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Abnormally increased surface expression of AMPA receptors in the cerebellum, cortex and striatum of *Cln3*^{-/-} mice

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Abstract

Mutations in the *CLN3* gene cause a fatal neurodegenerative disorder, juvenile CLN3 disease. Exploring the cause of the motor coordination deficit in the *Cln3*^{-/-} mouse model of the disease we have previously found that attenuation of AMPA receptor activity in 1-month-old *Cln3*^{-/-} mice significantly improves their motor coordination [20]. To elucidate the mechanism of the abnormally increased AMPA receptor function in *Cln3*^{-/-} mice, we examined the surface expression of AMPA receptors using surface cross-linking in brain slices from 1-month-old wild type (WT) and *Cln3*^{-/-} mice. In surface cross-linked brain samples, Western blotting for AMPA receptor subunits revealed significantly increased surface levels of GluA1 and GluA2 in the cerebellum, and of GluA2 in the cortex and striatum of *Cln3*^{-/-} mice as compared to WT mice. Expression levels of the GluA4 subunit were similar in the cerebellum of WT and *Cln3*^{-/-} mice. While intracellular GluA1 levels in the WT and *Cln3*^{-/-} cerebellum or cortex were similar, the intracellular expression of GluA1 in the *Cln3*^{-/-} striatum was decreased to 56% of the WT level.

Our results show a prominent increase in AMPA receptor surface expression in the brain of *Cln3*^{-/-} mice and suggest that CLN3 is involved in the regulation of AMPA receptor surface expression.

Keywords

juvenile neuronal ceroid lipofuscinosis; Batten disease; CLN3; glutamate receptor; AMPA receptor; surface expression

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Conflict of interest

None of the authors of this paper has any conflict of interest relating to the publication.

Author contributions

A.D.K. and D.A.P. designed the study; A.D.K. and C.H. performed the experiments; A.D.K. analyzed the data; A.D.K. and D.A.P. wrote the article.

1. Introduction

Ion channel-forming, ionotropic receptors mediate the fast excitatory neurotransmission by glutamate in the mammalian central nervous system. Named after their specific agonists, ionotropic glutamate receptors are classified into 3 groups: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), N-methyl-D-aspartate (NMDA) and kainate receptors [38]. AMPA receptors are heterotetramers formed by different combinations of four subunits (GluA1-4), and the subunit composition largely determines the functional properties of the receptor. For instance, the presence of the GluA2 subunit makes AMPA receptors calcium-impermeable [31]. Abnormal glutamate neurotransmission and glutamate receptor overactivation play a central role in the pathophysiology of a number of neurodegenerative disorders, e.g., Parkinson's disease, amyotrophic lateral sclerosis and Huntington's disease [6].

Neuronal ceroid lipofuscinoses, also called Batten disease, are a group of recessively inherited, fatal lysosomal storage disorders characterized by the intracellular accumulation of autofluorescent lipopigment and progressive neurodegeneration [32]. Batten disease mostly affects children. It is a rare disease with an estimated incidence of 1-2 in 50,000 live births in the US. Mutations of the *CLN3* gene cause the majority of the most prevalent, juvenile onset form of Batten disease, and this disorder is now called juvenile CLN3 disease to clearly identify the genetic cause and clinical form [11, 41]. The disease begins between 4 and 10 years of age, and reaches its terminal stage in the late teens or early 20s. *CLN3* encodes a putative lysosomal transmembrane protein but the exact function of *CLN3* and why *CLN3* mutations cause selective neurodegeneration are still unknown. Several studies indicated a role of *CLN3* in endocytosis and intracellular trafficking of membrane vesicles [15, 26, 27, 39].

