The major central endocannabinoid directly acts at GABA<sub>A</sub> receptors

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**GABA<sub>A</sub>** receptors are chloride ion channels composed of five subunits (1), mediating fast synaptic and tonic inhibition in the mammalian brain. A total of 19 different subunit isoforms have been identified, with the major receptor type in mammalian adult brain consisting of α<sub>1</sub>, β<sub>2</sub>, and γ<sub>2</sub> subunits (1, 2). GABA<sub>A</sub> receptors are the target of numerous sedating and anxiolytic drugs such as benzodiazepines (3). The currently known endogenous ligands include GABA, neurosteroids (4), and possibly drugs such as benzodiazepines (3). The pharmacological properties of this chloride ion channel strictly depend on receptor subunit composition (2) and arrangement (6).

Endocannabinoids (ECS) are lipid signaling molecules that modulate neurotransmission by acting presynaptically on CB<sub>1</sub> receptors. Thus, they act as retrograde signals. 2-AG is the major CB<sub>1</sub> receptor agonist (23), was similarly active at GABA<sub>A</sub> receptors containing α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>, whereas AEA and 2-methyl-2-<sup>5</sup>-hydroxy-5<sup>α</sup>-tetrahydrocannabinol (THC)D and modulates α-subunit–containing receptors, known to be located extrasynaptically and to respond to neurosteroids. 2-AG inhibits motility in CB<sub>1</sub>C57BL/6j mice show hypermotility. The identification of a functional binding site for 2-AG in the GABA<sub>A</sub> receptor may have far-reaching consequences for the study of locomotion and sedation.

Identification of Other Endocannabinoids That Target GABA<sub>A</sub> Receptors. To determine if this effect was only observed with 2-AG, we also evaluated physiologically relevant compounds sharing close structural similarity with 2-AG (Fig. 1C). The spontaneous isomerization product 3(3)-AG (stereoisomeric mixture produced by acyl migration) showed a similar potentiation as 2-AG. Whereas AEA and 2-methyl-2-<sup>5</sup>-hydroxy-5<sup>α</sup>-tetrahydrocannabinol (THC)D and arachidonic acid (AA) showed only a weak if any potentiation (Fig. 1D). Docosatetraenylethanolamide (DEA) and arachidonic acid (AA) showed only a weak if any potentiation (Fig. 1D). Not unexpectedly, oleamide, which was previously shown to only weakly potentiate GABA<sub>A</sub> receptors (5), was ineffective at 3 μM. The phytocannabinoid Δ<sub>2</sub>-tetrahydrocannabinol (THC)D at 3 μM only weakly potentiated the response to GABA, at 34 ± 10% (Fig. 1D). These data uncover the significant effect of 2-AG and NE at GABA<sub>A</sub> receptors and indicate the importance of the glyceral moiety for the GABA<sub>A</sub> receptor interaction.

Potentiation by 2-AG is Selective for GABA<sub>A</sub> Receptors Containing the β<sub>2</sub> Subunit. Next, we investigated the GABA<sub>A</sub> receptor subunit selectivity of 2-AG. The α<sub>1</sub> subunit in α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> was replaced by α<sub>2</sub>, α<sub>3</sub>, α<sub>5</sub>, or α<sub>6</sub>. This had little effect on the current potentiation by 3 μM 2-AG (Fig. 1E). However, drastic effects were observed upon replacement of the β<sub>2</sub> subunit by β<sub>1</sub> or β<sub>3</sub> (Fig. 1F). Whereas, in receptors containing β<sub>1</sub> potentiation was abolished, it was reduced to approximately one third in receptors containing β<sub>3</sub>. To test whether 2-AG shares the binding site with loreclezole (24), another allosteric activator of GABA<sub>A</sub> receptors, we evaluated the point mutation β<sub>2</sub>N265S in α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> receptors that abolishes the potentiation by loreclezole. As shown in Fig. 1F, this mutation only partially reduced the effect of 2-AG, thus indicating

Author contributions: E.S., A.Z., and J.G. designed research; R.B., I.R., and J.M. performed analgesia (20, 21), but the ECS has also been implicated in release (18, 19). The major central action of endocannabinoids is activation only partially reduced the effect of 2-AG, thus indicating

