating the effects of NAS on the efficacy of GABAergic inhibition. Selective mPR activation enhanced phosphorylation of Ser-408 and Ser-409 (Ser-408/9) within the GABA\(_A\)R β3 subunit, which depended on the activity of cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC). mPR activation did not directly modify GABA\(_A\)R activity and had no acute effects on phasic or tonic inhibition. Instead, mPR activation induced a sustained elevation in tonic current, which was blocked by PKA and PKC inhibition. Substitution of Ser-408/9 to alanine residues also prevented the effects of mPR activation on tonic current. Furthermore, this substitution abolished the effects of sustained NAS exposure on tonic inhibition. Interestingly, the allosteric effects of NAS on GABAergic inhibition were independent of Ser-408/9 in the β3 subunit. Additionally, although allosteric effects of NAS on GABAergic inhibition were sensitive to a recently developed “NAS antagonist,” the sustained effects of NAS on tonic inhibition were not. We conclude that metabotropic effects of NAS on GABAergic inhibition are mediated by mPR-dependent modulation of GABA\(_A\)R phosphorylation. We propose that this mechanism may contribute to the varying behavioral effects of NAS.

Neuroactive steroids (NASs)\(^{3}\) are synthesized within the brain from progesterone and exert powerful effects on brain excitability via their efficacy as endogenous positive allosteric modulators (PAMs) of γ-aminobutyric acid type A receptors (GABA\(_A\)Rs) (1). These Cl\(^-\)–preferring heteropentameric ligand-gated ion channels can be assembled from eight subunit classes with multiple members; α(1–6), β(1–3), γ(1–3), δ, ε, θ, π, and ρ(1–3) (2). Receptors that mediate phasic inhibition are enriched at inhibitory synapses and principally assembled from α1–3, β1–3, and γ2 subunits (3). Neurons also assemble specialized populations of extrasynaptic receptors composed of α4–6, β1–3, and δ subunits that mediate tonic, or sustained, inhibition (4). Importantly, GABA\(_A\)R subtypes that mediate phasic and tonic inhibition are all subject to allosteric potentiation by NAS, a phenomenon that is mediated by a highly conserved binding site centered on glutamine 241 within the α subunit isoforms (5, 6).

In addition to their accepted roles as GABA\(_A\)R PAMs, there is an accumulation of evidence that NAS can exert metabotropic effects on the efficacy of GABAergic inhibition (7, 8). Specifically, NAS enhance the phosphorylation of key regulatory residues within GABA\(_A\)Rs, including serines 408/409 in the β3 subunit. Enhanced phosphorylation of the β3 subunit correlates with increased receptor membrane accumulation and a sustained potentiation of GABAergic inhibition (9–11). Some studies have suggested that in addition to binding to GABA\(_A\)Rs, NASs such as allopregnanolone (ALLO), bind to and activate a family of membrane progesterone receptors (mPRs) (12, 13). These are a family of G protein–coupled receptors, of which some subtypes are highly expressed in the brain and have been shown to couple to G\(_{\alpha}\)o or G\(_{\beta}\) signaling pathways (12–17). However, the role that these receptors play in mediating the effects of NAS on GABAergic inhibition is unknown.

\(^{3}\) The abbreviations used are: NAS, neuroactive steroid; PAM, positive allosteric modulator; GABA\(_A\)R, GABA\(_A\) receptor; ALLO, allopregnanolone; mPR, membrane progesterone receptor; THDOC, tetrahydrodeoxycorticosterone; ORG, ORG OD 02-0; GFX, GF 109203X; KT, KT5720; TFR, transferrin receptor; sIPSC, spontaneous inhibitory synaptic current; ANOVA, analysis of variance; PKA, protein kinase A; PKC, protein kinase C; P4, progesterone; nACSF, normal artificial cerebrospinal fluid artificial cerebrospinal fluid; RT-qPCR, reverse transcriptase quantitative PCR; PFA, paraformaldehyde.

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Here we examine how NASs produce distinct metabotropic and allosteric effects on GABAergic inhibition. Our results demonstrate that NASs enhance tonic inhibition via mPR-dependent phosphorylation of Ser-408/9 in the GABA_A receptor beta3 subunit. Importantly, this mechanism is independent from the allosteric effects of NAS at the GABA_A receptor.

Results

**mPR agonists potentiate GABA_A receptor phosphorylation and plasma membrane accumulation**

Previous studies have shown that endogenous and selected synthetic neurosteroids, including ALLO, tetrahydrodeoxycorticosterone (THDOC), and SAGE-516, exert sustained effects on GABAergic inhibition. In parallel, these respective agents all enhance phosphorylation of key regulatory sites within the intracellular domain of the beta3 subunit of the receptor, serine residues 408 and 409 respectively (Ser-408/9) (9–11).

Neurosteroids have been reported to activate a putative family of G protein–coupled mPRs. Five mPRs (α, β, γ, δ, and ε) that couple to G_i (α, β, γ) or G_q (δ, ε) are expressed in mammals and at high levels in the brain (12–17). To examine the role that mPRs may play in mediating the effects of neurosteroids on GABAergic inhibition, we exposed hippocampal slices to ORG OD 02-0 (ORG), which selectively activates mPRs but not nuclear progesterone receptors (14).

