Supplementary information, Data S1

Methods and Materials

Cloning

The human α5 and β3 $GABA_A$ receptor genes (NCBI Reference Sequence: NM_000810.3 and NM_000814.5) were cloned into pEGBacMam vector containing either His_{10}-tag or Twin-Strep-tag at the C-terminus. Deletion constructs used for expression and structural analysis lack a large portion of the intracellular loop connecting TM3 and TM4. Residues from Arg347 to Ser423 in the α5 subunit, and that from Gly333 to Asn446 in the β3 subunit, were replaced with the short amino acid sequence SQPARAA, as previously described [1,2].

Expression and purification

The bacmids and baculoviruses were generated using standard methods. The P2 viruses of the same MOIs for the α5 and β3 subunits were used to infect HEK293S-GnTI$^-$ cell at 37 °C with 10 mM sodium butyrate. Cells were collected after ~72h post-infection by centrifugation, resuspended in buffer A (20 mM HEPES-NaOH, pH 7.4, 300 mM NaCl), and disrupted by sonication. The membranes were pelleted from the supernatant by centrifugation for 1 h at 48000*g*. Membrane pellets were mechanically homogenized and solubilized in buffer A supplemented with 1% (w/v) lauryl maltose neopentyl glycol (LMNG, Anatrace) and 0.1% (w/v) cholesteryl hemisuccinate (CHS, Sigma Aldrich), and by gentle agitation for 3 h on ice. Non-solubilized material was removed by ultracentrifugation (48000*g* and 40 min). The supernatant was incubated with Strep-

Tactin Sepharose (IBA Lifesciences) for 1 h with gentle rotation at 4°C. The resin was washed in buffer B (buffer A+0.006% LMNG+0.0006% CHS) and eluted in the same buffer containing 10 mM D-desthiobiotin (Sigma-Aldrich). The protein was further loaded onto a column containing 1 ml of the Co^{2+} affinity resin and eluted by 250 mM imidazole in buffer B. The nanobody Nb25, expressed and purified as described below, was added to the heteromeric α5β3 $GABA_A$ receptor at ten-fold molar excess and the complex were further purified by size-exclusion chromatography (SEC) in buffer C (20 mM HEPES-NaOH, pH 7.4, 100 mM NaCl, 0.006% LMNG and 0.0006% CHS,1 mM GABA). Fractions containing the receptor were pooled and concentrated to 2mg/ml.

Nanobody purification

The nanobody Nb25, was cloned into plasmid pET21a with an N-terminal PelB signal peptide and a C-terminal His_6-tag. The protein was expressed and purified from *E. coli*. Cultures were grown at 37 °C until their OD_{600} reached 0.6-0.8, at which point expression was induced with 1 mM IPTG. After induction, cells were grown at 28 °C overnight and harvested by centrifugation (20 min, 4,000 g). Nanobodies were released from the bacterial periplasm, purified using nickel affinity chromatography, and then subjected to size-exclusion chromatography on a Superdex 75 16/600 column (GE Healthcare) in buffer A.

Cryo-EM Sample preparation

2.5 μl samples of purified protein were applied to glow-discharged holey carbon grids

(Quantifoil R1.2/1.3 Au 200 mesh). Excess liquid was removed in a controlled environment (4 °C and 100% relative humidity) by blotting grids for 3s (with a blotting force of -15), then plunge-frozen into liquid ethane cooled by liquid nitrogen using a Vitrobot Mark IV (FEI).

Cryo-EM image collection and processing

CryoEM images were recorded on a Gatan K2 Summit direct electron detector in an FEI Titan Krios electron microscope (FEI) equipped with GIF quantum energy filter (Gatan) operated at 300 kV. Leginon [3] was used to semi-automatically record zero energy-loss (20 eV slit) image frames at a nominal magnification of 130,000 x (calibrated pixel size: 1.07Å/pixel on the sample) in counting mode. Images were recorded as movies consisting of 40 frames with total dose of 56 e-/Å^2 and an exposure of 8s. Preset defocus values ranged from 1.4 to 2 µm.

