Summary

Characterization of inhibitory connections originating from CCK/CB1-positive interneurons in mouse primary visual cortex

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Introduction

In the cerebral cortex, there are various subtypes of inhibitory interneurons present. Perisomatic inhibition is mediated by two classes of interneurons that express either the neuropeptide cholecystokinin (CCK) or the calcium-binding protein parvalbumin (PV). Inhibitory synaptic connections originating from PV interneurons are strengthened after eye-opening during postnatal development in the primary visual cortex (V1), leading to the opening of critical periods of experience-dependent plasticity. It has been reported that CCK-positive interneurons express cannabinoid receptor type 1 (CB1), which plays an important role in the development and plasticity of V1. However, functional synaptic properties due to the CCK/CB1-positive interneuron subtype in V1 and their developmental process remain unclear.

In this thesis, the proportion of interneurons expressing CCK and CB1 in each layer of mouse V1 were first examined by immunostaining for proCCK, a precursor of CCK, and CB1 using VGAT-Venus transgenic mice that had all interneurons labeled by Venus. The percentage of proCCK/CB1 double-positive cells were less than 5% in all layers at two different ages (postnatal 12–13 days, [P12–13] and P21). Additionally, some percentage of interneurons were proCCK-negative but CB1-positive P12–13, while these interneurons were almost undetectable at P21. These immunostaining results demonstrated that the proportion of CCK/CB1-positive interneurons was quite small in V1.

Materials and methods

All animal experiments were approved by the Animal Research Committee of the National Institutes of Natural Sciences.

Animals

To visualize GABAergic interneurons, VGAT-Venus mice (Wang et al., 2009) were used. To visualize CCK-expressing interneurons, CCK-IRES-Cre; Dlx5/6-Flpe; RCE-dual mice were generated by crossing CCK-IRES-Cre; Dlx5/6-Flpe mice with RCE-dual mice (Miyoshi et al., 2010; Taniguchi et al., 2011). RCE-dual mice were maintained without CCK-IRES-Cre or Dlx5/6-Flpe gene to avoid the possibility of recombination in germline cells (Luo et al., 2020). In one part of the histological experiments, wild-type mice (C57BL/6J) were used. Mice were housed on a 12-h light/dark cycle. Mice of either sex were used at postnatal 10–28 days (P10–28).

Slice preparations

All mice were euthanized with isoflurane and the brains were rapidly extracted in ice-cold artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 3 mM KCl, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose, saturated with 95% O_2 and 5% CO_2. The 300 μm thick slices of V1 were obtained on a vibrating microslicer (VT1200S; Leica) and recovered in an interface chamber at 25°C for 1 h. The slices were then transferred to a submerged chamber containing ACSF oxygenated with 95% O_2 and 5% CO_2 at 25 ± 1°C.

Electrophysiology

Brain slices were transferred into a submerge-type chamber filled with ACSF on the stage of a BX50WI microscope (Olympus). Fluorescent protein expression and cell shapes were visualized under fluorescent and infrared differential interference contrast optics with 40X, 0.8NA water immersion lens. Fluorescent-expressing cells and pyramidal-