Hippocampal slices were exposed to 300 nM ORG (its estimated EC_{50} concentration for mPRs) or vehicle for 10 min (14), and its effects on the phosphorylation of Ser-408/9 were determined. To do so, SDS-soluble lysates were immunoblotted with a phospho-specific antibody against phosphorylated Ser-408/9 (pS408/9), a phospho-independent antibody selective for the beta3 subunit, together with an antibody against actin to control for loading. The ratio of pS408/9/beta3 immunoreactivity was then determined and compared between treatments as detailed previously (10, 18–20). Under these conditions, ORG significantly increased Ser-408/9 phosphorylation to 138.9 ± 20.0% of control (Fig. 1A; p < 0.05; n = 6 mice). Published studies have shown that Ser-408/9 within beta3 are phosphorylated by a number of protein kinases, including both PKA and PKC (21). Thus, we examined the effects of ORG on GABA_A receptor phosphorylation in the presence of 10 μM GF 109203X (GFX), a selective inhibitor of PKC, and 1 μM KT5720 (KT), a selective inhibitor of PKA, for 10 min. Incubation with GFX or KT alone did not significantly modify ORG-induced phosphorylation. However, their co-application prevented ORG-induced phosphorylation (Fig. 1A; 94.7 ± 17.85% of control, p = 0.2811, n = 6). Under similar experimental conditions, exposure of slices to 100 nM ALLO significantly increased Ser-408/9 phosphorylation to 120.5 ± 3.2% of control (Fig. 1B; n = 3 mice, p < 0.01). Consistent with our studies using ORG, ALLO-induced potentiation of Ser-408/9 phosphorylation was abrogated using GFX/KT (Fig. 1B; 99.4 ± 2.8% of control, n = 3 mice, p = 0.7458). To further evaluate the role that mPRs may play in regulating GABA_A receptor phosphorylation, slices were exposed to progesterone (P4), which activates both mPRs and nuclear progesterone receptors. In keeping with our results, P4 significantly increased Ser-408/9 phosphorylation to 146.6 ± 21.9% of control (p < 0.05, n = 3).

Using biotinylation, we determined whether, in parallel with increasing Ser-408/9 phosphorylation, ORG treatment modifies accumulation of GABA_A receptors on the plasma membrane. Immunoblotting revealed that exposure to ORG significantly increased the plasma membrane levels of the beta3 subunit to 141.4 ± 11.52% of control (Fig. 2; p < 0.01, n = 6 mice) and the alpha4 subunit to 114.1 ± 4.2% of control (Fig. 2; p < 0.05, n = 5 mice), without affecting membrane levels of the transferrin receptor (TR). Importantly, our surface fractions were free of the cytosolic protein actin, demonstrating the robustness of our surface protein biotinylation procedure. Collectively, these experiments suggest that ORG and P4 mimic the effects of NAS on beta3 subunit phosphorylation and induce a selective increase in the plasma membrane accumulation of GABA_A receptors.

**ORG does not allosterically modulate GABAergic inhibition**

To examine the possible effects of mPR activation on GABAergic inhibition, we first assessed whether ORG directly modifies GABA_A activity. To test this, we expressed receptors composed of alpha4beta3 subunits in HEK293 cells. Using whole-cell patch-clamp recordings, the effects of ORG on the magnitude of GABA-induced currents (I_{GABA}) using an EC_{50} concentration of GABA (1 μM) were assessed. In contrast to ALLO, 100 nM ORG did not potentiate I_{GABA} (Fig. 3A; 116 ± 17% of control, p = 0.578, n = 3). Consistent with published studies, 100 nM ALLO potentiated I_{GABA} by 647 ± 118% of control (Fig. 3A; p < 0.01, n = 4). To extend our experiments using HEK293 cells, we used cells that stably express GABA_A receptors allowing the use of automated Q-patch technique to further examine the pharmacological effects of ORG on differing receptor subtypes as described previously (22). Here, we used LTK cells that...
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Figure 2. ORG enhances the plasma membrane accumulation of the GABA<sub>R</sub> α4 and β3 subunits B. Hippocampal slices were exposed to vehicle or 300 nM ORG and then labeled on ice with NHS-Biotin. After lysis and purification on immobilized avidin, surface and total fractions were immunoblotted with antibodies against the GABA<sub>R</sub> α4, and β3 subunits, TFR, or actin. The surface levels of α4, β3, and TFR were then determined and normalized to vehicle-treated controls. *, significantly different from control (p < 0.05, t test, n = 6–7 mice). The lines represent where individual lanes gel lanes were spliced together to make the respective image.