Individual movie frames were aligned, dose-weighted and summed using MotionCor2 [4]. Defocus and astigmatism parameters were estimated using Gctf [5]. Particle picking was performed in Gautomatch using projections from low pass filtered structure previously collected in-house. Total 699,673 particles were extracted from 3,724 micrographs using Relion 2.1 [6]. All subsequent image processing was carried out in cryoSPARC [7]. A set of 434,120 particles after 2D classification was selected to perform downstream image processing. 4 previous determined $GABA_A$ receptor structures with different number of nanobodies were used as initial models in the Heterogeneous refinement in cryoSparc. A set with the highest reported resolution was chosen and total 161,455 particles were subject to the final 3D refinement in cryoSPARC (Figure S5).

The final model was refined to an overall resolution of 3.51 Å (Figure S6). This model and the map sharpened by LocScale [8] were used for model building.

Model building and refinement

The atomic models of human $GABA_A$ receptor (PDB ID: 4COF) [1], and nanobody model (PDB ID: 5O8F) [2] and a homology model of α5 subunit generated by submitting the sequence to the swiss-model website [9] were fitted into the 3D density maps using UCSF Chimera [10] and used as initial models for atomic model building. Individual amino acid residues of each initial model were then manually adjusted in Coot [11], guided by well resolved features of side chain densities. Models were optimized with Phenix [12] and re-adjusted in Coot, iteratively, until no further improvement in model geometry could be obtained.

Reference:

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Supplementary Table and Figures:

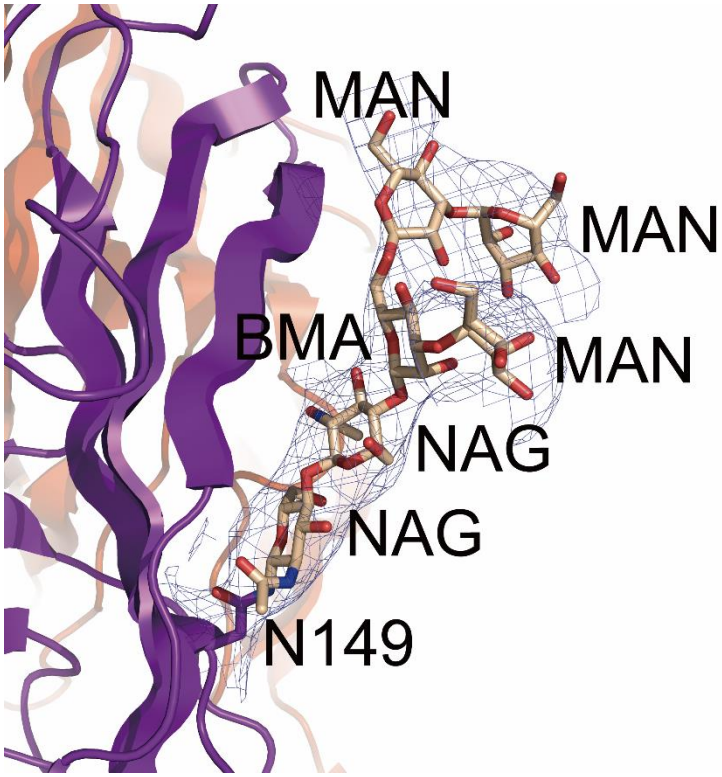

Figure S1. Cryo-EM map at 3σ contour level showing the N-linked glycan at Asn 149 of β subunit.