Results

**Endocannabinoid 2-AG Potentiates GABA<sub>A</sub> Receptors.** Recombinant α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> GABA<sub>A</sub> receptors were functionally expressed in *Xenopus* oocytes. We found that currents elicited by 1 μM GABA were potentiated by 2-AG in a concentration-dependent way (Fig. 1A and B). Fitting of the concentration–response curve indicated a maximal potentiation of 138 ± 21% (SEM) and an EC<sub>50</sub> value of 2.1 ± 0.5 μM (n = 5). The resulting Hill coefficient of 2.2 ± 0.2 indicates that more than one molecule of 2-AG interacts with one receptor. Potentiation by 3 μM 2-AG was determined at different GABA concentrations—0.5 μM (EC<sub>0.5</sub>), 1 μM (EC<sub>2.3</sub>), 10 μM (EC<sub>3.5</sub>) and 100 μM (EC<sub>92</sub>)—and amounted to 86 ± 13% (n = 3), 92 ± 25% (n = 6), 10 ± 6% (n = 3) and 2 ± 6% (n = 3), respectively. This shows that only currents elicited by low GABA concentrations are potentiated by 2-AG.

**Identification of Other Endocannabinoids That Target GABA<sub>A</sub> Receptors.** To determine if this effect was only observed with 2-AG, we also evaluated physiologically relevant compounds sharing close structural similarity with 2-AG (Fig. 1C). The spontaneous isomerization product 3(3)-AG (stereoisomeric mixture produced by acyl migration) showed a similar potentiation as 2-AG. Whereas AEA and 2-methyl-2-<sup>5</sup>-hydroxy-5<sup>α</sup>-tetrahydrocannabinol (THC)D and arachidonic acid (AA) showed only a weak if any potentiation (Fig. 1D). Not unexpectedly, oleamide, which was previously shown to only weakly potentiate GABA<sub>A</sub> receptors (5), was ineffective at 3 μM. The phytocannabinoid Δ<sub>2</sub>-tetrahydrocannabinol (THC)D at 3 μM only weakly potentiated the response to GABA, at 34 ± 10% (Fig. 1D). These data uncover the significant effect of 2-AG and NE at GABA<sub>A</sub> receptors and indicate the importance of the glyceral moiety for the GABA<sub>A</sub> receptor interaction.

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that endocannabinoids act through a different site. Potentiation was also strongly reduced upon omission of $\gamma_2$ in $\alpha_1\beta_2\gamma_2$ to give the dual combination $\alpha_1\beta_2$. Possibly, in receptors with two adjacent $\beta_2$ subunits the binding site for 2-AG is compromised in these subunits. Replacement of the $\beta_2$ subunit in dual subunit combinations by $\beta_1$ or $\beta_3$ in both cases led to an additional strong reduction of the potentiation (Fig. 1F). For all experiments, GABA was used at a concentration eliciting 1.0% to 3.2% of the maximal current amplitude in each receptor form.

We then investigated the concatenated receptors (25, 26) $\alpha_1\beta_1\gamma_1\gamma_2\beta_1$, $\alpha_1\beta_2\alpha_1\gamma_1\gamma_2\beta_2$, $\alpha_1\beta_3\alpha_1\gamma_1\gamma_2\beta_3$, and $\alpha_1\beta_2\alpha_1\gamma_1\gamma_2\beta_1$, in which the subunit arrangement is precluded by covalent linkage. These receptors have been described in detail previously (27). As shown in Fig. 1G, in receptors containing two $\beta_2$ subunits stimulation by 2-AG was strongly reduced compared with receptors containing two $\beta_2$ subunits. In the receptors containing one $\beta_2$ subunit, irrespective of the position, the potentiation was reduced by approximately 50%, indicating that two sites for 2-AG may be located on one $\alpha_1\beta_2\gamma_2$ receptor.

