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Short Communication

Gene expression profile in retinal excitotoxicity induced by L-glutamate in neonatal rats

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Abstract: In neonatal rats, glutamate could induce retinal thinning depending on the development stage, and the severity peaked at treatment on postnatal day (PND) 8. To elucidate the molecular mechanism of retinal thinning induced by L-glutamate in neonatal rats, we investigated the time-course gene expression profile in the developing retina in addition to initial histopathological changes. Histopathologically, apoptotic cells in the inner retina were observed at 6 hours after treatment on PNDs 4, 6 and 8, and inflammatory cell infiltration was noted at 24 hours. Comprehensive gene expression analysis conducted on PNDs 4 and 8 indicated that cell death/proliferation- and inflammation-related genes were upregulated and that neuron development- and neurotransmitter-related genes were downregulated. Furthermore, quantitative RT-PCR analysis of apoptosis- and inflammation-related genes performed on PNDs 4, 6, 8, 10 and 12 showed that the time-course changes of the gene expression ratios of *Gadd45b* and *Ccl3* seemed to be related to histopathological changes of the retina induced by L-glutamate. These results revealed that the association of initial histopathological changes with the gene expression profile in the retina induced by L-glutamate and that *Gadd45b* and *Ccl3* are considered to participate in retinal thinning induced by L-glutamate in neonatal rats. (DOI: 10.1293/tox.2018-0026; J Toxicol Pathol 2018; 31: 301–306)

Key words: gene expression profile, L-glutamate, neonatal rat, apoptosis, retinal excitotoxicity

Excitotoxicity is a phenomenon in which neuronal cells are damaged or killed by excessive stimulation with excitatory neurotransmitters such as glutamate and is linked to stroke, hypoglycemia, trauma, epilepsy, and chronic neurodegenerative diseases such as Huntington's disease, acquired immunodeficiency syndrome dementia complex, amyotrophic lateral sclerosis, and Alzheimer's disease[1, 2]. In the retina, excitotoxicity is believed to play an important role in retinal ischemia/reperfusion injury and neuronal loss in glaucoma[3–5].

Glutamate-induced retinal damage in neonatal rats is a well-known animal model of glutamate-induced excitotoxicity. The retina is not fully developed in newborn rats, taking approximately 3 weeks to mature[6], and the degree of retinal damage depends on the age at glutamate administration[7, 8]. Recently, the developing stage-dependent retinal thinning induced by L-glutamate in neonatal rats has been reported in detail[9]. Newborn rats received a single subcutaneous administration of L-glutamate on postnatal day (PND) 1 to 14. The inner retina on PND 21 exhibited thinning in rats treated after PND 2. The thinning was most marked in rats treated on PND 8: inner retina was almost lost. No thinning was observed in rats treated on PND 14. The neurotoxic effects of glutamate are mainly mediated by stimulation of N-methyl-d-aspartate (NMDA) receptors[10, 11], one of the ionotropic glutamate receptors. NMDA-type glutamate-gated channels have relatively high permeability to calcium ions and could induce apoptosis at high levels in the cytoplasm[12]. Retinal ganglion cells (RGC) are known to express NMDA receptors[13, 14], and glutamatergic excitotoxicity mediated by NMDA receptors has been demonstrated to significantly contribute to RGC injury both *in vitro* and in animal models[10, 15–17]. Furthermore, intravitreal injection of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainic acid, agonists of other types of ionotropic glutamate receptors, can also induce RGC injury[18, 19]. Hence, multiple types of glutamate receptors might participate in glutamate-induced RGC injury. However, it is difficult to clarify a pathogenesis of severe retinal thinning induced by L-glutamate.

Here, we investigated the initial histopathological changes and time-course gene expression profile in the retina of neonatal rats administrated L-glutamate subcutaneously as a single dose on each PND to reveal the molecular

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mechanism of retinal thinning induced by L-glutamate in neonatal rats.