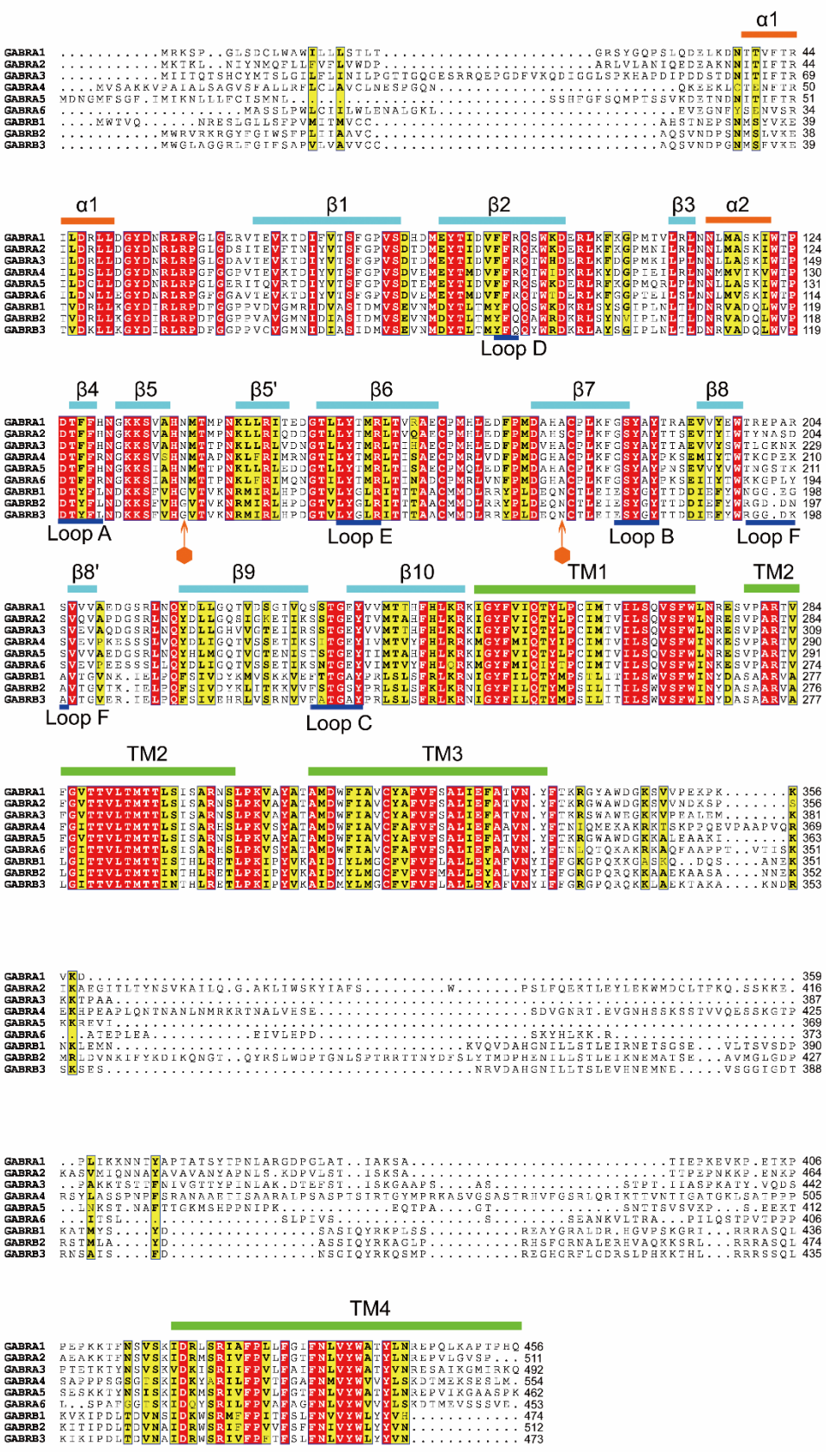

Figure S2. Sequence alignment of all α and β subunits of human $GABA_A$ receptor. Secondary structure elements were labeled.