**Identification of Amino Acid Residues Conferring Subunit Selectivity of 2-AG.** Next, we identified the amino acid residues (AARs) mediating the selectivity of 2-AG for the $\beta_2$ subunits. We aligned the protein sequences of the three $\beta$-subunits and identified homologous AAR in predicted transmembrane regions that were identical in $\beta_1$ and $\beta_3$, but different from those in $\beta_2$. These were the AAR corresponding to $\beta_2$M294 and $\beta_2$L301 in transmembrane region M3, and $\beta_2$V436 and $\beta_2$F439 in M4. Mutation of these residues in $\beta_2$ to the respective residues present in $\beta_1$ and $\beta_3$ were prepared and coexpressed with $\alpha_1$ and $\gamma_2$ subunits, and the resulting receptors screened for their potentiation by 3 $\mu$M of 2-AG (Fig. 2A and SI Appendix, Fig. S1). All mutations significantly reduced potentiation by 2-AG. In the mutant receptor $\alpha_1\beta_2$V436T$\gamma_2$, potentiation was even completely abolished, but the EC$_{50}$ of GABA was not significantly altered. In a second step, the corresponding residues in $\beta_1$ were mutated to the homologous residue present in $\beta_2$ (Fig. 2B). A critical residue located in the binding site would be expected to lead to a loss of potentiation in $\alpha_1\beta_2$V436T$\gamma_2$ and to a gain in potentiation in $\alpha_1\beta_2$M294$\gamma_2$ receptors. Our results clearly show that AAR V436 and, to a smaller extent, also F439 in M4 of $\beta_2$ mediate the functional effect of 2-AG, thus pinpointing the receptor binding site. Along the same line, both mutations combined resulted in almost complete recovery. Although allosteric effects by the mutations cannot be fully excluded, we consider this possibility unlikely. Both AARs are predicted to be located in cytoplasmic leaftlet of the $\alpha$-helix, in an angle of approximately 60° and a distance in the direction of the $\alpha$-helix of 4.5 Å. Intriguingly, in a homology model described by Ernst et al. (28), all four mentioned residues face the same cavity in the receptor.

**δ-Subunit–Containing Receptors Are Responsive to 2-AG.** In agreement with the finding that 2-AG mediates its effect via $\beta_2$, $\alpha_1\beta_2\delta$ receptors in which $\gamma_2$ in $\alpha_1\beta_2\gamma_2$ was replaced by the $\delta$-subunit also responded in a concentration-dependent way to 2-AG (Fig. 3A). Fitting of the concentration–response curve carried out with 1 $\mu$M GABA (EC$_{50}$) indicated a maximal potentiation of 150 ± 51% ($n = 3$), an EC$_{50}$ value of 2.9 ± 1.8 $\mu$M, and a Hill coefficient of 1.25 ± 0.15 (Fig. 3B). In additional experiments, $\alpha_1\beta_2\delta$ receptors responded with a potentiation of 114 ± 19%
GABA receptors. (n = 4; SI Appendix, Fig. S2) to 3 μM 2-AG. In αβ-δ receptors, the subunits may be arranged differently to form a pentamer, and the different receptor isoforms are characterized by distinct properties, some of them being strictly dependent on the presence of neurosteroids and low GABA concentrations (29). Together, these results establish 2-AG as an endogenous allosteric activator of GABA receptors and identify M4 of the β2 subunit as the primary molecular target for 2-AG.

2-AG Acts in a Superadditive Manner with Neurosteroids or Diazepam. Next, we investigated a possible functional interaction between 2-AG and neurosteroids at αβ-δ GABA receptors. Concentration–response curves for the potentiation by 2-AG were generated in the presence of 0.1 μM 3α, 21-dihydroxy-5α-pregnan-20-one (THDOC; Fig. 4A). GABA (0.5 μM) was applied alone and then in combination with 0.1 μM THDOC, followed by applications containing GABA, THDOC, and increasing concentrations of 2-AG. As shown in Fig. 4A potentiation by 0.1 μM THDOC alone amounted to 164 ± 44% (n = 4). As mentioned earlier, the maximal potentiation by 2-AG amounted to 138 ± 21 (n = 5). Combined application of 2-AG and THDOC resulted in a curve characterized by an EC50 of 1.7 ± 0.5 μM and a maximal potentiation of 1.04 ± 505% (n = 4). This indicates that THDOC acts by increasing the maximal potentiation without significantly affecting the EC50 for 2-AG. Combined application of 0.1 μM THDOC and 1 μM 2-AG resulted in a superadditive potentiation, thus suggesting a synergism between these two agents. Further experiments were performed to support a superadditivity between THDOC and 2-AG. Fig. 4B shows an experiment in which currents were potentiated by low concentrations of THDOC, 2-AG, or a combination of both. We observed a strong superadditive effect between the two endogenous modulators (Fig. 4C). Superadditivity was also observed in three additional experiments carried out at 0.05 μM THDOC (SI Appendix, Fig. S3). To assess whether the agonist site or the modulatory site for neurosteroids (30) was involved in the superadditivity, we applied 0.1 μM THDOC together with 1 μM 2-AG in the absence of GABA. Currents amplitudes amounting to less than 4 nA were elicited in oocytes expressing more than 10,000 nA maximal GABA current. It is therefore unlikely that the agonist site for neurosteroids is involved in the superadditivity of 2-AG and THDOC effects.