shape cells in layers 2/3–6 were targeted for whole-cell recordings. The distance between the two cell somas was less than 150 μm. Recorded fluorescent-expressing cells were examined for CB1-expression by post hoc immunostaining (see below). CB1-positive cells located in layer 2/3 were analyzed, and some CB1-negative cells in layers 5–6 were included in the analysis. Neurons were recorded from the soma located at least 50 μm below the cut surface of the slice. Patch pipettes (3–6 MΩ) were filled with an internal solution. The internal solution for interneuron recordings contained 130 mM K-gluconate, 8 mM KCl, 1 mM $MgCl_2$, 0.6 mM EGTA, 10 mM HEPES, 3 mM MgATP, 0.5 mM Na_2GTP, 10 mM Na-phosphocreatine, and 0.1% biocytin (pH 7.3 adjusted with KOH). The internal solution for pyramidal neuron recordings contained 130 mM Cs-gluconate, 8 mM CsCl, 1 mM $MgCl_2$, 0.6 mM EGTA, 10 mM HEPES, 3 mM MgATP, 0.5 mM Na_2GTP, 10 mM Na-phosphocreatine, and 0.1% biocytin (pH 7.3, adjusted with CsOH). Firing patterns evoked by depolarizing current injections to interneurons were measured in the current-clamp mode. To analyze inhibitory synaptic connections, the membrane potential of the recorded pyramidal cells was held at the reversal potential of excitatory postsynaptic currents (0 mV). In all paired recordings, the connections between neuron pairs were assessed by applying two brief (2 ms) depolarizing voltage pulses (interval of paired-pulse, 50 ms) to evoke action potentials in interneurons (minimum of 30 trials). All recordings were conducted using a Multiclamp 700B amplifier, and data were analyzed using pClamp9 software (Molecular Devices). Cells with a series resistance of fewer than 30 MΩ were selected for analysis and did not use series resistance compensation.

Drugs

For slice electrophysiology experiments, WIN 55,212-2, CB1 agonist (Cayman Chemical, 10009023) and AM251, CB1 antagonist (Cayman Chemical, 71670) were used.

Histology

Brain slices were fixed overnight in 4% paraformaldehyde at 4°C and then stored in PBS containing 30% sucrose. The slices were frozen and re-sectioned at 50 μm thickness using a microtome and then stored at 4°C in PBS until immunostaining. For immunostaining, free-floating brain sections were incubated in a blocking solution containing 2.5% normal goat serum, 0.1% Triton X-100, 0.2% λ-carrageenan, and 0.1% NaN_3 in PBS at 25 ± 1°C for 1 h. Sections were then incubated overnight at 4°C in a blocking solution containing following primary antibodies: chicken polyclonal anti-GFP antibody (1:1000, Abcam, ab13970), rabbit polyclonal anti-proCCK antibody (1:500, frontier institute, CCK-pro-Rb-Af350), rabbit polyclonal anti-CB1 antibody (1:500, frontier institute CB1-Rb-Af380), guinea pig polyclonal anti-CB1 antibody (1:500, frontier institute CB1-GP-Af530), mouse monoclonal anti-PV antibody (1:1500, Sigma-Aldrich, P3088), rabbit polyclonal anti-Somatostatin-14 antibody (1:1000, Peninsula Laboratories, T-4103), rabbit polyclonal anti-Neuropeptide Y antibody (1:500, Immuno Star, 22940), rat polyclonal anti-Ctip2 antibody (1:500, Abcam, ab18465), and rabbit polyclonal anti-CDP(Cux1) antibody (1:500, Santa CruzBiotechnology, sc-13024). Secondary antibody incubation was followed overnight at 4°C with 0.1% Triton X-100 in PBS containing the following secondary antibodies and dye: AlexaFluor-405-conjugated goat anti-rabbit (1:500, Invitrogen, A-31556), AlexaFluor-488-conjugated goat anti-chicken (1:1500, Invitrogen, A-11039), AlexaFluor-488-conjugated goat anti-rabbit (1:500, Invitrogen, A-

11034), AlexaFluor-568-conjugated goat anti-rabbit (1:500, Invitrogen, A-11011), AlexaFluor-594-conjugated goat anti-guinea pig (1:500, Invitrogen, A-11076), AlexaFluor-647-conjugated goat anti-rat (1:500, Invitrogen, A-21247), AlexaFluor-647-conjugated goat anti-guinea pig (1:500, Invitrogen, A-21450) and/or AlexaFluor-cy5-conjugated goat anti-mouse (1:1500, JacksonImmuno, 115-175-146) in addition to NeuroTrace™ 435/455 Blue Fluorescent Nissl Stain (1:1000, Invitrogen, N21479). For staining of biocytin followed by immunostainings, sections were incubated at 25 ± 1°C for 1.5 h with 0.1% Triton X-100 in PBS containing Alexa Fluor-568-conjugated streptavidin (1:1500, Invitrogen, S11226). Images were obtained using an A1R confocal microscope (Nikon).