Figure 3. ORG does not directly modify GABA<sub>R</sub> activity A, whole-cell currents in HEK293 cells expressing GABA<sub>R</sub>Rs composed of α4 and β3 subunits were measured in response to 1 μM GABA alone (red) or in the presence of 100 nM ALLO (black) or 300 nM ORG (black). The magnitude of I<sub>GABA</sub> was then normalized to that seen in cells exposed to GABA alone. B, LTr or CHO cells expressing GABA<sub>R</sub>Rs composed of α1β2γ2 or α4β3δ receptors, respectively, were subjected to Q-patch recording. The magnitude of I<sub>GABA</sub> induced by EC<sub>50</sub> GABA alone or in the presence of 1–1000 nM ORG was determined. Responses were then normalized to those seen with GABA alone.

express receptors composed of α1β2γ2 and CHO cells that express α4β3δ subunits, combinations that mimic the properties of receptor subtypes that mediate phasic and tonic inhibition, respectively. ORG from 0.01–1 μM did not significantly modify I<sub>GABA</sub> for either combination (Fig. 3B; n = 3). Thus, these experiments suggest that, in contrast to ALLO, ORG does not directly modify GABA<sub>R</sub> activity.

To further analyze the effects of ORG, we tested its allosteric effects on GABAergic inhibition in dentate gyrus granule cells using whole-cell patch-clamp recording. To measure effects on phasic inhibition, the effects of ORG on the properties of spontaneous inhibitory synaptic currents (sIPSCs) was evaluated. To do so, the effects of vehicle or 300 nM ORG on the amplitude, decay, and frequency of sIPSCs were examined. sIPSC amplitude (83.5 ± 7.9 pA for vehicle, 80.7 ± 6.9 pA for ORG, n = 7 cells), decay (8.7 ± 0.6 ms for vehicle, 8.9 ± 0.5 ms for ORG, n = 7 cells), and frequency (1.1 ± 0.2 Hz for vehicle, 0.9 ± 0.2 Hz for ORG, n = 7) were not significantly altered by ORG (Fig. 4A).

To measure possible effects on tonic inhibition, slices were exposed to drugs as outlined above, and the change in holding current was measured in 1 μM GABA in response to the GABA<sub>R</sub> antagonist, picrotoxin. Tonic current in the presence of 300 nM ORG was 75.4 ± 10.4 pA (n = 10 cells) compared with 67 ± 13.2 pA (n = 8 cells) in the presence of vehicle (DMSO) control (Fig. 4B). These results suggest that ORG does not have any direct or allosteric effects on GABAergic inhibition, which is consistent with our studies on recombinant receptors. In contrast to our results with ORG, published studies have shown that acute exposure of slices to NAS, including ALLO, slows sIPSC decay and increases tonic current (1, 8).

Sustained exposure of hippocampal slices to ORG selectively increases tonic current

To assess the effects of sustained exposure of ORG on GABAergic inhibition, slices were incubated with 300 nM ORG for 15 mins and then washed for a further 30–50 min before experimentation (Fig. 5A). Under these conditions, ORG significantly increased tonic inhibition in DGGCs from 52.6 ± 13 pA (n = 8 cells) in control to 91 ± 12 pA (Fig. 5B; p < 0.01, n = 10 cells) following ORG exposure. Consistent with our biochemical studies, the effects of ORG on tonic current were blocked by co-incubating slices with GFX/KT5720. (Fig. 5B; 54.2 ± 11 pA, n = 9 cells). In contrast to these effects on tonic current, sustained exposure to ORG did not affect the amplitude (106.9 ± 9.3 pA for vehicle, 105.1 ± 13 pA for ORG, n = 10 cells), frequency (1 ± 0.4 Hz for vehicle, 1.1 ± 0.4 Hz for ORG, n = 10 cells), or decay times (10.7 ± 0.7 ms for vehicle, 11 ± 0.8 ms for ORG) of sIPSCs (Fig. 5C; n = 10 cells).

The metabotropic effects of ORG on tonic inhibition are dependent upon Ser-408/9

Our electrophysiological and biochemical studies suggest that ORG exposure results in elevated phosphorylation of the β3 subunit, elevations in plasma membrane accumulation of GABA<sub>R</sub>Rs, and a sustained increase in tonic inhibition. To further assess whether Ser-408/9 directly contribute to the effects of ORG on tonic current, we prepared hippocampal slices from S408/9A homozygote mice. These mice have been shown to have reductions in tonic but elevated phasic inhibition in DGGCs. Thus, we examined the effects of ORG exposure on tonic inhibition in DGGCs from S408/9A mice. In contrast to WT mice (Fig. 5), exposure of hippocampal slices to ORG did
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Figure 4. ORG does not acutely modify phasic or tonic inhibition. A, the effects of a 5-min application of vehicle or 300 nM ORG on the efficacy of tonic inhibition were assessed using pertussis toxin (PTX). The effects of these treatments on the magnitude of tonic current were then determined. In these experiments, nASCF was supplemented with 1 μM GABA. B, sIPSCs were measured following a 5-min exposure to vehicle or 300 nM ORG. Sample traces and scale sIPSCs are shown in the right-hand panel. These data were then used to determine sIPSC amplitude, decay, and frequency. n = 8 cells for all experiments. sIPSC decays were determined using 200 events.

