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Developmental genetic profiles of glutamate receptor system, neuromodulator system, protector of normal tissue and mitochondria, and reelin in marmoset cortex: Potential molecular mechanisms of pruning phase of spines in primate synaptic formation process during the end of infancy and prepuberty (II)

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ABSTRACT

This is the second report of a series paper, which reports molecular mechanisms underlying the occurrence of pruning spine phase after rapid spinogenesis phase in neonates and young infant in the primate brain. We performed microarray analysis between the peak of spine numbers [postnatal 3 months (M)] and spine pruning (postnatal 6 M) in prefrontal, inferior temporal, and primary visual cortices of the common marmoset (*Callithrix jacchus*). The pruning phase is not clearly defined in rodents but is in primates including the marmoset. The differentially expressed genes between 3 M and 6 M in all three cortical areas were selected by two-way analysis of variance. The list of selected genes was analyzed by canonical pathway analysis using "Ingenuity Pathway Analysis of complex omics data" (IPA; Ingenuity Systems, Qiagen, Hilden, Germany). In this report, we discuss these lists of genes for the glutamate receptor system, G-protein-coupled neuromodulator system, protector of normal tissue and mitochondria, and reelin. (1) Glutamate is a common neurotransmitter. Its receptors AMPA1, GRIK1, and their scaffold protein DLG4 decreased as spine numbers decreased. Instead, GRIN3 (NMDA receptor) increased, suggesting that strong NMDA excitatory currents may be required for a single neuron to receive sufficient net synaptic activity in order to compensate for the decrease in synapse. (2) Most of the G protein-coupled receptor genes (e.g., *ADRA1D, HTR2A, HTR4,* and *DRD1*) in the selected list were upregulated at 6 M. The downstream gene *ROCK2* in these receptor systems plays a role of decreasing synapses, and *ROCK2* decreased at 6 M. (3) Synaptic phagosytosis by microglia with complement and other cytokines could cause damage to normal tissue and mitochondria. SOD1, XIAP, CD46, and CD55, which play protective roles in normal tissue and mitochondria, showed higher expression at 6 M than at 3 M, suggesting that normal brain tissue is more protected at 6 M. (4) Reelin has an important role in cortical layer formation. In addition, *RELN* and three different pathways of reelin were expressed at 6 M, suggesting that new synapse formation decreased at that age. Moreover, if new synapses were formed, their positions were free and probably dependent on activity.

1. Introduction

This is the third report of a three-series paper [1] that elucidates the mechanisms underlying spine pruning phase in the primate cerebral cortex during overshoot-type synaptic formation processes [2]. After birth, the cerebral cortex in primates, including humans, shows a rapid increase in the number of spines, which reaches a peak, and then a pruning phase occurs, which decreases the number of spines. In this series of three papers, we sampled three cortical areas (prefrontal, inferior temporal, primary visual cortices) of the common marmoset [3]. Sampling timing was at postnatal 3 months (M) and at postnatal 6 M. 3 M is the peak spine number time, and 6 M is the pruning phase in the three cortical areas [3]. After sampling, we performed microarray analysis and identified a list of genes differentially expressed between 3 M

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and 6 M commonly in the three areas (filtered by absolute fold change of >1.2) using two-way analysis of variance (ANOVA). These lists of genes were analyzed by canonical pathway analysis. In this study, (1) because glutamate is the most common excitatory neurotransmitter and is strongly related to formation and/or pruning of synapses [4], we start with the glutamate receptor system. (2) Next, we discuss the neuromodulator system (dopamine, noradrenaline, and serotonin), which affect dendritic spine numbers through G protein-coupled receptor genes (e.g., *ADRA1D, HTR2A, HTR4,* and *DRD1*) [5]. (3) Synaptic phagosytosis by microglia with complement and other cytokines could damage normal tissue and mitochondria [6,7]. We will discuss a protecting system for these structures and (4) reelin.

2. Materials and methods

2.1. Animals and tissue preparation

The animals used were the same as in the first report [1]. In short, we used common marmosets: each group consisted of three animals at 0 D, 2 M, 3 M, 6 M, and adult animals. All experimental and animal care procedures were performed in accordance with the NIH guidelines and with the Guide for Care and Use of Laboratory Primates published by the National Institute of Neuroscience, National Center of Neurology and Psychiatry (2005, 2008).

