Rem2 Effects on the Development of Neonatal Mouse Barrel Cortex

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Rem2 is a small Ras-like GTPase of the Rad/Rem/Rem2/Gem/Kir (RGK) family that is highly expressed in the central nervous system. Previous studies about Rem2 were conducted either in cultured neurons or in the visual system, while we applied Rem2 knockout on the barrel cortices of unilaterally sensory-deprived pups to see its effects on dendritic formation and arborization. We discovered that the loss of Rem2
resulted in decreases in both the quantity of primary neurites and the sizes of sensory-deprived cells, which was against the normal patterns of sensory deprivation model of the barrel cortex. This phenomenon suggests that Rem2 might play a crucial role specifically on the developmental regulation of deprived barrel cortex layer IV cells, and the underlying mechanisms might differ from those of other neuronal systems.
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Introduction

The development of mammalian nervous system is the basis of the formation of higher social and cognitive functions. One of the most efficient approaches to understand such development is to investigate the dynamic events that shape the unique structure and connectivity of one certain group of neurons. The motivations of these events can mainly be divided into two categories: the “nature” part which is destined by delicate inbuild genetic programming, and the “nurture” part which refine the former one by the regulation of experience-driven signaling pathways (Hubel & Wiesel, 1970). This research, however, will primarily focus on the “nurture” part through the study of a cytosolic signal molecule – Rem2 (Kenny et al., 2017), which may further our understandings on brain development through revealing its functions and underlying mechanisms.

Rem2 is a small Ras-like GTPase of the Rad/Rem/Rem2/Gem/Kir (RGK) family that is highly expressed in the central nervous system (Finlin, Shao, Kadono-Okuda, Guo, & Andres, 2000). Its function in the CNS may vary according to the expression sites: it was reported as a potent activity-dependent negative regulator of dendritic development and morphology both in vitro (Ghiretti & Paradis, 2011) and in vivo (Ghiretti et al., 2014), but a positive regulator of synapse formation in vitro (Moore,
Ghiretti, & Paradis, 2013). Nevertheless, previous studies were conducted either in cultured neurons or in the visual system, and it is of importance to know whether the same rules in visual cortex are common to all cortical areas. Thus, we chose the neonatal mouse barrel cortex from the somatosensory region as the developmental model and apply Rem2 knockout on unilaterally sensory-deprived pups to see its effects on dendritic formation and arborization.

The mouse barrel cortex refers to a region within cortical layer IV of the somatosensory cortex where blocks that are visibly darker than the surrounding area will appear after Golgi-Nissl staining (Woolsey & Van der Loos, 1970). These blocks, which were referred to as “barrels”, are a major target for the somatosensory input from thalamus, which receive raw information from relevant body parts. Of all those barrels, the ones that correspond to major facial whiskers are the largest and most elliptical in shape and have almost the same topographical organization as that of the whiskers, which provides convenience to delicate manipulation of sensory inputs to the barrel cortex. Besides, the critical period of barrel cortex starts right after an animal’s birth, and produces the greatest influence on cortex plasticity between postnatal day 0 (P0) and P4 (Fox, 1992). This time period is way behind the eye-opening time (about P14) of a mouse pup, which may provide more insights into the earliest stages of experience-dependent development of mammalian brains.

Based on the characteristics described above, we investigate Rem2 functions in
barrel cortex by performing unilateral whisker trimming on Rem2 knock out and wildtype neonatal mice, and assess the influences of Rem2 dysfunction on those unilateral sensory deprivation models. We found that the loss of Rem2 has resulted in significant decreases in both the neurite numbers and the sizes of sensory-deprived cells but not normal cells, suggesting that Rem2 might play a crucial role specifically on the developmental regulation of deprived barrel cortex layer IV cells.

**Materials and Methods**

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee at Brandeis University.

**Subjects**

The subjects involved in this research was a mouse strain provided by courtesy of Dr. Sarah E. Richards containing both heterozygous Rem2 “flxed” (i.e. flanked by loxP sites) gene and Ai9 TdTomato gene sets on the C57BL/6J genetic background. Since the parents were both Rem2 heterozygous, we took tissue sample from every neonatal pup for PCR followed by sequencing across the targeted Rem2 locus to confirm its genotype. As expected, the genotypes of pups were close to the Mendelian ratio, which was in accordance with the description of this strain (Moore et al., 2018). The TdTomato locus of those mice had a STOP cassette preventing the transcription of a red fluorescent protein tdTomato. This cassette was loxP-flanked as well, so a
neonatal AAV-Cre injection would activate both Rem2 deletion and tdTomato expression in selected regions, which provided the basis for further sensory manipulation.