Figure 5. ORG induces a sustained and selective increase in tonic current. A, the protocol used to measure the metabotropic effects of ORG on GABAergic inhibition is illustrated. B, sample traces are shown for tonic currents recorded from slices exposed to vehicle, 300 nM ORG, or 300 nM ORG + 10 μM GFX + 1 μM KT. These data were then used to determine tonic current under the varying conditions, and levels were compared with those seen in vehicle-treated controls. C, the effect of ORG or vehicle on sIPSCs was determined. Sample traces and individual events are shown in the right-hand panel. These data were then used to determine sIPSC amplitude, decay, and frequency. *, significantly different from control, p < 0.01 (ANOVA; n = 10 cells).

not significantly modify tonic current in DGCCs from S408/9A mice. Tonic current was 71.4 ± 6 pA (n = 10 cells) in control compared with 67.5 ± 10 pA (n = 10 cells) following exposure to ORG (Fig. 6A). This suggests that Ser-408/9 in the β3 subunit are critical determinants for the metabotropic effects of ORG on tonic current.

The metabotropic effects of neurosteroids on tonic current are mediated by Ser-408/9

In common with ORG, THDOC and ALLO have been shown to induce sustained increases in tonic inhibition that parallel increased Ser-408/9 phosphorylation (11). Therefore, we examined whether these metabotropic effects are dependent upon Ser-408/9. First, we examined the effects of vehicle or THDOC on tonic current as detailed above. In DGCCs from S408/9A mice, 100 nM THDOC did not significantly modify tonic current (Fig. 6B; control = 68.7 ± 9 pA, THDOC = 72.6 ± 13 pA, n = 9 cells). Likewise, 100 nM ALLO was without effect on this parameter in the mutant mouse (Fig. 6C; control = 74.7 ± 19 pA, ALLO = 90.4 ± 20 pA, n = 5–9 cells). However, consistent with published studies (11), 100 nM THDOC and ALLO significantly increased tonic current in DGCCs in WT mice (Fig. 6D; control = 71.1 ± 10.2 pA, ALLO = 95.2 ± 13.4 pA, THDOC = 96.8 ± 14.2 pA, p < 0.05 (ANOVA), n = 9–10 cells).

We also assessed the role that Ser-408/9 play in mediating the effects of NAS on GABAAR plasma membrane accumulation. To do so, slices from WT and S408/9A mice were incubated with 100 nM ALLO for 20 min, and slices were subject to biotinylation. Surface and total fractions were then immunoblotted with antibodies against the α4 and β3 subunits. In WT mice, ALLO significantly increased the plasma membrane levels of both subunits (Fig. 7; 142.5 ± 18.3 and 121 ± 8.5% of control, respectively; p < 0.005, n = 5 mice). In contrast to this, ALLO was without effect on these parameters in slices from S408/9A mutant mice (Fig. 7; 101.2 ± 8.3 and 105 ± 11.5% of control, respectively; p = 0.350, n = 6 mice). Collectively, these results suggest that the ability of NAS and mPR agonists to induce sustained effects on tonic current is dependent upon Ser-408/9 in the β3 subunit.

Ser-408/9 are not primary determinants for the effects of NAS on sIPSC

The role that Ser-408/9 plays in mediating the allosteric effects of ALLO on GABAergic inhibition was also tested. To do so, we compared the effects of ALLO on sIPSC properties measured in DGCCs from both genotypes (Table 1). sIPSC decay was significantly slowed to similar degrees in WT and S408/9A mice. sIPSC amplitude in S408/9A mice was reduced by ALLO,
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Figure 6. The ability of ORG and NAS to induce metabotropic effects on tonic current are dependent upon Ser-408/9. Hippocampal slices from S408/9A mice were incubated with vehicle, 300 nM ORG (A), 100 nM THDOC (B), or 100 nM ALLO (C). Slices were washed extensively, and the magnitude of tonic inhibition was then determined. Typical traces are shown in the left-hand panels, and these data were then used to determine the magnitude of tonic current under each condition, n = 8–10 cells. D, sample traces are shown for tonic currents recorded from WT hippocampal slices exposed to vehicle or 100 nM THDOC or ALLO. These data were then used to determine tonic current under the varying conditions, and levels were compared with that seen in vehicle-treated controls. *, significantly different from control, p < 0.05 (ANOVA, n = 9–10 cells).

Figure 7. The ability of NAS to modulate plasma membrane accumulation of GABA<sub>A</sub>Rs depends upon Ser-408/9. Hippocampal slices from WT and S408/9A mice were incubated with 100 nM ALLO and subject to biotinylation followed by immunoblotting with α4 and β3 subunit antibodies. Surface levels of both subunits were then determined and normalized to those in vehicle-treated controls. *, significantly different from control, p < 0.05 (t test; n = 6 mice).