2.2. Tissue processing

Tissue processing is the same as that performed previously [1]. In brief, the animals were deeply anaesthetized with ketamine hydrochloride (Ketalar, 25 mg/kg i.m.; Daiichi Sankyo, Tokyo, Japan) and overdosed with sodium pentobarbital (Somnopentyl, 100 mg/kg or i.p.; BCM Internationl, Hillsborough, NJ, USA). The animals were perfused intracardially with 0.1 M potassium phosphate-buffered saline (pH 7.2). Blocks of tissue from prefrontal cortex (PFC), inferotemporal cortex (IT), and primary visual cortex (V1) were excised, as performed previously [3], and immersed in RNAlater (Life Technologies Japan Ltd., Tokyo, Japan). Total RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

2.3. Generation of common marmoset cDNA sequences and construction of the microarray

We constructed the microarray using the GeneChip® CustomExpress® Array Program (Affymetrix, Santa Clara, CA, USA) by the same method as performed previously [1]. In brief, the arrays after hybridization were scanned with a GeneChip Scanner 3000 (Affymetrix), and the image files obtained were analyzed with Affymetrix microarray suite version 5 software (MAS5). We then obtained tab-delimited files containing data regarding the relative levels of transcript expression Signal and the reliability of detection (Detection Call). The derived signal values were globally normalized and controlled to all probe sets equal to 100.

2.4. High-density oligonucleotide microarray analysis

We performed high-density oligonucleotide microarray analysis, as performed previously [1].

2.5. Microarray data analysis

The data analysis is the same as that performed previously [1]. To examine genes specifically expressed in different cortical regions, average signal values of three replicates for each region were calculated. The gene expression data were analyzed using GeneSpring GX 11 software (Agilent Technologies). The signal intensity data were preprocessed using the RMA algorithm and normalized using MAS 5. To improve the false discovery rate when gene probe sets were turned on or off, we restricted probe sets ($\geqslant$50% present), as described by McClintick and Edenberg [8]. Differentially expressed genes among the three areas and five time points were analyzed using two-way ANOVA (p value computation: asymptotic; multiple testing correction: Benjamini and Hochberg false discovery rate; cutoff p value: 1%). In the two-way ANOVA, a main effect for gene expression of each gene was either only age or only brain region. Data showing a main effect of only age were analyzed using one-way ANOVA with a *post hoc* multiple comparison test (Tukey's HSD method) to find genes differentially expressed between 3 M and 6 M in all brain regions examined. To avoid false positive results, differentially expressed genes were further filtered so that the absolute fold change (FC) value of gene expression between 3 M and 6 M was >1.2 (Supplemental Table 1). FC was calculated as follows: when gene expression at 3 M was greater than that at 6 M, the former was divided by the latter, and when gene expression at 6 M was greater than that at 3 M, the former was divided by the latter and expressed as a negative value (−). Thus, negative FC values indicate that gene expression increased from 3 M to 6 M. Information on the selected genes is presented in Supplemental Table 1. To understand the biological implications of certain expression patterns, these data were analyzed with IPA software (Qiagen), which uses information gathered from the scientific literature (Table 1). We further confirmed the results with those reported in original literature.

3. Results and discussion

3.1. Glutamate receptor signaling

Glutamate and its receptor are important for excitatory transmission and functional and morphological plasticity [4]. Whether plasticity is potentiation or depression depends on many factors

Table 1
Canonical pathway most likely related to controlling spine number, based on differentially expressed genes between 3 months (3 M) and 6 months (6 M) in all areas.

Pathway	p-value	
Glutamate Receptor Signaling	7.24E−03	DLG4, GLS, GNG2, GNG7, GRIA1, GRIK1, GRID2, GRIN3A, SLC17A6, SLC1A6
G q signaling	2.95E−02	ADRA1D, GNA14, GNB4, GNG2, GNG4, GNG7, HTR2A, ITPR1, NAPEPLD, NFATC3, PIK3C2B, PIK3CG, PIK3R1, PLCB4, PPP3CA, PRKCG, ROCK2
G s signaling	2.55E−01	D1DR, HTR4, OPRK1
Amyotrophic lataral Scolerosis pathway	4.68E−05	CAPN5, PIK3C2B, RAB5C, CACNA1D, SOD1, CASP3, GRID2, GRIA1, PIK3R1, NEFH, CACNA1A, XIAP, GRIN3A, IGF1, PIK3CG, NEFM, BID, PPP3CA, GRIK1
Reelin signaling in neuron	2.09E−02	ARHGEF2, ARHGEF10, DAB1, DCX, ITGB2, PIK3C2B, PIK3CG, PIK3R1, RELN, VLDLR, YES1

Red letter means that expression at 3M was larger than that at 6M. Black letters mean vice versa.

such as timing and volume of release of glutamate, other neurotransmitters and neuromodulators, and downstream genes [4]. In this paper , we discuss the gene expression data of glutamate receptors, receptor scaffolding, and glutamate scavengers.