Female Sprague-Dawley (SD) rats at gestational day 13 purchased from Charles River Laboratories Japan (Shiga, Japan) were maintained under specific pathogen-free conditions, with *ad libitum* access to a commercial diet (CRF-1 30 kGy; Oriental Yeast, Tokyo, Japan) and water. Pregnant animals were housed individually in plastic cages with paper-chip bedding in an air-conditioned room at 23 ± 3°C and 55 ± 15% relative humidity with a 12-h light/dark cycle. Animals were maintained and treated in accordance with the Guide for the Care and Use of Laboratory Animals at our institution, which is certified by AAALAC. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc.

A total of 70 male and female neonatal SD rats were used in this study. Four neonatal SD rats on each of PNDs 4, 6, 8, 10 and 12 were given a single subcutaneous administration of monosodium L-glutamate (Sigma-Aldrich, St. Louis, MO, USA) at 10 μL of 2.4 mM glutamate/mg body weight. All rats were weighed at the time of L-glutamate treatment. Two rats were euthanized in each group by exsanguination under isoflurane anesthesia at 6 and 24 hours after administration of L-glutamate. The eyes were removed immediately after sacrifice, fixed with 4% phosphate-buffered glutaraldehyde, postfixed in 5% phosphate-buffered formalin, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin (HE) for histopathological examination. In addition, six neonatal SD rats on each of PNDs 4, 6, 8, 10 and 12 were given a single subcutaneous administration of monosodium L-glutamate (Sigma-Aldrich) at the same dosage. All rats were euthanized by exsanguination under isoflurane anesthesia at 6 hours after administration of L-glutamate. The eyes of six non-treated rats on PNDs 4, 6, 8, 10 and 12 served as normal controls. The posterior eyecups were stored in RNAlater® solution (Thermo Fisher Scientific, Waltham, MA, USA) at −80°C until use.

Total RNAs for GeneChip and quantitative RT-PCR (qRT-PCR) analyses were isolated from the eyecups using an RNeasy® Mini Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. The amounts of total RNA were determined using a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA).

The mRNAs of five animals per group from the control and treated groups were used for GeneChip analysis. The cDNA was synthesized using a GeneChip® 3′ IVT Reagent Kit (Affymetrix, Santa Clara, CA, USA) and analyzed using a GeneChip® Rat Genome 230 2.0 Array (Affymetrix), which contains 31,000 probe sets. We selected mRNAs showing more than 2-fold or less than 0.5-fold changes. QIAGEN's Ingenuity Pathway Analysis (IPA; QIAGEN) was used to analyze the function of selected mRNAs.

The mRNAs of six animals per group (five animals from the treated group on PND 8) were used for qRT-PCR analysis of *Gadd45b, Nfkbia, Xdh, Ccl2, Ccl3* and *Cxcl2*. The cDNAs were synthesized from 2.5 μg total RNA using SuperScript® VILO™ cDNA Master Mix (Thermo Fisher Scientific). TaqMan® Fast Advanced PCR Master Mix and TaqMan® Gene Expression Assays (Thermo Fisher Scientific) were added to the cDNA sample, and real-time PCR was performed using a QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific). A standard curve was prepared using a sample of normal retina, and the relative quantity for each sample was measured. The average relative quantity corrected using the levels of *Gapdh* was calculated, and the ratio of the values of the treated group to the normal control group was calculated. The assay IDs of the mRNAs are shown in Table 1.

Statistically significant differences in the gene expression ratio of each PND were analyzed using Tukey's multiple comparison test or Dunn's multiple comparison test after confirming equal variance using Bartlett's test. The cutoff for statistical significance was set at $p<0.05$.

As a result of histopathological examination, many pyknotic nuclei appeared in the inner retina at 6 hours after administration of L-glutamate on PNDs 4, 6 and 8.There were very few pyknotic nuclei in rats treated on PNDs 10 and 12. The majority of pyknotic nuclei were located in the inner area of inner nuclear layer. Furthermore, neutrophils and a few macrophages infiltrated in the inner retina at 24 hours after administration, and the inflammation was severer on PNDs 6 and 8 than on PND 4 (Fig. 1).

