Supplemental Material

Neurosteroid access to the GABA-A receptor

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1. Supplemental Table 1. Full details for data shown in Table 1.

Configuration	Preincubation	Pipette	Bath	OT1 (ms)	fr OT1	OT2 (ms)	fr OT2	OT3 (ms)	fr OT3	N
cell-attached	-	50 μM GABA	-	0.28±0.07	0.24±0.04	3.1±0.8	0.58±0.10	7.6±3.0	0.19±0.13	8
inside-out	-	50 μM GABA	-	0.32±0.11	0.31±0.06	2.4±0.4	0.47±0.14	5.9±1.2	0.22±0.13	4
cell-attached	-	50 μM GABA + 1 μM ACN	-	0.32±0.10 (NS: 1.0)	0.34±0.11 (NS: 0.2)	2.0±0.6 (NS: 0.8)	0.25±0.03 ***	19.8±3.4 *	0.42±0.08 *	3
cell-attached	-	50 μM GABA	1 μM ACN	0.40±0.13 (NS: 0.2)	0.37±0.06 *	5.5±3.4 (NS: 0.1)	0.28±0.06 ***	32.3±7.9 ***	0.35±0.12 (NS: 0.1)	4
cell-attached	1 μM ACN	50 μM GABA	-	0.37±0.09 (NS: 0.2)	0.28±0.09 (NS: 0.7)	2.4±1.2 (NS: 0.9)	0.27±0.09 ***	24.0±7.7 ***	0.46±0.08 ***	7
inside-out	1 μM ACN	50 μM GABA	-	0.35±0.06 (NS: 1.0)	0.36±0.07 (NS: 0.6)	4.6±2.8 (NS: 0.5)	0.27±0.05 (NS: 0.1)	15.5±1.9 **	0.37±0.10 (NS: 0.2)	5
inside-out	1 μM ACN	50 μM GABA	5 mM CDX	0.38±0.06 (NS: 0.6)	0.31±0.08 (NS: 1.0)	3.5±0.6 (NS: 0.9)	0.50±0.14 (NS: 1.0)	6.5±1.5 (NS: 1.0)	0.18±0.09 (NS: 1.0)	4
inside-out	-	50 μM GABA	1 μM ACN	0.44±0.07 (NS: 0.1)	0.41±0.06 (NS: 0.1)	9.3±4.1 **	0.30±0.18 (NS: 0.2)	19.4±6.1 **	0.29±0.14 (NS: 0.8)	4
cell-attached	-	50 μM GABA + 1 μM Alexa-3a5aP	-	0.33±0.08 (NS: 0.7)	0.25±0.06 (NS: 1.0)	2.7±1.0 (NS: 1.0)	0.59±0.14 (NS: 1.0)	8.5±2.9 (NS: 1.0)	0.16±0.13 (NS: 1.0)	7
inside-out	-	50 μM GABA	1 μM Alexa-3α5αP	0.36±0.05 (NS: 0.9)	0.34±0.03 (NS: 0.8)	3.3±1.3 (NS: 1.0)	0.17±0.07 **	15.2±5.7 *	0.49±0.07 **	4
cell-attached	-	50 μM GABA + 10 μM NBD-3α5αP	-	0.30±0.08 (NS: 1.0)	0.34±0.06 (NS: 0.1)	4.9±2.0 (NS: 0.2)	0.29±0.08 ***	21.3±8.4 **	0.38±0.11 *	5

Table 1A. The results from full kinetic analysis of data from the experimental conditions shown in Table 1. The durations (OT1-3) and fractions (fr OT1-3) of the open time components are given under different conditions. Statistical analysis was carried out using ANOVA with pairwise comparison to control group with two-tailed Dunnet's correction (Systat 7.0, SSPS, Chicago, IL). Symbols: * denotes significance at P<0.05, ** is for P<0.01 and *** is for P<0.001, NS is nonsignificant. CDX is methyl-β-cyclodextrin. Two control groups were used; for comparison to cell-attached records the data obtained with 50 μM GABA for cell attached records (line 1) was used, while analogous data for excised patches (line 2) was used as control for data from excised patches.

