Study of somatosensory circuit development using brainstem NMDAR-deficient mice

(Abridged version of the thesis)

Kimura, Chiemi

Doctor of Philosophy

Department of Genetics

School of Life Science

The Graduate University for Advanced Studies,

SOKENDAI

Laboratory of Mammalian Neural Circuits, National Institute of Genetics (NIG)

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Supervisor: Iwasato, Takuji

INTRODUCTION

Precise neural circuits emerge through an interplay between genetic programs and activity-dependent processes. A most attractive model for studying activity-dependent neural circuit formation is the rodent whisker-barrel system. In rodents, tactile information from whiskers sequentially reaches through the ventral brainstem principal sensory nucleus (vPrV), the thalamus ventroposteromedial nucleus (VPM) and finally to the layer 4 of the posteromedial barrel subfield (PMBSF) region of the somatosensory cortex. Along the trigeminal pathway, the afferent axons and the target cells are sequentially arranged in modules that represent whisker-related patterns, which are called "barrelettes" in the vPrV, "barreloids" in the VPM, and "barrels" in the cortex. These pattens are formed sequentially from the periphery to the center during the first few days after birth.

N-methyl-D-aspartate-type ionotropic glutamate receptors (NMDARs) are coincidence detectors of pre- and post-synaptic activities. During development, NMDARs play key roles in activity-dependent neural circuit refinement. It has been proposed that synaptic connections are strengthened by pre- and post-synaptic correlated activities, by a mechanism called "Hebb's rule". NMDAR-dependent long-term plasticity is important for this process. Previous studies in our lab and others have demonstrated the importance of NMDARs for the somatosensory neural circuit refinement. Most NMDARs are tetramer complexes composed of the principal NR1 subunits and modulatory NR2 subunits (NR2A-D). Global NR1 knockout (KO) mice that lack NR1 throughout the body die within 24 hours after birth because of respiratory failure. Even if the global NR1KO mice are attempted to survive to postnatal day 2 (P2) by pharmacological interventions and respiratory stimulation, barrelettes are still not detected. Similar results are subsequently demonstrated in global NR2BKO mice. Since both of the NR1KO and NR2BKO mice are perinatal lethal, it is still possible that barrelette formation is simply delayed rather than abolished. For the same reason, barreloids and barrels, which are formed later in development, are not examined in NR1KO and NR2BKO mice. To overcome these issues, Iwasato et al generated global NR1 knockdown (KD) mice, in which NR1 is minimally expressed for survival. They found that all of the barrelettes, barreloids and barrels were absent in the global NR1KD mice, which survive longer time. These results clearly demonstrate the importance of NMDARs in the somatosensory neural circuit refinement. However, it is difficult to determine the specific roles of NMDARs at each station of the trigeminal pathway because NMDARs were deficient all in the cortex, thalamus and brainstem in the global NR1KD mice. To delineate the region-specific roles of cortical NMDARs in the somatosensory neural circuit refinement, Iwasato et al generated cortex-restricted NR1KO (Cx-NR1KO) mice by developing Emx1-Cre mice, in which Cre-mediated recombination only occurs in excitatory neurons in the cortex. In the somatosensory cortex of Cx-NR1KO mice, thalamocortical axons (TCAs) formed only rudimentary patterns in regions corresponding large whiskers, and cortical layer 4 neurons failed to develop barrels, although barrelettes and barreloids normally developed. Arakawa et al generated thalamus-restricted NR1KO (Th-NR1KO) mice by using Sert-Cre mice, which induces Cre-mediated recombination only in the thalamus VPM. They report that barreloid formation is greatly perturbed and barrel patterning is blurred, meanwhile, barrelettes remain intact in the Th-NR1KO mice.

In contrast to the recent progress in understanding the roles of cortical and thalamic NMDARs in refinement of somatosensory neural circuits, the roles of brainstem NMDARs in development of somatosensory neural circuits are not well known. To uncover specific roles of brainstem NMDARs in the somatosensory circuit development, I here

generated two lines of mice that lack NR1, the essential NMDAR subunit, specifically in the brainstem (Brainstem-NR1 knockout (Bs-NR1KO) mice). In a line of mice, in which NR1 was supposed to be ablated in the majority of vPrV neurons, all barrelettes, barreloids and barrels were absent. These results suggest that the brainstem NMDARs are important for circuit development not only in the brainstem but also in the thalamus and cortex. In addition, during the analysis of another line of Bs-NR1KO mice, in which NR1 is supposed to remain in about half of vPrV neurons, I found that barrelettes were absent but barreloids and barrels were present, albeit partially, in these mice. Thus, barrel formation is robust, and the absence of barrelettes does not necessarily mean the absence of a template for barrel formation.