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses for cell densities were performed using Student's t test. Statistical analysis for inhibitory connections were performed by Fisher's exact test with Holm correction and Kruskal–Wallis test followed by Dunn's test for comparisons among more than two groups, and Wilcoxon matched pairs signed rank test for comparison between pre- and during drug applications. P values of < 0.05 were considered significant.

Results

Electrophysiological analysis targeting CCK/CB1-positive interneurons in V1 appears difficult, owing to the small number of this interneuron subtype. Thus, CCK-IRES-Cre; Dlx5/6-Flpe; RCE-dual mice were used, which were expected to express green fluorescent protein (GFP) only in CCK-positive interneurons. Immunostaining for

interneuron subtype marker proteins was conducted to confirm the subtypes of the GFP-positive cells found in these triple transgenic mice. At P21-24, cells expressing either proCCK or CB1 accounted for half of the total GFP-positive cell; however, PV, somatostatin, and neuropeptide Y (NPY) -positive cells were also present in a non-negligible proportion. None of the somatostatin-, PV-, or NPY-positive cells expressed CB1, suggesting that CCK/CB1-positive cells can be separated from other interneuron subtypes by CB1 immunostaining. The percentage of CCK/CB1 double-positive cells in the GFP-positive cells was much higher in the triple transgenic mice than in the VGAT-Venus transgenic mice. Therefore, to analyze functional synapses originating from CCK/CB1-positive interneurons in V1, GFP-positive cells in the triple transgenic mice were recorded using whole-cell patch-clamp techniques, and the recorded interneuron subtype was confirmed using post hoc immunostaining for CB1.

To investigate developmental changes in inhibitory postsynaptic currents (IPSCs) from CCK/CB1-positive interneurons to pyramidal cells, dual whole-cell patch-clamp recordings were conducted from these cells residing in layer 2/3 of V1 slices prepared from the mice at three developmental stages: P10–13, before eye-opening; P15–18, just after eye-opening and P21–25; during critical periods of visual response plasticity. The majority of recorded GFP-positive neurons demonstrated non-fast spiking (non-FS) firing patterns at all ages. Most of the non-FS cells were CB1-positive and some were somatostatin-positive. All GFP-positive cells with FS firing patterns were CB1-negative. Inhibitory connections from CCK/CB1-positive interneurons were detected in 41.4% of the recorded pairs at P11–13. The percentage decreased during development, and only 12.9% of the pairs were connected with inhibitory synapses at P21–25. In some experiments, the recorded cells were immunostained for proCCK in addition to CB1. The

connection probability from proCCK-negative but CB1-positive cells to pyramidal cells tended to be higher than that from proCCK/CB1-double positive cells, suggesting that the high connection probability at P11–13 may be owing to presence of many inhibitory connections from proCCK-negative but CB1-positive cells found only at P11–13. Finally, the IPSCs from the CCK/CB1-positive interneurons were characterized. There were no significant differences in amplitude, kinetics, and the paired-pulse ratio of the IPSCs among the three age groups. At all ages, the success rate of inhibitory transmissions was quite low. In contrast, IPSCs originating from FS cells or somatostatin-positive cells to pyramidal cells had high success rates. These results suggest that inhibition of CCK/CB1-positive interneurons is unreliable irrespective of age. The regulation of inhibitory synaptic transmission from CCK/CB1-positive cells by CB1 activation was also examined. It was observed that the application of CB1 agonist significantly decreased IPSC amplitude and success rate, while CB1 antagonist tended to have opposite effects. These electrophysiological results suggested that CCK neurons were involved in perisomatic inhibition in V1 when inhibitory connections from PV neurons were immature before eye-opening.

Conclusion

In conclusion, a method to identify CCK/CB1-positive interneurons in mouse V1 by combining the use of transgenic mice with marker protein expression analysis was established. Using this method, it was detected that inhibitory connections from CCK/CB1-positive interneurons to pyramidal neurons decrease during postnatal development and that synaptic transmissions are less efficient at all ages and modified by CB1.