Configuration	Preincubation	Pipette	Bath	CT1 (ms)	fr CT1	CT2 (ms)	fr CT2	CT3 (ms)	fr CT3	N
cell-attached	-	50 μM GABA	-	0.17±0.02	0.57±0.06	1.7±0.7	0.14±0.05	13.5±5.3	0.29±0.02	8
inside-out	-	50 μM GABA	-	0.22±0.06	0.57±0.02	2.8±1.8	0.21±0.08	11.0±2.7	0.21±0.10	4
cell-attached	-	50 μM GABA + 1 μM ACN	-	0.17±0.02 (NS: 1.0)	0.62±0.06 (NS: 0.9)	1.5±0.1 (NS: 1.0)	0.32±0.04 **	19.7±5.6 (NS: 0.1)	0.06±0.03 ***	3
cell-attached	-	50 μM GABA	1 μM ACN	0.21±0.11 (NS: 0.6)	0.70±0.09 (NS: 0.1)	2.1±1.5 (NS: 0.9)	0.24±0.07 (NS: 0.1)	27.0±4.0 ***	0.06±0.02 ***	4
cell-attached	1 μM ACN	50 μM GABA	-	0.21±0.07 (NS: 0.4)	0.69±0.06 *	1.7±0.7 (NS: 1.0)	0.23±0.04 (NS: 0.1)	19.8±3.1 *	0.08±0.03 ***	7
inside-out	1 μM ACN	50 μM GABA	-	0.18±0.06 (NS: 0.9)	0.53±0.12 (NS: 0.7)	1.2±0.4 (NS: 0.2)	0.36±0.10 *	14.1±2.7 (NS: 0.2)	0.12±0.02 *	5
inside-out	1 μM ACN	50 μM GABA	5 mM CDX	0.17±0.03 (NS: 0.7)	0.55±0.06 (NS: 1.0)	2.7±2.2 (NS: 1.0)	0.17±0.08 (NS: 0.9)	11.0±1.7 (NS: 1.0)	0.28±0.05 (NS: 0.2)	4
inside-out	-	50 μM GABA	1 μM ACN	0.38±0.10 *	0.60±0.03 (NS: 1.0)	2.1±0.4 (NS: 0.9)	0.30±0.04 (NS: 0.3)	12.8±1.6 (NS: 0.7)	0.10±0.01 *	4
cell-attached	-	50 μM GABA + 1 μM Alexa-3α5αP	-	0.18±0.04 (NS: 1.0)	0.56±0.09 (NS: 1.0)	2.0±1.4 (NS: 1.0)	0.21±0.09 (NS: 0.3)	13.0±2.3 (NS: 1.0)	0.23±0.03 *	7
inside-out	-	50 μM GABA	1 μM Alexa-3α5αP	0.25±0.09 (NS: 0.9)	0.55±0.04 (NS: 1.0)	1.5±0.4 (NS: 0.5)	0.36±0.05 *	13.9±2.9 (NS: 0.3)	0.09±0.01 **	4
cell-attached	-	50 μM GABA + 10 μM NBD-3α5αP	-	0.20±0.04 (NS: 0.8)	0.60±0.15 (NS: 1.0)	1.7±0.4 (NS: 1.0)	0.28±0.10 **	11.9±2.7 (NS: 0.9)	0.13±0.07 ***	5

Table 1B. The results from full kinetic analysis of data from the experimental conditions shown in Table 1. The durations (CT1-3) and fractions (fr CT1-3) of the closed time components are given under different conditions. Statistical analysis was carried out using ANOVA with pairwise comparison to control group with two-tailed Dunnet's correction (Systat 7.0, SSPS, Chicago, IL). Symbols: * denotes significance at P<0.05, ** is for P<0.01 and *** is for P<0.001, NS is nonsignificant. CDX is methyl-β-cyclodextrin. Two control groups were used; for comparison to cell-attached records the data obtained with 50 μM GABA for cell attached records (line 1) was used, while analogous data for excised patches (line 2) was used as control for data from excised patches.