3.1.1. GRIA1

GRIA1 belongs to the AMPA receptor family [9]. GRIA1 was downregulated (FC: 1.21) at 6 M, which may be related to the decrease in synapse number [3].

3.1.2. GRIN3A

GRIN3A represents 90% of NMDA glutamate receptors. Heteromeric NMDA receptor, co-expressed with GRIN3A, shows reduced NMDA synaptic currents [10], whereas *GRIN3A* knockout mice show increased receptor current and density of dendritic spines [11]. *GRIN3A* decreased at 6 M (FC: 1.89), which may cause an increase in NMDA current, suggesting that strong NMDA excitatory currents may be required so that a single neuron could receive sufficient net synaptic activity to compensate for the synaptic decrease.

3.1.3. GRIK1

GRIK1 belongs to the kainate receptor family, which does not have calcium permeability. Kainate receptors exhibit more limited distribution than AMAP receptors and have different modulators. *GRIK1* expression decreased (FC: 1.27) at 6 M. Again, this may be related to decreased synapse number.

3.1.4. DLG4

DLG4 encodes PSD-95, which is almost exclusively located in the postsynaptic density of neurons and is involved in anchoring synaptic proteins. Its direct and indirect binding partners include neuroligin, NMDA receptors, AMPA receptors, and potassium channels. *DLG4* was downregulated (FC: 1.20) at 6 M.

3.1.5. GLS

GLS encodes glutaminase, which generates glutamate from glutamine. The glutamate released from presynaptic boutons is taken up by astrocytes and immediately converted to glutamine. GLS modulates this glutamine-to-glutamate transition to pack presynaptic vesicles. *GLS* was upregulated (FC: −1.28) at 6 M, suggesting that a more active synaptic release occurs at 6 M.

The ratio of differentially expressed *GRIN3A* (calcium-permeable NMDA receptor; FC: 1.89) between 3 M and 6 M was larger than that of receptors belonging to the AMPA and kainate receptor families, and the results obtained for its binding partner DLG4 (1.21, 1.27, and 1.20, respectively) suggest a lower density of glutamate-dependent stable synapses at 6 M. However, it may be balanced by an increase in glutamate release mediated by *GLS* expression (see above).

3.2. G protein-coupled receptors and their ligands

G protein-coupled receptors and their ligands are related to synaptic plasticity, and thus synapse number.

3.2.1. Gαq-type

ADRA1D (adrenaline receptor) and HTR2A (serotonin receptor) upregulate a heterotrimeric G protein subunit that activates phospholipase C (PLC). PLC, in turn, hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacyl glycerol and inositol triphosphate in a signal transduction pathway. *ADRA1D* and *HTR2A* were upregulated (−1.30 and −1.22, respectively) at 6 M. *ROCK2*, which acts downstream of these receptors [12], was downregulated (FC: 1.27) at 6 M, suggesting that it helps decrease synapse number at 6 M [12].

3.2.2. Gαs-type

DRD1 (a dopamine receptor), HTR4 (a serotonin receptor), and OPRK1 (a mu-opioid receptor) are heterotrimeric G protein subunits that activate the cAMP-dependent pathway by activating adenylate cyclase, which causes long-term potentiation, long-term depression, and depotentiation depending on the final effect of the cAMP-responsive element-binding protein [13]. *DRD1* and *HTR4* were upregulated (FC: −1.44 and −1.44, respectively) at 6 M. *OPRK1* expression decreased (FC: 1.29) at 6 M. The effect of these receptors on synaptic number should be confirmed during the pruning phase.

3.3. Protectors of normal tissue and mitochondria and anti-inflammation

Immunoglobulins, HLA, and complement-tagged synapses were phagocytized at 6 M. Phagocytosis can simultaneously damage non-tagged normal tissue and mitochondria, particularly by superoxide, the caspase system, and the complement system [14,15]. From the IPA (Qiagen) canonical pathway sets, the second most plausible (second smallest p value) canonical pathway based on the selected gene list was the amyotrophic lateral sclerosis system. In this disease, tissue and mitochondria are damaged by the superoxide and caspase systems [15]. We analyzed protector genes against the superoxide and caspase systems using this canonical pathway. Genes belonging to other systems will also be considered.