The results of comprehensive analysis of gene expression using the samples on PNDs 4 and 8 are shown in Fig. 2. We revealed that the expression levels of 112 and 174 mRNAs differed significantly between the normal and treated groups on PNDs 4 and 8, respectively. Among them, the levels of 68 mRNAs changed on both PNDs 4 and 8. The expression levels of 20, 45 and 41 genes were upregulated over 2-fold, and those of 24, 61 and 27 genes were downregulated less than 0.5-fold on PND 4, 8, and both PNDs 4

Table 1. Assay IDs of mRNAs Used for TaqMan qRT-PCR

Symbol	Name	Assay ID
Gadd45b	growth arrest and DNA-damage-inducible, beta	Rn01452530_g1
Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	Rn01473658_g1
Xdh	xanthine dehydrogenase	Rn00567654_m1
Ccl2	chemokine (C-C motif) ligand 2	Rn01456716_g1
Ccl3	chemokine (C-C motif) ligand 3	Rn01464736_g1
Cxcl2	chemokine (C-X-C motif) ligand 2	Rn00586403_m1

and 8, respectively. In addition, when the functions of these genes were analyzed with IPA, upregulated genes were mainly cell death/proliferation- and inflammation-related genes and that downregulated genes were related to neuron development and neurotransmitter.

To further investigate the expression of genes linked to the histopathological changes, time-course changes of the expression ratios of apoptosis- and inflammation-related genes were analyzed on PNDs 4, 6, 8, 10 and 12 using qRT-PCR. We selected 3 genes each for apoptosis- and inflammation-related genes for a qRT-PCR analysis based on pathway analysis with IPA: *Gadd45b, Nfkbia* and *Xdh* related to apoptosis, and *Ccl2, Ccl3* and *Cxcl2* related to inflammation (Table 1). The results of qRT-PCR analysis are shown in Fig. 3. The expression ratio of *Gadd45b* on PND 8 peaked and was significantly different from the ratio on PNDs 4 and 12, corresponding to the severity of retinal thinning in histopathological examination[9]. The mean values for the expression ratios of *Nfkbia* and *Xdh* was highest on PNDs 10 and 6, respectively. The expression ratio of *Ccl3* showed higher values on PNDs 6 and 8 than PNDs 4, 10 and 12, which also corresponded to the severity of retinal thinning[9]. The expression ratios of *Ccl2* on PNDs 10, 12 and *Cxcl2* on PNDs 8, 10 and 12 showed high values and great variations. There was no difference in gene expression levels between male and female rats.

The present study indicated the initial histopathological changes and time-course gene expression profile related