Next, we investigated a possible interaction of 2-AG with the benzodiazepine diazepam. Concentration–response curves for the potentiation by 2-AG were generated in the presence of 0.3 μM diazepam (SI Appendix, Fig. S4). GABA (0.5 μM) was applied alone and then in combination with 0.3 μM diazepam, followed by several applications containing GABA, diazepam, and increasing concentrations of 2-AG. In these experiments, potentiation by 0.3 μM diazepam alone amounted to 128 ± 7% (n = 3). The EC50 for 2-AG in the presence of diazepam was 0.8 ± 0.3 μM, indicating a small increase in apparent affinity for 2-AG, and the maximal additional potentiation relative to this level achieved with diazepam alone was 210 ± 35% (n = 3). In additional experiments, currents were potentiated by 0.3 μM diazepam or 2-AG, respectively, or by a combination of both. Again, we observed a significant superadditivity between the two modulators (SI Appendix, Fig. S5). These results clearly suggest a superadditivity between the modulation by diazepam and 2-AG.

Locomotor Activity Is Suppressed by 2-AG and NE in CB1/CB2 Double-KO Mice, and j2 KO Mice Show Hypermotility. The sedating effects of GABAα receptor activation can be readily determined in vivo by evaluating exploratory locomotor behaviors (31). To eliminate the cannabinoid receptor-mediated effects by 2-AG, the experiments were carried out in cannabinoid receptor (Cnr1−/−/Cnr2−/−) double-KO mice. Additionally, WT mice were investigated. Vehicle-injected control animals of both genotypes showed a characteristically open-field exploratory behavior. Locomotor activity was initially high and gradually decreased over time, as the animals habituated to the new environment (Fig. 5A and B). As anticipated, 2-AG–treated animals (10 mg·kg−1·i.v.) showed a strong hypomotility in WT and in KO animals (P < 0.0001; Fig. 5A and
Similarly, NE (10 mg·kg\(^{-1}\) i.v.), a metabolically stable ether-linked analogue of 2-AG (23), led to potent hypomotility (Fig. 5 C and D). After 2-AG treatment in several cases, a brief anesthetic effect (i.e., loss of righting reflex) was detected (WT, three of eight; KO, five of eight). NE treatment in all cases led to a brief loss of righting reflex. Thus, these data provide evidence for the cannabinoid receptor-independent sedative effect of 2-AG and NE.

Next, we addressed the potential pharmacological superadditivity between 2-AG and THDOC. Given that 2-AG injection leads to rapid degradation of 2-AG in the brain (32) we used an indirect approach through pharmacological inhibition of 2-AG metabolism. Treatment of WT and cannabinoid receptor (Cnr1\(^{-/-}\)/Cnr2\(^{-/-}\)) double-KO mice with the selective monoacylglycerol lipase inhibitor JZL184 (16 mg·kg\(^{-1}\) i.p.), a compound that inhibits the enzymatic hydrolysis of 2-AG (32, 33), or THDOC (2 mg·kg\(^{-1}\) i.v.) alone, only partially inhibited locomotor activity after habituation to the test cage (\(P = 0.08\) and \(P = 0.156\), respectively; Fig. 5 E and F). However, when the animals received THDOC (2 mg·kg\(^{-1}\) i.v.) 2 h after the JZL184 treatment, they presented strong hypolocomotion after habituation (\(P < 0.0001\)). These data show that elevated endogenous 2-AG metabolism led to potent hypomotility in WT and KO mice. 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AG in brain mediates a superadditive effect with externally administered THDOC, independent of CB receptors (Fig. 5 E and F). In WT mice, the superadditive effect was more pronounced than in KO mice, and may also involve CB receptors. JZL184 (16 mg·kg⁻¹ i.p.) did not trigger the full effect on 2-AG elevation, which is approximately 10-fold (33), and showed significant hypomotility only during the habituation phase (Fig. 5 G and H).