Table 1 Ser-408/9 are dispensable for effects of ALLO on sIPSCs

|          | WT    | S408/9A |
|----------|-------|---------|
|          | Control | ALLO    | Control | ALLO    |
| Amplitude (pA) | 49 ± 1  | 49.1 ± 0.9 | 60.3 ± 1  | 52.9 ± 1*  |
| Frequency (Hz)  | 4.3 ± 1.4 | 3.2 ± 1.4  | 5 ± 1.1  | 7 ± 1.2  |
| Decay (ms)      | 13.7 ± 1.1 | 20.3 ± 1.5b | 14.8 ± 0.5 | 18.6 ± 1c  |

*p < 0.0001,  
**p = 0.0053,  
*p = 0.009.

we used GR3027, a compound that has been shown to block allosteric effects of NAS on GABA<sub>A</sub>R receptor activity but not compromise receptor agonist activation (23, 24). Consistent with this notion, 10 μM GR3027 blocked the effects of 100 nM ALLO on sIPSC decay, 8.9 ± 0.6 ms before and 8.1 ± 0.8 ms after (n = 6 cells) the acute co-application of ALLO and GR3027 (Fig. 8A). However, GR3027 did not impact the metabotropic effects of ALLO on tonic inhibition. Co-incubation of hippocampal slices with ALLO plus GR3027 increased tonic current to 97 ± 10.6 pA (n = 11 cells) compared with 60.6 ± 7.5 pA (n = 9 cells) in vehicle control (Fig. 8B). This result provides further evidence that distinct molecular mechanisms mediate the metabotropic and allosteric effects of NAS on GABAergic inhibition.

mPRs mediated the effects of ORG and NAS on GABA<sub>A</sub>R phosphorylation

Our results suggest that ORG and NAS exert sustained effects on tonic inhibition by promoting the phosphorylation of GABA<sub>A</sub>Rs on residues that include Ser-408/9 in the β3 subunit. Due to the lack of specific mPR antagonists, we examined the role these GPCRs play in mediating the effects of NAS on GABA<sub>A</sub>R phosphorylation using recombinant expression in HEK293 cells. First, we used reverse transcriptase quantitative
PCR (RT-qPCR) to examine which mPRs were endogenously expressed in this cell line. The relative levels of mPR mRNAs were then compared by reference to a tubulin standard, and the results were then expressed as arbitrary units, as described previously (25). 2.2 ± 0.4 and 5.9 ± 0.8 arbitrary units of mPRα and mPRβ mRNAs were detected in HEK293 cells, whereas those corresponding to γ, δ, and ε were not. The effect of ALLO on phosphorylation of GABA_ARs composed of α4 and β3 subunits was then tested when transiently expressed in HEK293 cells. 100 nM ALLO was without effect on the phosphorylation of Ser-408/9, as measured using immunoblotting (Fig. 9A; 105.4 ± 8.4% of control, p = 0.488, n = 5). The subcellular localization of mPRs has been widely debated due to studies describing both intracellular (26, 27) and surface expression of mPR (28, 29). mPRs were found in intracellular compartments in HEK293 cells and COS-1 cells. Thus, to investigate whether the lack of ALLO-mediated phosphorylation of β3 subunits was due to the absence of mPRs at the plasma membrane, we used a murine mPRα construct modified at its C terminus with the FLAG epitope (mPRα-FLAG) that demonstrated localization to the plasma membrane (Fig. 9B). Exposure of ALLO to HEK293 cells transfected with GABA_ARs αβ/3 and mPRα-FLAG resulted in increased phosphorylation of Ser-408/9 (Fig. 9; 146 ± 21.0% of control, p < 0.01, n = 5).

300 nM ORG also increased phosphorylation of these residues to 139.2 ± 11.6% of control (Fig. 9D; p < 0.01, n = 3), an effect that was occluded by inhibitors of PKA and PKC (Fig. 9D; 92.06 ± 17.1% of control, p = 0.460, n = 3). Consistent with the effect of ORG on β3 surface expression in hippocampal slices, ORG increased GABA_AR plasma membrane accumulation of β3 subunits in HEK293 cells expressing GABA_ARs αβ/3 and mPRα-FLAG (Fig 10; 117.2 ± 8.0% of control, p < 0.05, n = 3).

Thus, these results suggest that NAS and ORG modulate GABA_AR phosphorylation dependent upon the activation of mPRs.

**Discussion**

NAS are accepted to play key roles as endogenous modulators of GABAergic inhibition, in part by acting as GABA_AR PAMs (1, 8). Recent studies have suggested that, in addition to their role as PAMs, NASs exert sustained metabolic effects on GABAergic inhibition via an underlying mechanism that remains ill-defined (10, 30). In addition to its role as a GABA_AR PAM, ALLO has been shown to bind and activate mPRs, which have been proposed to be a novel family of GPCRs that are highly expressed in the brain (12–17). Although tools to manipulate the activity of this family of G protein-coupled receptors are limited, we made use of the only available selective agonist, ORG (14). ORG increased Ser-408/9 phosphorylation dependent upon PKA/PKC in acute hippocampal slices. Under the same experimental conditions, ALLO induced similar PKA/PKC-dependent enhancement on Ser-408/9 phosphorylation. Moreover, ORG also increased the plasma membrane accumulation of GABA_ARs. However, in contrast to ALLO, ORG did not exhibit any efficacy as a GABA_AR PAM. Consistent with these results, acute exposure of hippocampal slices to ORG did not modify tonic current or the properties of sIPSCs.