2. Membrane pre-loading: other steroids and concentration dependence

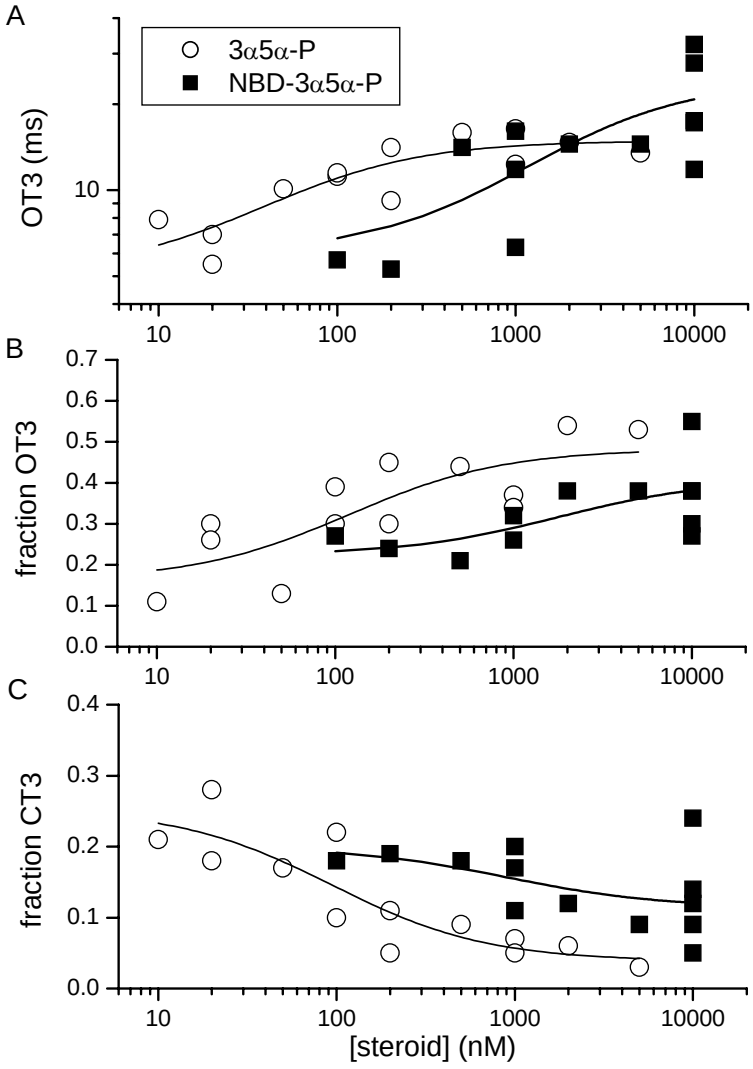
The data presented in the manuscript demonstrate that membrane-accumulated ACN can act on the GABA-A receptor potentiating its activation by GABA. We investigated whether structurally unrelated steroids can have similar effects. We first exposed the cells to (3α,5β,17β)-3-hydroxy-18-norandrostance-17-carbonitrile (B285). When applied in the pipette solution to the extracellular side of the membrane, the dose-response curves for this steroid exhibit widely different EC50-s for effects mediated through Sites A and B (Akk et al., 2004). For example, 1 μM B285 saturates the effects mediated through Site A (fraction OT3 and fraction CT3) but has practically no effect on the duration of OT3 (Site B effect).

Cells pre-incubated with 1 μM B285 and patched with 50 μM GABA following steroid washout from the bath also demonstrate steroid effects on fraction OT3 and fraction CT3 but a relatively minor effect on the duration of OT3. The fraction of OT3 increased to 0.27±0.06 (N = 3 patches) and the fraction of CT3 decreased to 0.15±0.03. In contrast the duration of OT3 increased only slightly to 10.0±2.0 ms. These findings suggest that B285 similarly affects receptor function when applied in the pipette solution to the extracellular side of the cell membrane or through membrane loading.