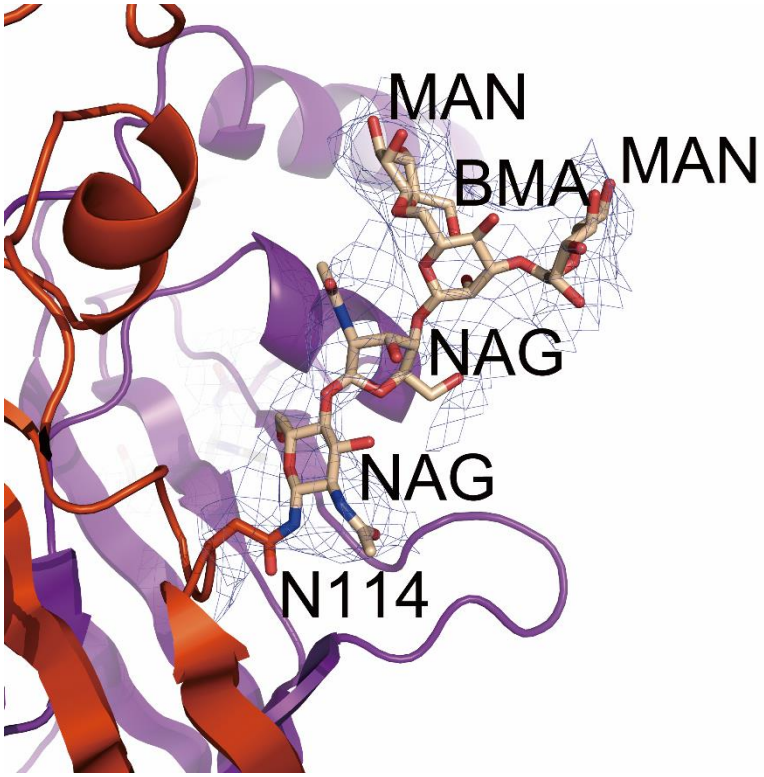

Figure S3. Cryo-EM map at 3σ contour level showing the N-linked glycan at Asn 114 of α subunit.

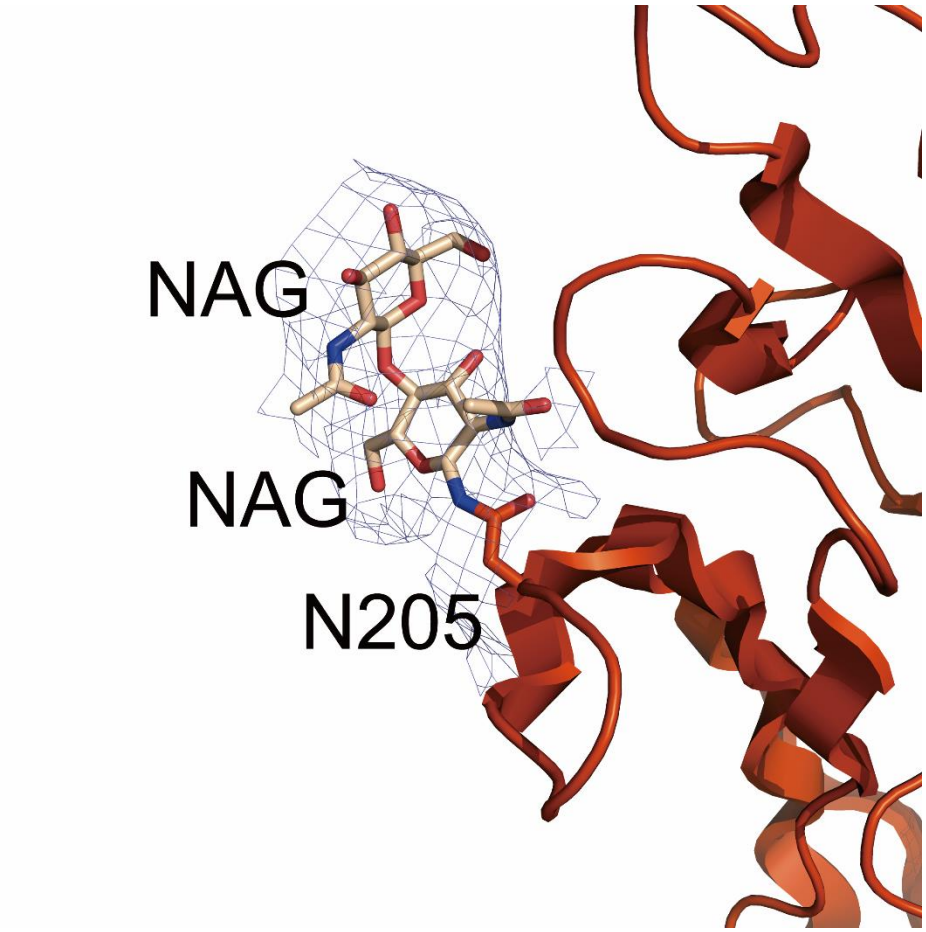

Figure S4. Cryo-EM map at 3σ contour level showing the N-linked glycan at Asn 205 of α subunit.