Next, we assessed whether ORG exerts any metabolic effects on GABAergic inhibition by exposing slices to this agent, followed by extensive washing to measure effects on tonic and phasic inhibition. Consistent with our biochemical analysis, ORG increased tonic current in a mechanism that depended upon PKA and PKC. In contrast to this sustained exposure, ORG did not have a significant impact on sIPSCs. To assess whether the ability of ORG to exert its metabolic effects is dependent upon its ability to modulate receptor phosphorylation, we made use of S408/9A mice. In contrast to WT mice, the ability of ORG to exert sustained effects on tonic current was lost in these mutant mice. Similar experiments revealed that Ser-408/9 in the GABA_AR β3 subunit are critical in mediating the metabolic effects of ALLO on tonic inhibition and their ability to modulate receptor trafficking. In contrast to this, and its ability of ALLO to slow sIPSC decay (1), a hallmark of their efficacy as GABA_AR PAMs, was maintained in the mutant mice. To further evaluate the relationship between the metabolic and allosteric signaling mechanism of NAS, we used GR3027, a neurosteroid antagonist that ablates the efficacy as PAMs (23, 24). As predicted, this agent blocked the effects of ALLO on sIPSC kinetics but not on its ability to induce metabolic effects on tonic inhibition.

Finally, we directly tested whether the ability of ORG and ALLO to modulate phosphorylation of GABA_ARs is actually dependent upon mPRs, a task that is complicated by the lack of specific mPR antagonists. Given the heterogeneity of mPR structure and the lack of knockout mice, we tested this hypothesis using recombinant expression. We found that HEK293 cells express mPRs, but these proteins are largely restricted to intracellular compartments. Accordingly, NAS did not significantly modify GABA_AR phosphorylation in these cells. Previous studies exploring the subcellular localization of mPRs have
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Figure 9. mPRs mediate the effects of NAS and ORG on GABA<sub>R</sub> phosphorylation. A, HEK293 cells transfected with α4/β3 GABA<sub>R</sub>s were treated with either vehicle or 100 nM ALLO for 5 min and immunoblotted with ps408/9, β3, and actin antibodies. The ratio of ps408/9/β3 immunoreactivity was then determined and normalized to control (100%). B, HEK293 cells transfected with mPRα-FLAG or empty plasmid were labeled on ice with NHS-Biotin. After cells were lysed and protein was purified on immobilized avidin, surface and total fractions were immunoblotted with antibodies against FLAG, tubulin, or TfR. C, HEK293 cells co-transfected with α4/β3 GABA<sub>R</sub>s and mPRα-FLAG were treated with 100 nM ALLO (D) or 300 nM ORG (E) for 5 min or PKA and PKC inhibitors (10 μM GFX, 1 μM KT) with vehicle or 300 nM ORG for 15 min (10-min pretreatment with inhibitors). The ratio of ps408/9/β3 immunoreactivity was then determined and normalized to control or GFX/KT alone (100%). *, significantly different from control, p < 0.05, n = 3–5.

Figure 10. ORG increases surface expression of β3 receptors in HEK293 cells co-expressing mPRα. HEK293 cells transfected with GABA<sub>R</sub>s α4/β3 and mPRα-FLAG were treated with vehicle or 300 nM ORG for 10 min. Cells were fixed with PFA (no permeabilization), and surface β3 subunits were labeled using β3 antibodies conjugated to GFP secondary antibodies. In each panel the scale bar represents 20 μm. Cells were refixed and permeabilized, and intracellular β3 subunits were labeled with β3 antibodies conjugated to RFP secondary antibodies. Surface β3 levels were quantified by dividing surface levels by the sum of surface and intracellular levels of β3, *: significantly different from control p < 0.05 (t test, n = 20 cells from four independent transfections).

Proven controversial, with some studies suggesting that their expression is restricted to intracellular compartments (26, 27), whereas others suggest plasma membrane expression (28, 29). However, in cells expressing mPRα-FLAG, accumulation of mPR immunoreactivity was evident on the plasma membrane and facilitated NAS- and ORG-dependent phosphorylation of the β3 subunit on Ser-408/9, via a PKA- and PKC-dependent mechanism, similar to events seen in brain slices. Clearly, further experimentation is required to determine the mechanisms that regulate mPR signaling and their roles in determining the effects of NAS on neuronal excitability.

Collectively, our experiments suggest that neurosteroids exert profound metabotropic effects on tonic inhibition by promoting the phosphorylation of Ser-408/9 in the β3 subunit via the activation of mPRs via a mechanism distinct from their actions as GABA<sub>R</sub> PAMs. NASs also potentiate the phosphorylation of Ser-443 in the α4 subunit, which at least in expression systems enhances the plasma membrane accumulation of GABA<sub>R</sub>s (10, 31). It will be of interest to examine the role that Ser-443 plays in mediating the metabotropic effects of NAS on tonic inhibition. However, such studies await the production of suitable phospho-specific antibodies and mutant mice.