As a negative control, we examined whether pre-incubation with 3β5αP affects receptor currents elicited by GABA. This steroid is known to be inactive when applied from the extracellular side or inhibits responses to GABA at higher concentrations (Wang et al., 2002). In our hands, pre-incubation with 1 μM 3β5αP did not affect subsequent channel activation elicited by 50 μM GABA (data not shown).

We also examined whether steroid applied through pre-incubation exhibits concentration dependence in its effects. To do so, we pre-incubated cells expressing GABA-A receptors in 10 nM ACN. The experimental protocol was the same as what is described in the manuscript for 1 μM ACN pre-incubation. When co-applied with GABA in the pipette solution, 10 nM ACN does not affect single-channel currents (Akk and Steinbach, 2003). Similarly, our present data show that pre-incubation of cells in 10 nM ACN did not affect currents recorded following the washout of steroid. The mean duration of OT3 was 5.2±1.3 ms (N = 3 patches), the fraction of OT3 was 0.34±0.21 and the fraction of CT3 0.23±0.20.

3. Supplemental Figure 1. Kinetic analysis of the effects of 3α5αP and NBD-3α5αP. The effects of 3α5αP (open circles) and NBD-3α5αP (filled squares) on the duration of OT3 (A), the fraction of OT3 (B) and the fraction of CT3 (C). Each point represents data from one patch. The curves were fitted using: $Y([steroid])=Y_0 + (Y_{max} - Y_0)$ $[steroid]/([steroid] + EC_{50})$. (A) The best-fit parameters for 3α5αP were: Y_0=5.2±2.3 ms, Y_{max}=14.9±1.1 ms, EC_{50}=66.2±54.3 nM, for NBD-3α5αP: Y_0=6.1±5.4 ms, Y_{max}=24.3±7.0 ms, EC_{50}=2.3±4.1 μM. (B) The best-fit parameters for 3α5αP were: Y_0=0.16± 0.08, Y_{max}=0.48±0.06, EC_{50}=119±125 nM, for NBD-3α5αP: Y_0=0.22±0.07, Y_{max}=0.41±0.07, EC_{50}=1.8±3.3 μM. (C) The best-fit parameters for 3α5αP were: Y_0=0.25± 0.04, Y_{max}=0.04±0.03, EC_{50}=94±73 nM, for NBD-3α5αP: Y_0=0.20±0.06, Y_{max}=0.11±0.03, EC_{50}=0.9±2.2 μM.



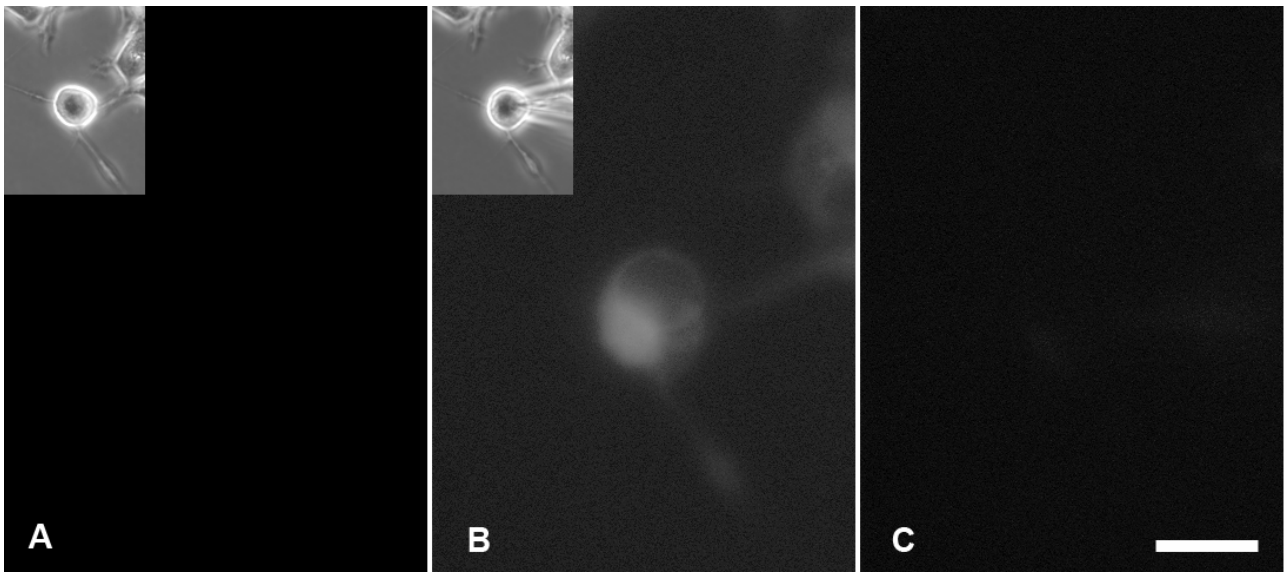
4. Supplemental Table 2: Direct gating by pre-incubation