**Neonatal Virus Injection**

The AAV-Cre constructs were provided by courtesy of Dr. Sarah E. Richards as well. The mothers of the neonatal mice pups were kept together with the pups for 24 hours after parturition to ensure that the pups receive colostrum before any treatment, while the fathers were moved to other chambers to prevent harms to the pups and pregnancy during the nurturing period. After this time period, pups were anesthetized by cooling on ice covered by aluminum foils for 6-8 minutes. The foils were placed to prevent cold lesions caused by direct contact with ice. After the pups stopped response to most of the external stimulus, they were mounted on a stereotaxic frame with light source downwards. Since neonatal pups were somewhat translucent, strong light would be able to penetrate the brain and reveal the cleavage between parietal and interparietal bones as well as the cleavage between two symmetrical parietal bones. 1μl of AAV (diluted 1:200) was then delivered below the dural surface at the position indicated by Figure 1 on both hemispheres and toe-clipping was performed for identification purpose. After all these manipulations, pups recovered on a heating pad and were returned to the animal facility until use.

**Neonatal Sensory Deprivation by Whisker Trimming**
For all the mice pups involved in the research, all whiskers on the right side were carefully trimmed once a day from the date of birth (P0) to postnatal day 14 (P14) with a curved eye scissor. The trimming was considered as complete when all whiskers were no longer than the facial fur of the animal. Since the animals didn’t open their eyes for most of the trimming days, they were rather docile and didn’t require any anesthesia during the operation. The pups were returned to their dams after every trimming until P14.

**Brain Extraction and Immunocytochemistry**

Reared P14, mice pups were deeply anesthetized with ketamine/xylazine cocktail (ketamine 50mg/kg, xylazine 5mg/kg) and perfused first with 0.1M PBS followed by 4% paraformaldehyde in 0.1M sodium phosphate buffer. Brains were then extracted and sectioned to two hemispheres. For each hemisphere, the thalamus and olfactory bulb were carefully removed, leaving only the cortex for the next step. The cortices were soaked in 4% paraformaldehyde in 0.1M sodium phosphate buffer, with a slide gently placed on top of them for 24 hours. The weight of the slide would flatten the cortex without causing any harm, and the flattened and fixed cortices were then transferred to 30% sucrose for preservation.

After placed in 30% sucrose for at least 24 hours, cortices were mounted on a cryomicrotome and cut horizontally to produce 150μm thick sections. They were then transferred to well plates with 3 slices in 1 well and washed for 3 times with 0.1M PBS.
on the shake bed to get rid of the formalin and sucrose, with 10 minutes each time. Slices were washed with the washing buffer (0.1M PBS + 3% Triton) with the same process, and soaked in the blocking buffer (0.1M PBS + 3% Triton + 10% donkey serum) on the shake bed for 3 hours after the washing. The blocked sections were incubated with the 1st antibody (1:4000 guinea pig anti-VgluT2 in 0.1M PBS) overnight in a 4°C refrigerator. On the next day, the 1st antibody was washed away with 0.1M PBS for 10x3 minutes, and the 2nd antibody (1:500 goat anti-guinea pig CF633 in the blocking buffer) was added for incubation on the shake bed for 2 hours. The 2nd antibody was washed away with the same process of the 1st antibody, and the slices were incubated with DAPI (1:500 DAPI in 0.1M PBS) for 10 minutes. The DAPI solution was washed away with the same process. The sections were finally mounted on slides and coverslipped with Permount (Fisher Scientific) for microscopy.

Three-dimensional Cell Reconstruction and Analysis

Slides containing tissue sections were imaged under immunofluorescence mode with a Zeiss LSM 880 AxioObserver microscope. The laser wavelengths used were 633nm (for anti-VGlut2 antibody), 561nm (for TdTomato) and 405nm (for DAPI). The barrel overview and target cell positions were first recorded under 5x to 20x objectives, with z-stacks of individual neurons sampled with a 40x oil objective from layer IV of the barrel cortex. 3D reconstruction with scalebars was done by the inbuild function “3D” of ZEN from the stacks of images.
The 3D model of the neuron was then projected to a 2D surface by the “image” command. The 2D projection, together with the “cell maps” (pictures from 5x to 20x view that showed the exact positions of the target neurons and the barrel outlines), were opened in Adobe Photoshop CS5 for quantitative analysis as described in Figure 2. First, the boundaries of the barrels that were relevant to target cells were plotted with both the outlines of bright regions in the VgluT2 channel and the bright “grids” that were formed by the aggregation of somas in the DAPI channel. Second, 2D projections of the target neurons were adjusted according to their relative sizes to the cell maps until they overlapped perfectly with the target neurons on the maps, and the exchange ratios from pixels to micrometers were calculated by the scalebars on those projections as well. Third, neurites were marked manually on the 2D projections according to observations in the 3D models, with each primary neurite (defined as neurites directly protruded from the soma) and its branches marked with a different color. The number of branch tips within/without the barrels and the number of primary neurites were counted during this step. Last, bounding boxes (defined as the ellipse with minimum area that can include the target) of neurons and relevant barrels were drawn with the lengths, heights and bounding box angles (defined as the angle rotated from horizontal position between -90° and 90°; clockwise rotations were defined as positive) recorded for statistical analysis.