Besides modulating GABA<sub>ergic</sub> inhibition, NASs have also been suggested to exert broader effects on neuronal survival, neuroprotection, and promoting neurogenesis (12, 32, 33). It is tempting to speculate that in part, these effects may be mediated by the ability of NAS to modulate mPR activation and perhaps the recently described efficacy of these agents to alleviate mood disorders (34). Finally, alterations in NAS signaling may also contribute to altered neuronal excitability seen in Fragile X-syndrome, where compromised tonic inhibition and modified GABA<sub>R</sub> phosphorylation has been demonstrated (35).

Experimental procedures

Animals

8–12-Week-old C57BL/6J mice (The Jackson Laboratory) were housed under constant temperature and humidity on a 12-h light/dark cycle with standard rodent food and water ad
**Antibodies and constructs**

The following antibodies were used for Western blotting: polyclonal anti-phospho-Ser-408/9 GABA_A β3 (18, 19) (1:5000–1:10,000); polyclonal anti-GABA_A β3 (PhosphoSolutions, catalogue no. 863-GB3C, 1:1000); polyclonal anti-α4 (31) (1:10,000); monoclonal anti-transferrin (Thermo Fisher Scientific, catalogue no. 13-6800, 1:1000); monoclonal anti-β-actin (Sigma, catalogue no. A1978, 1:25,000); polyclonal peroxidase AffiniPure goat anti-rabbit (Jackson Immunoresearch, 111-035-144, 1:7000); polyclonal peroxidase AffiniPure goat anti-mouse (Jackson Immunoresearch, 115-035-003, 1:7000–1:12,000).

**Cell culture and transfection**

Human embryonic kidney 293 cells containing the SV40 T-antigen (HEK293T) were passaged in 1× Dulbecco’s modified Eagle’s medium + 4.5 g/liter D-glucose + 1-glutamine (Thermo Fisher Scientific) with 10% fetal bovine serum (Fisher) as detailed previously (10, 18–20). These values were then used for statistical analysis (Student’s t test or one-way ANOVA) on GraphPad Prism 7.

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MM NaHCO_3, 1.25 mm NaH_2PO_4, 2.5 mm KCl, 2 mm CaCl_2, 2 mm MgCl_2, 10 mm D-glucose, 1.5 mm pyruvate, 1 mm L-glutamine (300–310 mosm) at 31 °C. After recovery, slices were treated with either control (DMSO) or neurosteroids in oxygenated ACSF for 20 min in bacterial centrifuge tubes. For kinase inhibitor experiments, slices were pretreated for 10 min with GFX and KT 5720 followed by treatment with neurosteroids for 10 min as described previously (36, 37).

**Western blotting**

Slices treated with neurosteroids were snap-frozen, briefly thawed on ice, and homogenized using a hand-held motorized tissue grinder (Fisher, catalogue no. 12-1413-61) in lysis buffer containing the following: 20 mm Tris-Cl, pH 8.0, 150 mm NaCl, 5 mm EDTA, pH 8.0, 10 mm NaF, 2 mm sodium orthovanadate, 10 mm sodium pyrophosphate, 0.1% SDS, 2% Triton X-100 (v/v) was added to the samples before solubilizing on a rotating wheel at 4 °C for at least 2 h. HEK293T cells treated with neurosteroids were solubilized in lysis buffer (as described above) with no SDS and 1% Triton X-100. Samples were centrifuged at 15,000 rpm for 15 min, and protein concentration was determined using the Bradford assay. Samples were boiled in 4× sample buffer for 1–3 min at 95 °C. 25 μg of protein was loaded on 8–10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes overnight at 4 °C. Membranes were incubated with 6% milk in PBS-T for all antibodies except anti-phospho-Ser-408/9 GABA_A β3, for which 6% BSA in PBS-T was used. Blots were incubated with primary antibodies over-night at 4 °C and secondary antibodies at room temperature for 1.5 h. Blots were developed using chemiluminescence on the charge-coupled device–based ChemiDoc XRS system (catalogue no. 1708265) and quantified using densitometric analysis (ImageJ). After controlling for gel loading with reference to actin immunoreactivity, the ratio of pS408/9/β3 immunoreactivity was then determined and compared between treatments as described previously (10, 18–20). These values were then used for statistical analysis (Student’s t test or one-way ANOVA).

**RT-qPCR**

RNA was extracted from HEK293T cells using the RNasy Plus Mini Kit (Qiagen). cDNA was synthesized using the Super-
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Script® IV First-Strand Synthesis kit (Life Technologies) with random hexamers following the manufacturer’s instructions. The gene dose was calculated based on the standard curve method relative to tubulin as described previously (25).

Immunocytochemistry

Coverslips with transfected HEK293T cells were treated with ORG OD 02-0 for 5 min and fixed in 4% PFA/PBS for 20 min. PFA was quenched with 0.1 M glycine for 5 min and washed three times with 1× PBS. Surface and intracellular staining was then performed as described by Connolly et al. (38) using anti-GABA_A β2,3 clone BD17 antibody (Millipore).

Image analysis

The outline of cells was drawn using the drawing tool in ImageJ, and the average integrated density of fluorescence for each channel (emission 488 and 568 nm) was determined. Surface levels were quantified as follows: surface fluorescence/(surface fluorescence + intracellular fluorescence). Surface levels were normalized to those in control-treated cells (38).