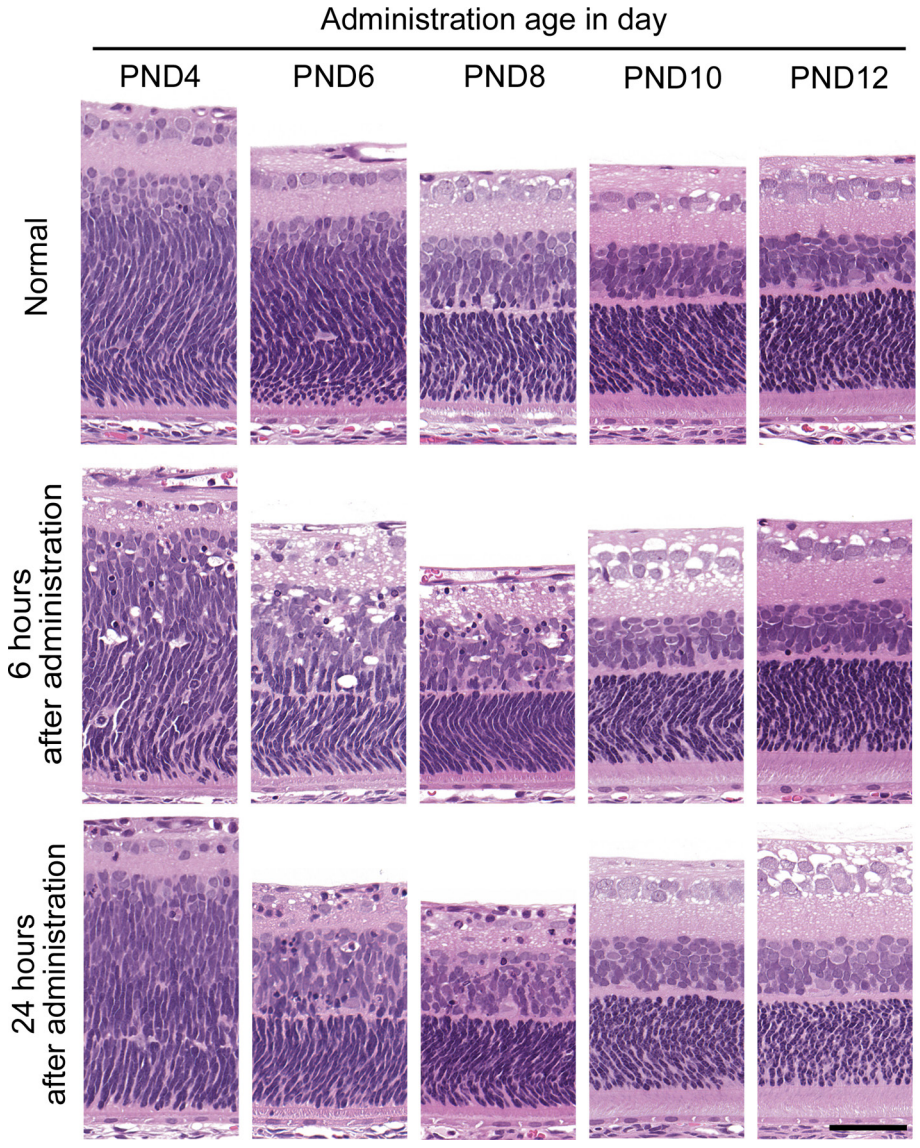
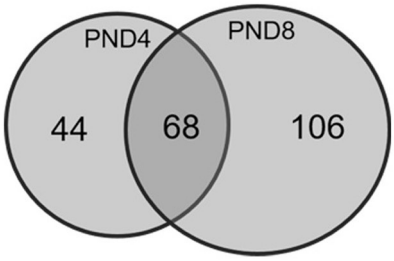


Fig. 1. Initial changes induced by L-glutamate in the inner retina on postnatal days (PNDs) 4, 6, 8, 10 and 12. Many pyknotic nuclei appeared in the inner retina at 6 hours after administration of L-glutamate on PNDs 4, 6 and 8.There were very few pyknotic nuclei in rats treated on PNDs 10 and 12. Pyknotic nuclei were localized in the inner area of inner nuclear layer. Neutrophils and a few macrophages infiltrated in the inner retina at 24 hours after administration on PNDs 4, 6 and 8, and the inflammation was severer on PNDs 6 and 8 than on PND 4. HE stain. Bar = 50 µm

A

	2-fold↑	0.5-fold↓
PND4	20	24
PND8	45	61
PND4 and 8	41	27



B

		cell death/ proliferation	inflammation	neuron development	neuro transmitter
PND4	2-fold↑	11	9	3	1
	0.5-fold↓	2	1	4	3
PND8	2-fold↑	15	16	5	2
	0.5-fold↓	7	6	16	11
PND4 and 8	2-fold↑	22	24	5	2
	0.5-fold↓	4	3	9	5

Fig. 2. Overview of the analyses of mRNA levels in the retina. The number of mRNAs with ratios of changes upregulated over 2-fold or downregulated less than 0.5-fold (A) and the number of mRNAs related to cell death/proliferation, inflammation, neuron development and neurotransmitter (from IPA) (B). The expression levels of 112 and 174 mRNAs differed significantly between the normal and treated groups on postnatal days (PNDs) 4 and 8, respectively. Among them, the levels of 68 mRNAs changed on both PNDs 4 and 8. The expression levels of 20, 45 and 41 genes were upregulated over 2-fold and those of 24, 61 and 27 genes were downregulated less than 0.5-fold on PND 4, 8, both PNDs 4 and 8, respectively (A). When the functions of these genes were analyzed with IPA, cell death/proliferation- and inflammation-related genes were mainly upregulated, and neuron development- and neurotransmitter-related genes were downregulated (B).