For the statistical analysis, effects of Rem2 knock-out were primarily quantified by two major groups of factors. The first group was the neurite features, which included
the number of primary neurites (PN, defined as the number of protrusions originated directly from the soma), the number of neurite branch tips (BT, defined as the number of all neurite endings from a single cell) and the BT/PN ratios. The second group was the cell size features, which included the cellular bounding box areas (CA, defined as the area of the smallest oval (the “bounding box”) that can contain the 2D projection of the cell), the barrel bounding box areas (BA, defined as the bounding box area of the barrel in which a cell resided) and the BA/CA ratios.

Statistics

Data recording and preliminary data processing were done via MICROSOFT EXCEL 2016. Major data analysis and plotting of analysis results were done via MATLAB R2017b. Data analysis included Kruskal-Wallis tests and Mann-Whitney U-tests, with significance defined as p<0.05.

Results

Rem2 knock out lowered the primary neurite numbers in sensory-deprived cell development

Based on the neurological properties and treatments elaborated in the previous paragraphs, the cells observed could be mainly divided into three pairs of categories: whether they were inside (I)/outside (O) a barrel, sensory-deprived (D)/non-deprived (ND) or Rem2 knocked-out (KO)/remained wildtype (WT). During the analysis of
neurites, I/O factor was found to be insignificant in the comparison between numbers of primary neurites (PN, $p = 0.12$), number of neurite branch tips (BT, $p = 0.95$) and the BT/PN ratios ($p = 0.40$). Due to these results, we combined the I/O cells together in the following analysis of this part.

We then compared PN, BT and BT/PN values among the cell groups. As predicted by former reports, sensory deprived cells had far larger PN (Fig. 3a, $p < 0.01$) than the non-deprived ones in WT animals. However, this was not the case in KO animals since their neurons had almost the same PN (Fig. 3a, $p = 0.44$). Further analysis revealed that the PN of ND cells did not vary much (Fig. 3a, $p = 0.36$) under genetic manipulation; it was the PN of deprived cells that dropped significantly (Fig. 3a, $p < 0.01$) compared to their wild type counterparts. Same series of analyses were done on BT (Fig. 3b with $p$-values listed below) and BT/PN (Fig. 3c with $p$-values listed below) but found no significance. This implied that Rem2 might be altering more general patterns of neurites rather than detailed distributions of every single branch tip.

**Rem2 knock out reduced the absolute and relative cell sizes of the deprived cells**

After the explorations with the neurite morphologies, another approach was made by analyzing the effects of Rem2 knock out on the overall sizes of cell groups. This approach was essential because skipping the details such as branch tips might reduce the error brought by marking these details manually, thus reflect the actual functional status of cells more accurately.
In order to evaluate cell sizes, we defined the bounding box of a cell/barrel as the smallest oval that could contain the 2D projection of the entire cell/barrel and recorded the cell bounding box areas (CA), the barrel bounding box areas (BA) and the BA/CA ratios for comparison. Rem2 showed stronger effects on cell sizes than on neurites, since the sizes of D-KO cells were not only far smaller than the D-WT cells (Fig. 4a, p < 0.001), but also smaller than the ND-KO cells (Fig. 4a, p < 0.05). This was different from what PN data have shown and reversed the relationship that existed in wild type cells.

Nevertheless, it was natural to realize that the sizes of cells were largely affected by the sizes of barrels they were in. To exclude such influence, we picked out all the cells and recorded the BA of their residing barrels. All variances between barrel sizes didn’t exceed the confidence interval (Fig. 4b with p-values listed below), which meant previous conclusion on cell sizes was reliable. Relative cell sizes calculated by BA/CA confirmed previous observations except an interesting fact that the difference within wild type cells vanished (Fig. 4c, p = 0.2403), indicating that barrel cells might have some mechanisms to counteract the effects of sensory deprivation on their occupying areas, and those mechanisms might as well be disrupted by loss of Rem2.

**Conclusion and Discussions**

With clues from both dendritic morphology and overall cell sizes, it would be proper to conclude that Rem2 might pose positive regulatory effects on the development of
mouse barrel cortex cells under the pressure of sensory deprivation, and this phenomenon could possibly be achieved though promoting the generation of primary neurites and elongation of neurites.