Together, this study demonstrates the importance of brainstem NMDARs in the somatosensory system development and the aspects of thalamic NMDAR function in the cortical circuit refinement.

MATERIALS AND METHODS

Animals

All experiments were performed according to the guidelines for animal experimentation of the National Institute of Genetics (NIG) and were approved by the animal experimentation committee of the NIG.

LacZ staining

Mice were perfused with 0.9% NaCl solution. Brains were removed and post-fixed for 2 hours in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) and cryoprotected in 30% sucrose in 0.1 M PB. Brains were sectioned in the coronal plane on a freezing microtome (ROM-380, Yamato) at 200-μm thickness. Slices were stained in LacZ solution [5 mM K3FeCN6, 5 mM K4FeCN6, 2 mM MgCl2, 1 mg/ml X-gal, 0.05 M PR (7.4)] at 37°C overnight. Slices were washed with 0.1 M PB, post-fixed in 4% PFA in 0.1 M PB and kept in 4°C. Imaging was acquired using the M205 FCA microscope (Leica) and DFC7000T camera (Leica).

Cytochrome oxidase (CO) staining

Mice were anesthetized with pentobarbital injection and perfused transcardially with 0.9% NaCl solution and subsequently with 4% PFA in 0.1 M PB. The brains were post-fixed in 4% PFA in 0.1 M PB for 1 day at 4°C. Cortices were flattened between two slide glasses and transferred to 30% sucrose in 0.1 M PB for cryoprotection at 4°C. Hindbrains and thalamus were also cryoprotected in 30% sucrose in 0.1 M PB. Tangential slices (100-μm thickness) and coronal slices (50-μm thickness) were obtained with a freezing microtome. Sections were incubated with CO reaction solution (4 g of sucrose, 50 mg of cytochrome C, and 50 mg of diaminobenzidine in 100 ml of phosphate buffer) free-floating for 3-4 hours at 37°C. Sections were washed with 0.1 M PB, mounted on slides (S2441, Matsunami). The slices were dried overnight at room temperature, and coverslipped (24×60 NEO, Matsunami) with EUKITT (ORSAtec). Imaging was acquired using the M205 FCA microscope (Leica) and DFC7000T camera (Leica).

Immunohistochemistry

Primary antibodies were as follows: guinea pig anti-VgluT1 (1:1000, AB5905, Millipore), rabbit anti-VgluT2 (1:1000, 135403, Synaptic Systems), rat anti-Tenascin (1:250, T2413, Sigma), mouse anti-NeuN (1:750, MAB377, Millipore), rabbit anti-β-gal (1:1000, Z3781, Promega), and rabbit anti-cFos (1:1000, PC38, MilliporeSigma). Second antibodies were as follows: Alexa 488 goat anti rabbit IgG (1:1000, A11034, Invitrogen), Alexa 568-goat anti guinea pig IgG (1:1000, A11075, Invitrogen), Alexa 568 goat anti mouse IgG (1:1000, A11004, Invitrogen), Alexa 647 goat anti rabbit IgG (1:1000, A21244, Invitrogen), and biotinylated goat anti rat IgG (1:200, BA9400, Vector laboratories). For immunofluorescence, frozen sections (50 μm-thickness) were prepared and incubated in 0.3% Triton X-100 and 3% goat serum for 1 hour, followed by

incubation with the primary antibodies 2-3 days overnight at 4°C. Next, the sections were incubated with secondary antibodies and counterstained with DAPI (2 μg/ml, 10236276001, Roche) before mounting. Sections were washed with 1×PBS, and mounted on slides (PLATINUM PRO, PRO-04, Matsunami). Before sections were dried up, the slides were coverslipped (24×60 NEO, Matsunami) with 0.2% n-PG in 90% glycerol in 1× PBS. Images were acquired by a confocal microscope (Leica, TCS SP5) and a 10X lens. For tenascin immunostaining, frozen sections (50 μm-thickness) were incubated in 3% H_2O_2 for 10 minutes to inactivate endogenous peroxidases. After the incubation with 10% goat serum, free-floating sections were incubated with the primary antibody for 1 day overnight at 4°C. Immunoreactivity for tenascin was detected with biotinylated secondary antibodies and ABC reagent (Vector laboratories) and visualized with diaminobenzidine. Sections were washed with 0.1M PB and mounted on slides (S2241, Matsunami). The slides were dried overnight at room temperature, and coverslipped (24×60 NEO, Matsunami) with EUKITT (ORSAtec). Images were taken using the M205 FCA microscope (Leica) and DFC7000T camera (Leica).