to retinal damage induced by L-glutamate in neonatal rats. Histopathologically, many pyknotic nuclei appeared in the inner retina at 6 hours after administration of L-glutamate on PNDs 4, 6, and 8. The number of pyknotic nuclei peaked on PND 8. They were localized in the inner area of inner nuclear layer. It is suggested that pyknotic nuclei indicate apoptosis because we have previously reported that there were many TUNEL-positive cells in the inner area of inner nuclear layer[9]. It has previously been shown that glutamate-induced retinal damage correlates with apoptosis as inferred from caspase-3 and caspase-9 activation and DNA fragmentation[8, 10, 20]. Furthermore, inflammatory cell infiltration was noted in the inner retina at 24 hours after administration on PNDs 4, 6 and 8, and it was severer on PNDs 6 and 8 than on PND 4 and was considered a reactive changes against apoptosis. Apoptosis caused by L-glutamate followed by inflammation induced thinning of the inner retina on PND 21[9]. It was considered that there was no difference in histopathological changes in glutamate-induced retinal damage between males and females because no gender-related differences have been observed in retinal excitotoxicity[19, 21].

The comprehensive analysis of the gene expression using mRNA isolated from the retina of the rats treated with L-glutamate on PNDs 4 and 8 revealed upregulated cell death/proliferation- and inflammation-related genes and downregulated neuron development- and neurotransmitter-related genes. Since treatment of the neonatal rats with L-glutamate on PNDs 4 to 8 induced apoptosis of retinal cells followed by inflammatory cell infiltration, upregulated genes on PNDs 4 and 8 are considered to be closely related to the pathogenesis of histopathological changes. Furthermore, since retinal thinning was severer in the retina on PND 4 than that on PND 8[9], the difference in the severity of the retinal thinning might depend on the genes with different expression profiles between PNDs 4 and 8. It was thought that neuron development and neurotransmitter-related genes were downregulated because functions of developing retinal cells were deteriorated by L-glutamate treatment in neonatal rats.

We also investigated the time-course changes of the apoptosis- and inflammation-related genes using qRT-PCR analysis. The expression ratio of *Gadd45b* peaked on PND 8, and the ratio of *Ccl3* indicated high values on PNDs 6 and 8, corresponding to the severity of the retinal thinning in histopathological examination[9]. Therefore, *Gadd45b* and *Ccl3* might be related to severity to the retinal thinning induced by L-glutamate in neonatal rats. *Gadd45* was implicated in stress signaling in response to physiological or environmental stressors, which results in cell cycle arrest, DNA repair, cell survival and senescence, or apoptosis[22]. The function of *Gadd45b* in this model was not clear in detail; however, it was indicated that *Gadd45b* might be involved in apopto-