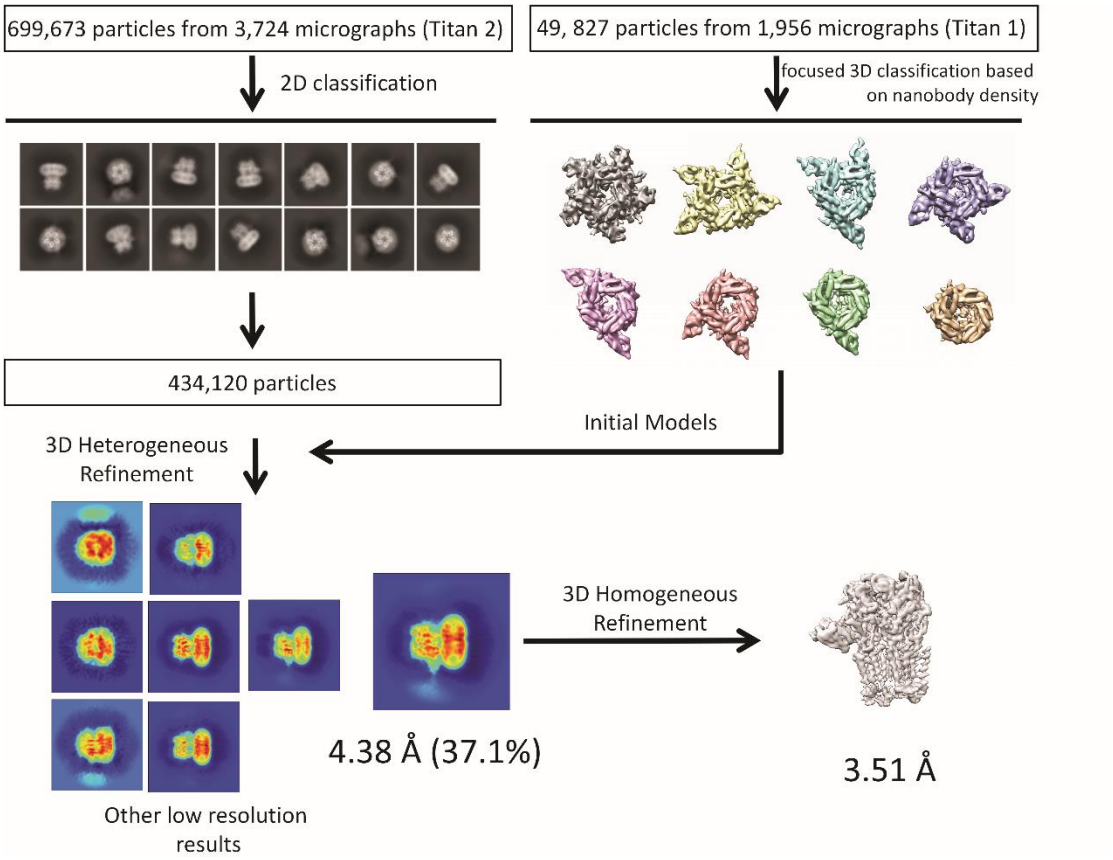

Figure S5. Flow chart of cryo-EM data processing

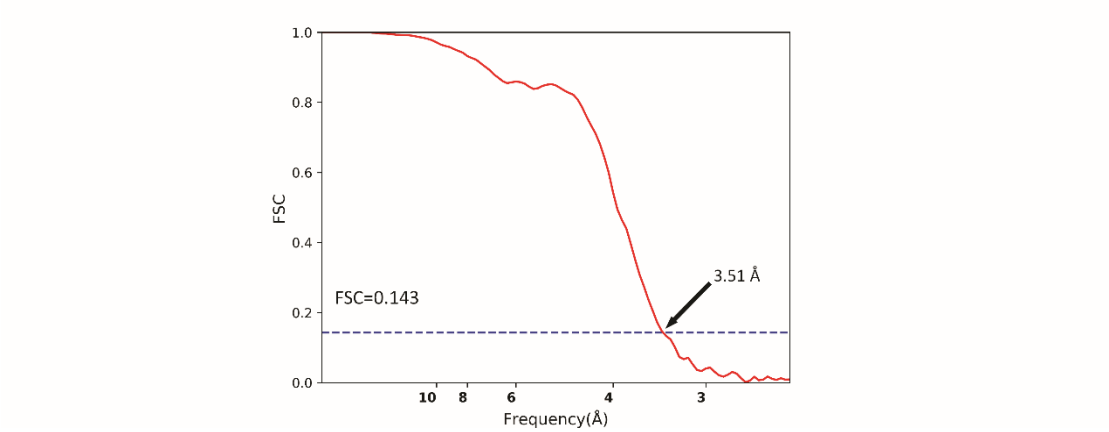

Figure S6. “Gold-standard” FSC coefficient curve of the final reconstruction