Our data demonstrate that pre-incubation of cells with 1 μM ACN and subsequent washout of bath steroid results in channel activity even when the cells are patched with no active drug in the patch pipette. Such channel currents have a single-channel conductance similar to what we observe when α1β2γ2-expressing cells are exposed to GABAergic agonists (data not shown). The channel openings have characteristics (number of components, durations and fractions) which are similar to those observed when α1β2γ2 receptors are exposed to ACN through the patch pipette medium (Table 1) leading us to conclude that the openings arise from GABA-A receptors activated by membrane-accumulated steroid. This suggests that similar to the sites responsible for potentiation of the GABA-A receptor, the sites involved in direct gating are accessible from the cell membrane. These data do not address the question whether the same site is responsible for both potentiation and direct gating.

Preincubation	Pipette	OT1 (ms)	fraction OT1	OT2 (ms)	fraction OT2	N
1 μM ACN	-	0.24±0.04	0.73±0.14	1.42±0.48	0.27±0.14	3
-	5 μM ACN	0.48±0.27	0.75±0.10	1.39±0.69	0.25±0.10	3

Table 2. The properties of single-channel activity under two experimental conditions. The upper row gives the parameters of open time components observed following pre-incubation of cells with 1 μM ACN for 8 min followed by washout of bath steroid. The cells were then patched without any GABAergic compounds in the pipette solution. The lower row gives the parameters of open time components observed during patching untreated cells with 5 μM ACN in the patch pipette. Due to low open probability, single-channel clusters could not be observed with steroid direct gating. Hence, the analysis of closed time was not carried out.

5. Supplemental Figure 2. Ineffectiveness of whole-cell loading of steroids. **A.** Lack of fluorescence of a target HEK cell before approach with a whole-cell pipette containing 10 μM NBD-3α5αP. The inset shows a phase-contrast photomicrograph of the cell. **B.** With approach of a whole-cell pipette (open tip resistance 2.8 MΩ, with positive pressure maintained), the target cell and surrounding bath become detectably fluorescent. The inset shows the phase-contrast view, with the whole-cell pipette present. **C.** Image taken after 3 min of active washing with extracellular saline flow with pipette sealed to the cell but before membrane rupture, followed by 10 min of dialysis of the cell interior in the whole-cell mode. Although the cell was still viable (not shown), there was no detectable accumulation of fluorescent steroid in the intracellular compartment. Similar results were obtained in 3 neurons. Scale bar indicates 20 μm.



6. Supplemental Figure 3. Wash-dependent loss of steroid fluorescence. Cells were pre-incubated in 10 μM NBD-3α5αP for 10 min, followed by 5 complete bath exchanges with saline solution. **A1.** The first 4 images were collected every 10 s in static bath, the condition in which patch experiments were performed in the present studies. There was little loss of fluorescence. **A2.** The next 4 images were obtained immediately following switching on local perfusion, used in whole-cell experiments. Images are pseudocolored for fluorescence intensity, with hot colors representing high intensity and cool colors representing low fluorescence intensity. **B.** Summary of results from 7 cells. Intracellular fluorescence was measured near the nucleus. The slow decay of ~10% before perfusion (arrow) represents the combination of diffusion of steroid out of the cell into the saline bath and perhaps some degree of fluorescence bleaching. Local solution flow speeded steroid removal. Solid lines are linear regressions through the indicated points.