Nicotinic α5 subunits drive developmental changes in the activation and morphology of prefrontal cortex layer VI neurons

Supplemental Information

Supplemental Methods and Materials

Brain slice preparation

Coronal slices 400 µm thick of the medial prefrontal cortex (mPFC) were prepared from mice at four distinct postnatal developmental ages: week 2 (P11-13), week 3 (P14-20), week 4 (P21-27) and young adulthood (P60-115). Mice were sacrificed by decapitation under chloral hydrate anesthesia (approximately 1 g/kg) administered i.p. in a 16% (w/v) solution. Brains were excised as rapidly as possible and cooled in 4°C oxygenated sucrose artificial cerebrospinal fluid (ACSF) (254 mM sucrose, 10 mM D-glucose, 24 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 3 mM KCl, 1.25 mM NaH_2PO_4, pH 7.4). Prefrontal slices were cut using a Dosaka Linear Slicer (SciMedia, Irvine, California) from anterior to posterior using the appearance of white matter and the corpus callosum as anterior and posterior guides (1,2). Slices were transferred to oxygenated ACSF (composition listed above except 128 mM NaCl was substituted for sucrose) maintained at 30°C in a prechamber (Automate Scientific, Berkeley, California) and allowed to recover for at least 2 hr before the beginning of an experiment. For whole-cell recording, slices were placed in a modified chamber (Warner Instruments, Hamden, Connecticut) mounted on the stage of either an Olympus BX50WI or Olympus FV1000 microscope (Olympus, Markham, Ontario). Regular ACSF was bubbled with 95% oxygen and 5% carbon dioxide and flowed over the slice at room temperature at a rate of 3-4 mL/min.

Electrophysiology

Whole-cell patch electrodes (2–4 MΩ) contained 120 mM K-gluconate, 5 mM KCl, 2 mM $MgCl_2$, 4 mM K_2-ATP, 400 µM Na_2-GTP, 10 mM Na_2-phosphocreatine and 10 mM HEPES buffer

(adjusted to pH 7.3 with KOH). mPFC layer VI pyramidal neurons were patched under visual control using infrared differential interference contrast microscopy. All recordings were made using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, California), acquired at 20 kHz and lowpass filtered at 2 kHz using a Digidata 1440A data acquisition system (Molecular Devices), and corrected for the liquid junction potential. Patched neurons were first recorded in current clamp mode at their resting membrane potential. Membrane potential responses to positive and negative current steps were recorded in order to measure inherent electrophysiological properties. Experiments were not performed on fast-spiking interneurons or on burst-firing neurons because they have been demonstrated to respond predominantly to indirect effects of nicotinic stimulation (3).

Neurons were held at -75 mV under voltage clamp mode. All experiments were performed in the presence of atropine (200 nM, continuous application) in the ACSF bath to block the muscarinic subtype of acetylcholine (ACh) receptors. ACh was bath-applied to the slice in oxygenated ACSF after a stable baseline period of at least one minute, and peak inward current responses were measured using Clampfit software (Molecular Devices) by subtracting the mean inward current at the peak of the response from the mean holding current at baseline. ACh was allowed to wash out of the slices for at least five minutes before reapplication. Atropine and ACh chloride were purchased from Sigma-Aldrich (Oakville, Ontario) and stored in stock solutions at -20°C before being diluted and applied to the slice.

Immunohistochemistry

Immunohistochemistry was performed for the exogenous yellow fluorescent protein (YFP) motif in the mice containing α4-YFP nicotinic acetylcholine receptor (nAChR) subunits in order to avoid potential problems with antibody specificity that may occur when targeting endogenous nAChR subunits (4). A detailed protocol for immunohistochemical labeling, visualization and analysis of neurons in the α4-YFP mice has been described previously (5).

Briefly, brain slices containing the mPFC were collected as described above for electrophysiology experiments and fixed in 4% (w/v) paraformaldehyde. A three-step immunohistochemical protocol was employed using a primary antibody targeting GFP (Invitrogen, Carlsbad, California; which also recognizes YFP), a biotin-conjugated secondary antibody (Invitrogen), and streptavidin Alexa Fluor 594 (Invitrogen). Slices were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) to identify nuclei. Multiphoton imaging of the immunostained sections was performed at a consistent depth in the Z-axis using a Ti:sapphire laser tuned to wavelength 780 nm to collect overlapping images throughout the mPFC that were then stitched together to create a montage of the mPFC for each mouse. Images were then analyzed to examine the overall morphology of the mPFC and also to identify the localization of neurons expressing α4* nAChRs within the mPFC of each mouse.

Supplemental References

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4. Moser N, Mechawar N, Jones I, Gochberg-Sarver A, Orr-Urtreger A, Plomann M, *et al.* (2007): Evaluating the suitability of nicotinic acetylcholine receptor antibodies for standard immunodetection procedures. *J Neurochem* 102:479-492.
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