Upon completion of the experiments, the JZL184-treated (Ctrl1−/−/Ctrl2−/−) double-KO mice showed a fourfold increase of 2-AG in brain, and the free AA was significantly reduced (SI Appendix, Fig. S6). As expected, a more potent superadditive effect was observed in the late phase in animals treated with combinations of low doses of NE (5 mg·kg⁻¹ i.v.) and THDOC (2 mg·kg⁻¹ i.v.; P < 0.0001; Fig. 5 G and H1), as a result of the better metabolic stability of NE vs. 2-AG (34). It should be noted that nontreated KO mice (vehicle controls in Fig. 5 B, D, F, and H) display a lower motility than WT mice (vehicle controls in Fig. 5 A, C, E, and G; P < 0.0001).

As 2-AG acts via the β2 subunit, we studied β2 KO mice. These mice displayed a very pronounced hypermotility (P < 0.0001; Fig. 6A), which is in agreement with a previous report (35). This may indicate the involvement of a tonic activation of β2 subunit-containing GABA_A receptors by 2-AG in WT C57BL/6j/129SvEv mice. When 2-AG levels were increased by JZL184 and CB1 receptors simultaneously blocked by low concentrations of the CB1 antagonist SR141716 at which GABA_A receptors were not affected (36), WT mice showed hypomotility, whereas the β2 KO mice showed hypermotility during the habituation phase (Fig. 6B). This clearly indicates that physiological concentrations of 2-AG (as modulated by JZL184) can exert hypolocomotion in these mice via both β2 and CB1 receptors. However, treatment of β2 KO mice with NE (10 mg·kg⁻¹ i.v.) and SR141716 (3 mg·kg⁻¹ i.v.) likewise induced strong hypermotility (SI Appendix, Fig. S7), thus indicating additional receptors for NE.

Discussion

In addition to the well known retrograde GABAAergic signaling of endocannabinoids mediated via the CB1 receptor at central synapses (19), we show an unexpected direct molecular interaction between 2-AG and GABA_A receptors. 2-AG is an endogenous ester formed from the omega-6 fatty acid AA and glycerol by different biosynthetic pathways (16). 2-AG, unlike AEA, is present at high levels in the central nervous system; it is the most abundant molecular species of monacylglycerol found in mouse and rat brain (~5–10 μmol/kg tissue) (16). Our finding that elevated 2-AG levels upon JZL184 treatment inversely correlate with free AA levels is consistent with a previous study (32) and clearly indicates that 2-AG is a major AA metabolite in mouse brain. AEA levels in brain are generally low (up to 0.1 μmol/kg wet weight) (17), and at 1 μM of AEA, only minor and irrelevant effects on GABA_A receptors were detected in this study. This is in line with our previous observation that AEA fails to modulate motility in Cnr1−/−/Cnr2−/− double-KO mice (37) even though it inhibits locomotion in CB1 KO mice (38). In this study, NE produced stronger pharmacological effects in Cnr1−/−/Cnr2−/− double-KO mice than 2-AG, which may be explained by its better metabolic stability (34) or additional receptor targets. The latter is also indicated by the fact that, in β2 KO mice, NE showed similar effects as in WT mice in the presence of CB1 receptor blockade by SR141716 (SI Appendix, Fig. S7). In agreement with a previous study (39), we were not able to detect NE in mouse brain. Other groups have reported only low levels (in nmol/kg) of NE in rat brain (40). This suggests that NE is not a major AA brain metabolite, and physiological interactions with GABA_A receptors are therefore rather unlikely.

The superadditivity between the effects of THDOC and 2-AG at GABA_A receptors is intriguing and may suggest that 2-AG can modulate the action of neurosteroids at GABA_A receptors. A study showing that 2-AG distribution in rat brain does not match the CB1 receptor sites describes high amounts of 2-AG in brainstem and hippocampus (41). We also explored the superadditivity of 2-AG and THDOC in animal experiments to obtain additional evidence for the physiological relevance of our finding. Low concentrations of THDOC in combination with partially elevated 2-AG levels or low concentrations of NE administered i.v. resulted in significant superadditive inhibition of locomotion both in WT and KO mice. Intriguingly, β2 KO mice showed an overall pronounced hyperlocomotion compared with WT mice, indicating a role for β2 in 2-AG-mediated locomotion behavior.