In utero electroporation (IUE)

IUE was conducted at E15 morning. Timed-pregnant female mice were anesthetized with an intraperitoneal injection of a combination anesthetic (medetomidine (0.3 mg/kg. Zenoaq, Domitor), midazolam (4 mg/kg. Maruishi Pharmaceutical, Dormicum), and butorphanol (5 mg/kg; Meiji Seika Pharma, Vetorphale) in 0.9% NaCl. To label layer 2/3 neurons in the cortex, pK064 (CAG-GFP, Mizuno et al., 2014, 1000 ng/ul) was used. DNA solution (diluted in Milli-Q water and < 5% trypan blue; Sigma T8154) was injected into lateral ventricles of embryos via a pulled glass capillary (Drummond, 2-000-050), and three to five times square electric pulses (35-40 V; 50 ms) were delivered by tweezer-electrodes (NepaGene, CUY650P5) and an electroporator (NepaGene, CUY21SC). When a combination anesthetic was used, atipamezole (0.3 mg/kg. Zenoaq, Antisedan) in saline was administered as an intraperitoneal injection after IUE. For observation of barrel net, tangential sections (50-μm thickness) were prepared. To visualize barrels, sections were stained with DAPI (2 μg/ml, 10236276001, Roche). Sections were rinsed with 0.1 M phosphate buffer, and mounted on coated slides (PLATINUM PRO, PRO-04, Matsunami). Slides were coverslipped (24×60 NEO, Matsunami) with 0.2% n-PG in 90% glycerol in 1×PBS. Images were taken by a confocal microscope (Leica, TCS SP5) and a 10X lens.

Enriched environment procedure

P20-P21 mice were anesthetized with a combination anesthetic and all but two whiskers (D1 and D3, or B1 and D1) on both sides of the face were cut close to the skin. Mice were placed on 37°C heater for recovery from anesthesia. 1 day after the trimming on bilateral side, mice were placed in the cage of enriched environment that was equipped with gadgets such as pipes, marbles, tree branches, acorns and stones, and left in darkness. After 1 hour-long exploration period, mice were perfused and processed for c-Fos immunostaining. To analyze c-Fos-positive cell density in barrels, circles of 270 μm diameter were placed in the center of D1, D2 and D3 barrels using Adobe Photoshop 2022. c-Fos-positive cells were counted within outline regions.

RESULTS

Absence of whisker-related patterns in the brainstem vPrV in Bs[K]-NR1KO mice

To examine the role of brainstem NMDARs in somatosensory circuit development, I generated Brainstem (Bs)[K]-NR1KO mice in which the gene for NR1, the essential NMDAR subunit, was disrupted specifically in the brainstem using the Cre/loxP recombination approach. For this purpose, I used K-Cre knock-in mice, in which Cre-mediated recombination was detected in the brainstem ventral PrV (vPrV) but not in the thalamus VPM or somatosensory cortex in the neonatal stage. I crossed K-Cre mice and NR1-flox mice to obtain K-Cre; NR1flox/flox and control mice. Hereafter I refer to K-Cre; NR1flox/flox mice as Bs[K]-NR1KO mice.

To examine the barrelette patterning in the brainstem vPrV in Bs[K]-NR1KO mice, I performed cytochrome oxidase (CO) staining at P7. CO staining is a commonly used technique to visualize the whisker-related patterns. In control mice, CO staining clearly visualized barrelettes, which are patches corresponding to the facial whiskers. On the other hand, Bs[K]-NR1KO mice displayed a uniform staining in the vPrV, indicating the absence of barrelette patterns.

To further verify the absence of barrelettes in Bs[K]-NR1KO mice, I performed VgluT1 immunostaining, which stains the presynaptic terminals of trigeminal axons. VgluT1 signals were also uniform and did not show any patches in the vPrV of Bs[K]-NR1KO mice at P7-P10.

Partial impairment of whisker-related patterns in the thalamus in Bs[K]-NR1KO mice

To analyze whisker-related patterns in VPM in Bs[K]-NR1KO mice, I first performed CO staining. Control mice showed clear patterning of barreloids at P7. In contrast, Bs[K]-NR1KO mice showed blurred patterning of individual patches. To analyze the presynaptic trigemino-thalamic axon (TTA) patterns in barreloids, I performed immunostaining for VgluT2, a TTA marker, at P7. The TTA clusters corresponding to the whiskers were present albeit obscure in Bs[K]-NR1KO mice. I also examined the postsynaptic patterns in VPM by DAPI staining at P7. Cells were arranged around TTA clusters in control mice. On the other hand, cells were distributed uniformly across multiple barreloids in Bs[K]-NR1KO mice.