This conclusion, however, matched with some of the previous studies but not all of them. Such incongruities might as well be valuable since Rem2 was a part of multiple complicated and overlapping regulatory pathways, and further inspections into those issues including behavioral, electrophysiological, genetic and molecular approaches might provide better understandings on Rem2 functions within different parts of the brain. For instance, further experiments might include gene rescue experiment on Rem2 knock out animals to see if it can offset the effects described in this study, or behavioral tests described in previous studies (Krupa, Matell, Brisben, Oliveira, & Nicolelis, 2001) to evaluate the actual performance of altered cortices.

Besides, this research reached some interesting aspects that were not listed as conclusions above. One of them was that there seems to be differences in cell forms as well, i.e. the number of levels of bifurcations. KO cells seem to have fewer levels, which means one must “walk past” only 1 or 2 bifurcations from the soma to one branch tip. Another was that the inward cells almost showed (p = 0.062) significance in BT/PN ratios, which suggested that the branching tendencies of target cells might also be a point worth noticing in the following researches.
References


Figure 1. The virus injection sites. The figure was modified from the mouse skull picture of *The Anatomy of the Laboratory Mouse* by Margaret J. Cook. The injection sites were indicated by black dots on the figure.

a)  
- anti-VGlut2
- DAPI
- combined

b)
Figure 2. An example of the cell analysis process. The example cell used in the pictures was the inward cell 6 (Icell6) from the right hemisphere of the injected mouse 1 (I1R). (a) The barrel outlines over the anti-VGluT2 channel (left), DAPI channel (middle) and combined picture(right). (b) The original cell map before (left) and after (middle) the 2D projection of Icell1 was located on the map. It was worth noticing that not only the microstructures of Icell6 matched the cell map perfectly, so did the other cells appear on the 2D projection. An inscribed circle within the 280*280nm square (right) of the 2D projection had a diameter of 661px, indicating that 100px equaled to 42.36nm in this cell map. (c) The 2D projection with 5 neurites and all branches marked, each neurite and its relevant branches were marked with different colors. Barrel outlines were turned off during the plotting to avoid subjective interference. (d) Bounding boxes of Icell6 (left) and its residing barrel (right) were drawn and relevant data were acquired.
a) Comparisons between Numbers of Primary Neurites

b) Comparisons between Numbers of Branch Tips
Figure 3. Rem2 knock out decreased the primary neurite numbers in sensory-deprived cell development. Circles (“o”) indicated cells from animal KO-1, asterisks (“*”) from KO-2, squares (“□”) from WT-1, and crosses (“+”) from WT-2. N = 12 for each animal while dots might pile up to show less than 6 dots on the plot. 

a) Comparisons between numbers of primary neurites. p(D-KO/ND-KO) = 0.4377; p(D-KO/D-WT) = 0.0046; p(ND-KO/ND-WT) = 0.3574; p(D-WT/ND-WT) = 0.0072. Other p-values were not calculated since comparison between data with no relation would be meaningless.

b) Comparisons between numbers of cell branch tips. p(D-KO/ND-KO) = 0.1188; p(D-KO/D-WT) = 0.0994; p(ND-KO/ND-WT) = 0.1254; p(D-WT/ND-WT) = 0.0828.

c) Comparisons between BT/PN ratios. p(D-KO/ND-KO) = 0.2364; p(D-KO/D-WT) = 0.7727; p(ND-KO/ND-WT) = 0.2143; p(D-WT/ND-WT) = 0.5634.
a) Comparisons between Cell Sizes

b) Comparisons between Residing Barrel Sizes
Figure 4. Rem2 knock out reduced the absolute and relative cell sizes of the deprived cells.  

a) Comparisons between all cell sizes. $p(D\text{-KO/ND\text{-KO}}) = 0.0120$; $p(D\text{-KO/D\text{-WT}}) = 7.3148e^{-4}$; $p(ND\text{-KO/ND\text{-WT}}) = 0.2145$; $p(D\text{-WT/ND\text{-WT}}) = 0.0086$.  

b) Comparison between the sizes of residing barrels of I cells. It is worth noticing that since inward cells only counted for half of the cells recorded, relevant data would have only half of the data points (N = 6 for each animal) as well. $p(D\text{-KO/ND\text{-KO}}) = 0.1255$; $p(D\text{-KO/D\text{-WT}}) = 0.3701$; $p(ND\text{-KO/ND\text{-WT}}) = 0.6667$; $p(D\text{-WT/ND\text{-WT}}) = 0.3723$.  

c) Comparison between relevant inward cell sizes. “Relevant” was used to stress that BA/CA was used to symbolize “true” cell sizes in this subplot, with greater values for smaller relevant sizes. $p(D\text{-KO/ND\text{-KO}}) = 0.0411$; $p(D\text{-KO/D\text{-WT}}) = 0.0043$; $p(ND\text{-KO/ND\text{-WT}}) = 0.8182$; $p(D\text{-WT/ND\text{-WT}}) = 0.2403$. 