Blocking of 2-AG degradation in the presence of SR141716 in β2 KO mice resulted in hyperlocomotion during the habituation phase, which was not observed in WT mice. On the contrary, WT mice showed inhibition of locomotion during this phase, which is in agreement with the hypothesis that 2-AG can mediate behavioral effects also via β2. Nevertheless, the expected lack of locomotion inhibition by 2-AG in the later phase in β2 KO mice was not observed. Although our data with β2 KO mice demonstrate the involvement of β2-containing GABA_A receptors during locomotion habituation, the overall strong hypolocomotion induced by NE in the β2 KO mice suggests the involvement of additional receptors, possibly from the cys-loop family.

2-AG is biosynthetically generated in postsynaptic neurons directly from constituents of the cell membrane where GABA_A receptors are located (42). Given that 2-AG is synthesized in the membrane and cannot easily cross the aqueous barrier, it is tempting to speculate that 2-AG accumulates and interacts locally with GABA_A receptors within the postsynaptic neuron (Fig. 7). Based on our finding that 2-AG acts exclusively at low concentrations of GABA_A and that it synergizes with neurosteroids, it is likely that extrasympathetic GABA_A receptors may be preferentially targeted by 2-AG. As it has been shown in hippocampus, not all synapses are silenced by 2-AG to the same degree, and residual synaptic activity, especially in dendritic synapses, may

Fig. 6. Hypermotility of β2-KO mice and effects of JZL184 and SR141716. The spontaneous locomotor activity was determined before (A) and after (B) treatment with JZL184 (16 mg·kg⁻¹ i.v.) plus SR141716 (3 mg·kg⁻¹ i.v.) in WT (open circles) and β2-KO mice (open squares). Data are shown as the mean distance traveled in 1-min time bins ± SEM (n = 6 each).

Fig. 7. Hypothetical scheme showing how newly synthesized 2-AG may diffuse laterally and act in the postsynaptic membrane at extrasympathetic and, to some degree, also at synaptic β2 subunit-containing GABA_A receptors. Only after diffusion or transport to the presynaptic membrane, 2-AG inhibits the release of GABA upon activation of CB1 receptors.
additionally underlie modulation by 2-AG (43), at least during the late phase of the inhibitory postsynaptic current. Fig. 7 summarizes these possible actions of 2-AG. The fact that modulation of GABA_A receptors by 2-AG is exclusively observed at low concentrations of GABA may be the reason why we were not able to establish conditions to measure robust effects of 2-AG in brain slice experiments.

In this study, we have identified the AAR V436 and F439 in the inner membrane leaflet of the M4 helix of the β2 subunit as the molecular site of action of 2-AG. The discovery of a modulatory site for 2-AG on a specific set of GABA_A receptor subtypes adds another level of complexity to endocannabinoid and GABA action and provides important insight into their molecular mechanisms.

Materials and Methods

Materials. 2-AG was obtained from Cayman. According to GC-MS analyses, 2-AG was 92% pure. The remaining 8% was determined as the spontaneous breakdown product 1(3)-AG. 2-AG was prepared as a 10-mM stock solution in DMSO and was dissolved in the experimental solutions, resulting in a maximal final DMSO concentration of 0.1%. 1-AG/3-AG, AEA, t-AEA, oleamide, and DEA were obtained from Cayman and were at least 95% pure. THC was obtained from THC Pharm (>95% purity). THDOC was purchased from Fluka.

Electrophysiological Experiments. Preparation of RNA, isolation of Xenopus oocytes, culturing of the oocytes, injection of cRNA, defolliculation, and two-electrode voltage-clamp measurements were performed as described earlier (44). 2-AG was preapplied for 30 s. Relative current potentiation by 2-AG was determined as \( \frac{I_{2-AG} \times \text{GABA}_\text{max}}{I_{\text{GABA}} 	imes \text{GABA}_\text{max}} \times 100\% \). Unless indicated otherwise, potentiation by 2-AG was determined at all receptors at a similar relative GABA concentration in the each receptor form. Concentration response curves were fitted with the following equation:

\[
P(c) = \max(1-(c/EC_{50})^n)
\]

where P is the current potentiation, c, the concentration of 2-AG, max is the maximal current potentiation, the EC_{50} is the concentration of 2-AG at which half-maximal potentiation was observed, and n is the Hill coefficient. The perfusion system was cleaned between two experiments by washing with 100% DMSO after application of 2-AG or THDOC to avoid contamination.

Behavioral Experiments and Quantification of 2-AG and AA. Methods of behavioral experiments and quantification of 2-AG and AA are provided in the SI Appendix.

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