Partial impairment of whisker-related patterns in the cortex in Bs[K]-NR1KO mice

I assessed the barrel formation in Bs[K]-NR1KO mice by CO staining. In Bs[K]-NR1KO mice, barrels were detected in posteromedial barrel subfield (PMBSF), although individual patches were slightly obscured and smaller compared to those of control mice. Patches in the anterior snout-corresponding and lower lip-corresponding regions in the cortex in Bs[K]-NR1KO mice were similar to those in control mice.

I examined the TCA patterning by immunostaining using VgluT2 antibody for thalamocortical axons. VgluT2-positive presynaptic afferent terminals formed patches in both control and Bs[K]-NR1KO mice. On the other hand, the boundaries of individual TCA patches in Bs[K]-NR1KO mice appeared blurred compared to that in control mice. I next analyzed barrel patterns using DAPI staining. In control mice, layer 4 neurons were

arranged around the presynaptic terminals to form the postsynaptic patterns. In control mice, layer 4 neuron density between barrels were low and septa were clearly observed. In Bs[K]-NR1KO mice, layer 4 neuron arrangement around the presynaptic terminals were found. However, layer 4 neuron density between barrels appeared to be uniform and septa were not observed in Bs[K]-NR1KO mice. In septa of normal mice, expression of the extracellular matrix molecules is generally increased during early postnatal week. I performed immunostaining for tenascin, which is an extracellular matrix molecule, at P7-P10. In control mice, staining of tenascin was found between barrels and patterning of extracellular matrix was clearly detected. In Bs[K]-NR1KO mice, staining of tenascin was also found between individual barrels, but the staining was appeared to be blurred and the staining region was wider than control mice. In the septal region of the somatosensory cortex layer 4, the axons of layer 2/3 neurons run through and form the structure called “barrel net”, which is visible only after P10, while barrels already exist at P5. I labeled layer 2/3 neurons with GFP by *in utero* electroporation at E15.5 and observed barrel net at P14-P15. In control mice, GFP-positive axons of layer 2/3 neurons showed mesh-like distributions located in the septal region and formed barrel net. In Bs[K]-NR1KO mice, axonal trajectories of layer 2/3 neurons were found between the barrels in the cortex layer 4 to form a barrel net as in control mice. However, the mesh-like distributions in the cortex layer 4 in Bs[K]-NR1KO mice appeared to be wider and fuzzier than those in control mice.

In summary, barrels were detected in Bs[K]-NR1KO mice, although there were some differences from control mice. These results indicate that barrels can develop even without barrelettes in Bs[K]-NR1KO mice.

One-to-one functional relationships between whiskers and barrels in Bs[K]-NR1KO mice were largely maintained, albeit not normal

There are one-to-one functional relationships between whiskers and barrels. When a whisker is stimulated in an adult mouse, immediate early gene c-Fos is upregulated in the corresponding barrel within a short time after the increase of neuronal activity. To assess the functional relationships between whiskers and barrels in Bs[K]-NR1KO mice, I performed c-Fos immunohistochemistry following the whisker stimulation. I trimmed all large whiskers except D1 and D3 whiskers bilaterally. One day after the trimming, I put mice in enriched environment filled with many gadgets under the dark, which induced voluntary whisker stimulation during tactile exploration. Following this whisker stimulation for one hour, I collected the brains and analyzed c-Fos upregulation in the barrel cortex layer 4 by immunohistochemistry using tangential sections. In control mice, strong c-Fos labeling was observed in D1 and D3 barrels, but in other area, for example in D2 barrel, only a few c-Fos-positive cells were found. In Bs[K]-NR1KO mice, c-Fos was strongly activated around D1 and D3 barrels, but c-Fos expression appeared to be more widespread than in control mice.

Correctively, these results indicated that the functional relationships between whiskers and barrels in the Bs[K]-NR1KO mice were largely formed, albeit not perfect. These results also showed that one-to-one functional relationships between whiskers and barrels were present in Bs[K]-NR1KO mice even in the absence of barrelette patterns.