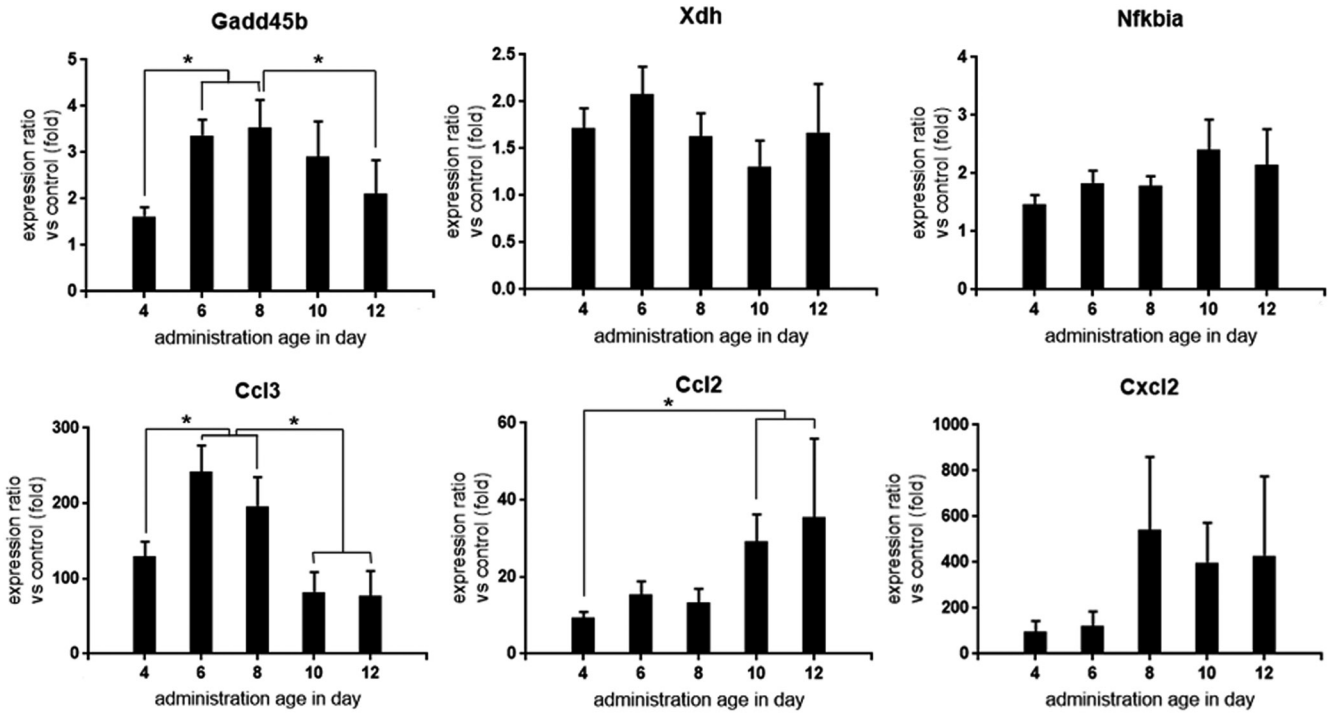


Fig. 3. Expression ratios of *Gadd45b, Nfkbia, Xdh, Ccl3, Ccl2* and *Cxcl2* mRNA in the retina of rats treated with L-glutamate on each postnatal day (PND). In the apoptosis-related genes, the expression ratio of *Gadd45b* peaked on PND 8, corresponding to the severity of the retinal thinning. The mean values of the expression ratios of *Nfkbia* and *Xdh* were highest on PNDs 10 and 6, respectively. In the inflammation-related genes, the expression ratio of *Ccl3* showed higher values on PNDs 6 and 8 than PNDs 4, 10 and 12, corresponding to the severity of the retinal thinning. The ratios of *Ccl2* on PNDs 10, 12 and *Cxcl2* on PNDs 8, 10, 12 showed high values and great variations. *Statistically significant difference in gene expression ratio ($p<0.05$).

sis due to retinal excitotoxicity. *Ccl3* is a chemokine mainly produced from macrophages, and its receptors are Ccr1 and Ccr5. Ccr1 and Ccr5 are expressed in various immune cells, T and B cells, neutrophils, macrophages, dendritic cells, or NK cells, and it was noted that an inflammatory response could be induced by *Ccl3* stimulation[23, 24]. It was shown that *Ccl3* might contribute to inflammatory cell infiltration in this model.

In summary, we demonstrated that initial histopathological changes and time-course gene expression profile in a retinal excitotoxicity model using neonatal rats. It was suggested that the thinning of the inner retina was attributed to apoptosis and inflammation induced by L-glutamate. Cell death/proliferation- and inflammation-related genes were rapidly upregulated, and neuron development- and neurotransmitter-related genes were downregulated. Since retinal thinning was severer in the retina on PND 4 than that on PND 8[9], the difference in the severity of the retinal thinning might depend on the genes with different expression profiles between PNDs 4 and 8. The expression ratio of *Gadd45b* and *Ccl3* corresponded to the degree of retinal thinning in the histopathological examination. Therefore, *Gadd45b* and *Ccl3* are considered to be related to retinal thinning induced by L-glutamate. These findings would be helpful in understanding the retinal degenerative diseases related to excitotoxicity and in developing a therapy for these diseases. More detailed investigations of the molecular analysis uncovered in this study will provide a better understanding of glutamate-induced excitotoxicity in the retina.

Disclosure of Potential Conflicts of Interest: The authors have no conflicts of interest to be disclosed in relation to this paper.

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