3.3.1. Sod1

SOD1 is a soluble cytoplasmic protein that acts as a homodimer to convert naturally occurring but harmful superoxide radicals to molecular oxygen and hydrogen peroxide [16]. Another isozyme protects mitochondria. *SOD1* was upregulated (FC: −1.27) at 6 M, suggesting that SOD1 plays a more protective role in neuronal structures at 6 M, when pruning occurs, than at 3 M.

3.3.2. Xiap

XIAP inhibits at least two members of the caspase family of cell death proteases: caspase-3 and caspase-7 [17]. *XIAP* expression increased (FC: −1.32) at 6 M, suggesting that this gene inhibits caspase activity to protect brain cells.

3.3.3. CD46 and CD55

As described in the complement system section in our previous study, these molecules inhibited inflammation at 6 M.

3.4. Reelin system

Reelin plays an important role in layer formation [18]. However, reelin has several receptors, and each receptor exhibits a different function. *RELN* itself was downregulated (FC: 1.33) at 6 M.

3.4.1. Reelin–ApoER2 pathway

ARHGEF2 and ARHGEF10, which act downstream of this pathway, are involved in cytoskeletal rearrangement [19,20]. *ARHGEF2* and *ARHGEF10* were downregulated (CF: 1.23 and 1.33) at 6 M, suggesting a lower number of new synapses at this time point.

3.4.2. Reelin-VLDLR pathway

VLDLR was downregulated (FC: 1.27) at 6 M. DAB1, which acts downstream of this pathway, accelerates dendritic spine formation [21,22]. *DAB1* expression decreased (FC: 1.23) at 6 M.

3.4.3. Reelin–CNR1/2 pathway

Doublecortin acts downstream of this pathway through Fyn and contributes to determine axonal connections [12]. Doublecortin

was downregulated (FC: 1.67) at 6 M, suggesting that axonal connections exhibited a less determined position and probably occurred at more appropriate locations at 6 M.

4. Conclusions

In summary, glutamate is the most common neurotransmitter. Its receptors *AMPA1, GRIK1*, and their scaffold protein *DLG4* decreased as dendritic spine number decreased. *GRIN3A* (NMDA receptor) increased, but the NMDA complex containing GRIN3A decreased NMDA synaptic currents. However, *GLS* upregulation suggested that there was more net glutamate release at 6 M probably to compensate synaptic currents enough for single neurons to fire appropriately. (2) Gαs protein-coupled receptor genes (*ADRA1D* and *HTR2A*) in a selected list were upregulated at 6 M. The downstream *ROCK2* of these receptor systems plays a role decreasing synapses, and *ROCK2* decreased at 6 M. Gαs protein-coupled receptor genes (*DRD1, HTR4,* and *OPRK1)* had a bidirectional effect on synaptic number. (3) *SOD1, XIAP, CD46*, and *CD55*, which have a protective role in normal tissue and mitochondria, showed higher expression at 6 M, suggesting that normal brain tissue is more protected at 6 M, probably against active pruning. (4) *RELN* expression and three different pathways to reelin at 6 M suggested that new synapse formation decreased at this age. Moreover, axonal connections exhibited a less determined position and probably occurred at a more appropriate location at 6 M.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.023.