Brain slice preparation for electrophysiology

Brain slices were prepared from 3–5-week-old male C57BL/6J mice. Mice were anesthetized with isoflurane and decapitated, and brains were rapidly removed and submerged in ice-cold cutting solution containing 126 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl_2, 2 mM MgCl_2, 26 mM NaHCO_3, 1.25 mM NaH_2PO_4, 10 mM glucose, 1.5 mM sodium pyruvate, and 3 mM kynurenic acid. Coronal 310-μm-thick slices were cut with the vibratome VT1000S (Leica Microsystems, St. Louis, MO). The slices were then transferred into an incubation chamber filled with prewarmed (31–32 °C) oxygenated nACSF of the following composition: 126 mM NaCl, 2.5 mM KCl, 2 mM CaCl_2, 2 mM MgCl_2, 26 mM NaHCO_3, 1.25 mM NaH_2PO_4, 10 mM glucose, 1.5 mM sodium pyruvate, 1 mM glutamine, 3 mM kynurenic acid, and 0.005 M GABA bubbled with 95% O_2, 5% CO_2. Exogenous GABA was added to standardize ambient GABA in the slice and provide an agonist source for newly inserted extrasynaptic GABA_A Rs. Slices were allowed to recover at 32 °C for at least 30 min before exposure to neurosteroids. Hippocampal slices were incubated for 15 min in a chamber containing either control or neurosteroids dissolved in nACSF that did not contain kynurenic acid. Following this incubation, slices were transferred to a submerged, dual-perfusion chamber containing either control or neurosteroids. Hippocampal slices were incubated for 15 min in a chamber containing either control or neurosteroids dissolved in nACSF that did not contain kynurenic acid. Following this incubation, slices were transferred to a submerged, dual-perfusion recording chamber (Warner Instruments, Hamden, CT) on the stage of an upright microscope (Nikon FN-1) with a ×40 water immersion objective equipped with differential interference contrast/infrared optics. Slices were maintained at 32 °C and gravity superfused with nACSF solution (with kynurenic acid) throughout experimentation and perfused at a rate of 2 ml/min with oxygenated (O_2/CO_2, 95%/5%) nACSF. Slices were perfused for 30–60 min before recordings were started.

Electrophysiological recordings

Whole-cell currents were recorded from the dentate gyrus granule cells in 310-μm-thick coronal hippocampal slices. Patch pipettes (5–7 megaohms) were pulled from borosilicate glass (World Precision Instruments) and filled with intracellular solution of the following composition: 140 mM CsCl, 1 mM MgCl_2, 0.1 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, 4 mM NaCl, and 0.3 mM Na-GTP (pH 7.2 with CsOH). A 5-min period for stabilization after obtaining the whole-cell recording configuration (holding potential of −60 mV) was allowed before currents were recorded using an Axopatch 200B amplifier (Molecular Devices), low-pass-filtered at 2 kHz, digitized at 20 kHz (Digidata 1440A; Molecular Devices), and stored for offline analysis. For tonic current measurements, an all-points histogram was plotted for a 10-s period before and during 100 μM picrotoxin application, once the response reached a plateau level. Recordings with unstable baselines were discarded. Fitting the histogram with a Gaussian distribution gave the mean baseline current amplitude, and the difference between the amplitudes before and during picrotoxin was considered to be the tonic current. The negative section of the all-points histogram that corresponds to the inward inhibitory synaptic currents was not fitted with a Gaussian distribution (39, 40). Series resistance and whole-cell capacitance were continually monitored and compensated throughout the course of the experiment. Recordings were eliminated from data analysis if series resistance increased by >20%. sIPSCs were analyzed using the mini-analysis software (version 5.6.4; Synaptosoft, Decatur, GA). Minimum threshold detection was set to 3 times the value of baseline noise signal. To assess sIPSC kinetics, the recording trace was visually inspected, and only events with a stable baseline, sharp rising phase, and single peak were used to negate artifacts due to event summation. Only recordings with a minimum of 200 events fitting these criteria were analyzed. sIPSC amplitude and frequency from each experimental condition were pooled and expressed as mean ± S.E. To measure sIPSC decay, we averaged 100 consecutive events and fitted the decay to a double exponential and took the weighted decay constant (η). Statistical analysis was performed by using Student’s t test (paired and unpaired where appropriate), where p < 0.05 is considered significant.

Analysis of GABA-induced currents in HEK293T cells

HEK293T cells expressing GABA_A Rs composed of α4β3 subunits were superfused, at a rate of 2 ml/min at 32–33 °C, with an extracellular solution containing 140 mM NaCl, 5 mM KCl, 1.2 mM MgCl_2, 2.5 mM CaCl_2, 10 mM HEPES, and 11 mM glucose and adjusted to pH 7.4 with NaOH as detailed previously (31). Experiments were started 3–5 min after achieving the whole-cell configuration at −60 mV. GABA (1 μM, ~EC_50) and other drugs were applied via a fast-step perfusion system (Warner Instruments).

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