Absence of whisker-related patterns in the brainstem, thalamus and cortex in Bs[KH]-NR1KO mice

Although there were some abnormalities in Bs[K]-NR1KO mice, these abnormalities were much milder than I expected. I speculated that the recombination efficiencies in vPrV in K-Cre mice were not high enough, which may have caused the mild phenotypes in these mice. Previously, Dr. Ayumi Suzuki in our lab found that if K-Cre mice were crossed with H-mice, which show brainstem-specific Cre recombination, the Cre-mediated recombination efficiency increases. Based on these findings, I generated K-Cre; H-Cre; NR1 flox/flox mice by crossing K-Cre; NR1 flox/flox and H-Cre; NR1 flox/flox mice. Hereafter I refer K-Cre; H-Cre; NR1 flox/flox mice as Bs[KH]-NR1KO mice.

Barrelette patterns revealed by CO staining were missing in Bs[KH]-NR1KO mice at P7, similar to the observation in Bs[K]-NR1KO mice. In VPM, CO staining density and VgluT2 staining density was uniform in Bs[KH]-NR1KO mice, indicating that barreloid patterns were absent in Bs[KH]-NR1KO mice. These results showed that barreloids were severely impaired in Bs[KH]-NR1KO mice compared to that in Bs[K]-NR1KO mice. PMBSF region in the somatosensory cortex was almost uniformly stained by CO in Bs[KH]-NR1KO mice at P7, implying that barrel patterns were almost absent in Bs[KH]-NR1KO mice. In addition, patterns in anterior snout-related region and lower lip-corresponding region were also absent in the Bs[KH]-NR1KO mice. Barrel patterns revealed by CO staining were almost absent in the Bs[KH]-NR1KO mice even at postnatal 3 weeks, similarly at P7. Hence the disrupted pattern of cortical maps in Bs[KH]-NR1KO mice was not due to developmental retardation. Barrel patterns by VgluT2 immunostaining, DAPI staining or tenascin immunostaining were almost absent at P7-P10 in Bs[KH]-NR1KO mice.

Overall, barrels were not formed when NR1 is extensively deleted in the brainstem.

Absence of whisker-related patterns in the cortex in Bs/Th[KS]-NR1KO mice

The results that barrels and barreloids were formed to some extent even without detectable barrelettes in Bs[K]-NR1KO mice was somewhat unexpected because barrels and barreloids are thought to be formed using barrelettes in the vPrV as the template. This suggests a possibility that although barrelettes are absent, there is still a rudimentary template for constructing barrels in Bs[K]-NR1KO mice. I hypothesized that thalamic NMDARs may contribute to the development of barrels and barreloids without detectable barrelettes in Bs[K]-NR1KO mice. To explore this possibility, I used Sert-Cre mice previously generated by our lab, in which Cre-mediated recombination is observed in VPM, but not in vPrV or cortex. I crossed K-Cre; NR1 flox/flox and Sert-Cre; NR1 flox/flox mice to obtain K-Cre; Sert-Cre; NR1 flox/flox mice. Hereafter I refer K-Cre; Sert-Cre; NR1 flox/flox mice as Bs/Th[KS]-NR1KO mice.

I analyzed the barrel formation by CO staining. In Bs/Th[KS]-NR1KO mice, barrels were almost absent at P8. TCA patterns by VgluT2 immunostaining were almost absent in Bs/Th[KS]-NR1KO mice. Patterning of extracellular matrix was not detected in Bs/Th[KS]-NR1KO mice by tenascin immunostaining at P7-P10.

These results indicate that thalamic NR1 is required for barrels to development from rudimentary templates

in the brainstem in Bs[K]-NR1KO mice.

DISCUSSION

In this study, I have shown the followings. First, in Bs[KH]-NR1KO mice, in which NR1 was supposed to be ablated in the majority of vPrV neurons, all barrelettes, barreloids and barrels were absent. These results demonstrate that the brainstem NMDARs are important not only for barrelette formation but also for barreloid and barrel formation. Thus, brainstem NMDARs distantly regulate neural circuit refinement in the cortex. Second, in Bs[K]-NR1KO mice, barrelettes were absent but barrels were still present to some extent and one-to-one functional relationships between whiskers and barrels were largely maintained. These results indicated that barrel formation is robust. These results also demonstrate that even if there are no barrelettes, it is possible that a trace level template still exists and supports development of barrels. Third, barrels were absent when Bs[K]-NR1KO mice lack thalamic NMDAR function in Bs/Th[KS]-NR1KO mice. These results indicated that the developing thalamus may serve for cortical circuit refinement in Bs[K]-NR1KO mice by compensating brainstem-derived templates, in which thalamic NMDARs play an important role. Taken together, the present study demonstrates the importance of brainstem NMDARs throughout the somatosensory neural circuit development and highlights the aspects of thalamic NMDAR function in the cortical circuit refinement.