References

[1] T. Sasaki, T. Oga, K. Nakagaki et al., Developmental expression profiles of axon guidance signaling and the immune system in the marmoset cortex: Potential molecular mechanisms of pruning of dendritic spines during primate synapse formation in late infancy and prepuberty (I), Biochem Biophys Res Commun. (accompanying paper-a).
[2] M. Missler, A. Wolff, H.J. Merker, J.R. Wolff, Pre- and postnatal development of the primary visual cortex of the common marmoset. II. Formation, remodelling, and elimination of synapses as overlapping processes, J. Comp. Neurol. 333 (1993) 53–67.
[3] T. Oga, H. Aoi, T. Sasaki, I. Fujita, N. Ichinohe, Postnatal development of layer III pyramidal cells in the primary visual, inferior temporal, and prefrontal cortices of the marmoset, Front. Neural Circuits 7 (2013) 31.
[4] C. Lohmann, H. Kessels, The developmental stages of synaptic plasticity, J. Physiol. 592 (2014) 13–31.
[5] P. Licznerski, R.S. Duman, Remodeling of axo-spinous synapses in the pathophysiology and treatment of depression, Neuroscience 251 (2013) 33–50.
[6] J. Bylund, K.L. Brown, C. Movitz, C. Dahlgren, A. Karlsson, Intracellular generation of superoxide by the phagocyte NADPH oxidase: how, where, and what for?, Free Radic. Biol. Med. 49 (2010) 1834–1845.
[7] P. Dibaj, H. Steffens, J. Zschüntzsch, F. Nadrigny, E.D. Schomburg, F. Kirchhoff, C. Neusch, In Vivo imaging reveals distinct inflammatory activity of CNS microglia versus PNS macrophages in a mouse model for ALS, PLoS One 6 (2011) e17910.
[8] J.N. McClintick, H.J. Edenberg, Effects of filtering by present call on analysis of microarray experiments, BMC Bioinformatics 7 (2006) 49.
[9] R.L. Huganir, R.A. Nicoll, AMPARs and Synaptic Plasticity: The Last 25 Years, Neuron 80 (2013) 704–717.
[10] N.J. Sucher, S. Akbarian, C.L. Chi, C.L. Leclerc, M. Awobuluyi, D.L. Deitcher, M.K. Wu, J.P. Yuan, E.G. Jones, S.A. Lipton, Developmental and regional expression pattern of a novel NMDA receptor-like subunit (NMDAR-L) in the rodent brain, J. Neurosci. 15 (10) (1995) 6509–6520.
[11] L. Olalla, A. Gutiérrez, J.A. Campos, et al., Nuclear localization of L-type glutaminase in mammalian brain, J. Biol. Chem. 277 (2002). 38939-38344.
[12] Z. Zhou, Y. Meng, S. Asrar, Z. Todorovski, Z. Jia, A critical role of Rho-kinase ROCK2 in the regulation of spine and synaptic function, Neuropharmacology 56 (2009) 81–89.
[13] H. Wang, M. Zhang, The role of Ca^{2+}-stimulated adenylyl cyclases in bidirectional synaptic plasticity and brain function, Rev. Neurosci. 23 (2012) 67–78.
[14] Y. Wang, Y. Li, S.L. Dalle Lucca, M. Simovic, G.C. Tsokos, J.J. Dalle Lucca, Decay accelerating factor (CD55) protects neuronal cells from chemical hypoxia-induced injury, J. Neuroinflammation. 7 (2010) 24.
[15] J.D. Rothstein, Current hypotheses for the underlying biology of amyotrophic lateral sclerosis, Ann. Neurol. 65 (Suppl. 1) (2009) S3–S9.
[16] E.F. Barrett, J.N. Barrett, G. David, Mitochondria in motor nerve terminals: function in health and in mutant superoxide dismutase 1 mouse models of familial ALS, J. Bioenerg. Biomembr. 43 (2011) 581–586.
[17] T. Nakamura, D.H. Cho, S.A. Lipton, Redox regulation of protein misfolding, mitochondrial dysfunction, synaptic damage, and cell death in neurodegenerative diseases, Exp. Neurol. 238 (2012) 12–21.
[18] M. Frotscher, Role for Reelin in stabilizing cortical architecture, Trends Neurosci. 33 (2010) 407–414.
[19] N.B. Myant, Reelin and apolipoprotein E receptor 2 in the embryonic and mature brain: effects of an evolutionary change in the apoER2 gene, Proc. Biol. Sci. 277 (2010) 345–351.
[20] S.S. Reddy, T.E. Connor, E.J. Weeber, W. Rebeck, Similarities and differences in structure, expression, and functions of VLDLR and ApoER2, Mol. Neurodegener. 6 (2011) 30.
[21] S. Niu, A. Renfro, C.C. Quattrocchi, M. Sheldon, G. D'Arcangelo, Reelin Hellwig, et al., Reelin promotes hippocampal dendrite development through the VLDLR/ApoER2-Dab1 pathway, Neuron 41 (2004) 71–84.
[22] S. Niu, O. Yabut, G. D'Arcangelo, The Reelin signaling pathway promotes dendritic spine development in hippocampal neurons, J. Neurosci. 28 (2008) 10339–10348.