The *Cln3*-knockout (*Cln3*^{-/-}) mouse model of juvenile CLN3 disease displays several characteristic pathological features of the human disorder including accumulation of autofluorescent storage material in lysosomes [29] and the presence of circulating autoantibodies [8, 23]. *Cln3*^{-/-} mice, similarly to patients with juvenile CLN3 disease, show an early deficit in motor coordination as measured by the rotarod test [22, 40]. Exploring the possible cause(s) of the motor incoordination in *Cln3*^{-/-} mice we have previously found that *Cln3*^{-/-} cerebellar granule cells in dissociated cultures and in organotypic cerebellar slice cultures are significantly more sensitive to AMPA, but not NMDA, receptor-mediated toxicity than their wild type counterparts [22], indicating an abnormally enhanced AMPA receptor function. Attenuation of AMPA receptor activity in 1-month-old *Cln3*^{-/-} mice by a single intraperitoneal injection of a specific AMPA antagonist resulted in an immediate improvement of their motor skills [20], whereas acute inhibition of NMDA receptors in 1-month-old *Cln3*^{-/-} mice had no effect [21]. These results demonstrated that a selectively enhanced AMPA receptor activity largely contributes to the motor coordination deficit of *Cln3*^{-/-} mice. Since the surface expression level of glutamate receptors primarily determines their activity, in the present study we examined the surface expression of AMPA receptors in the brain of 1-month-old *Cln3*^{-/-} mice.

2. Materials and methods

2.1. Animals

In this study, 1-month-old 129S6/SvEv wild type (WT) male and homozygous *Cln3*-knockout (*Cln3*^{-/-}) male mice [29] inbred on the 129S6/SvEv background were used. All procedures were carried out according to the guidelines of the Animal Welfare Act, NIH policies, the University of Rochester (NY) Animal Care and Use Committee, and the Sanford Research Animal Care and Use Committee.

2.2. Surface cross-linking in acute brain slices

Surface cross-linking in acutely isolated brain slices was carried out as we previously described [13, 14].

2.3. Western blotting

For Western blotting, samples (30- or 50-μg total protein) were loaded and electrophoresed on a 6% Tris-HCl gel under reducing conditions; proteins were then transferred onto nitrocellulose membranes. Western blotting with antibodies to AMPA receptor subunits [rabbit anti-GluA1 (Cat. No. AB1504), 1:1,000; rabbit anti-GluA2 (Cat. No. AB1768), 1:500; rabbit anti-GluA4 (Cat. No. 06-308), 1:500; all from Millipore (Temecula, CA)] was carried out as we previously described [13, 14]. Densities of surface and intracellular bands in each lane were determined using the ImageJ program (NIH). Care was taken to avoid measuring the density of saturated bands; for the visibility of some bands, figures were made using images with longer exposure times. Band intensities in each lane were normalized to total protein determined by Ponceau S staining of the membranes as described previously [4, 5, 10, 14]. The use of total protein stains as an alternative to blotting for housekeeping proteins has been investigated and was found better than high-abundance loading controls at detecting known differences in protein level [1].

2.4. Statistical analysis

Two-way ANOVA with Bonferroni's post-test for pairwise multiple comparisons was performed using GraphPad Prism version 5.04.

3. Results

To examine if the surface expression of AMPA receptors is increased in the brain of *Cln3*^{-/-} mice, we used the method of surface cross-linking in acutely isolated brain slices from 1-month-old WT and *Cln3*^{-/-} male mice. Treatment of acutely isolated brain slices with the cell impermeable cross-linking agent, BS³ (bis[sulfosuccinimidyl] suberate), results in covalent linkage of neighboring cell surface proteins, producing large protein complexes, while intracellular proteins are not modified [5, 10, 14]. Following the BS³ treatment, surface-expressed AMPA receptors, as high-molecular-weight, cross-linked complexes, can be separated from intracellular receptor subunits by SDS-PAGE and quantified by Western blotting using antibodies that recognize the C-terminal (intracellular) part of AMPA receptor subunits [5, 10, 14] (see Fig. 1A). The surface cross-linking method has successfully been

used to study AMPA receptor surface expression in different brain regions [4, 5, 10, 13, 14, 17, 30].

Our surface cross-linking experiments revealed significantly increased surface expression of the GluA1 and GluA2 AMPA receptor subunits in the cerebellum of *Cln3*^{-/-} mice (Fig. 1A-B). GluA1 and GluA2 surface levels were increased to 157% and 234% of the WT levels. At the same time, the intracellular GluA1 and GluA2 levels in the WT and *Cln3*^{-/-} cerebellum were similar. Besides the GluA1 and GluA2 subunits, the GluA4 AMPA receptor subunit is also highly expressed in the cerebellum [31]. Therefore, we compared its cerebellar expression levels and found that surface and intracellular GluA4 levels were similar in WT and *Cln3*^{-/-} mice (Fig. 1C).

We also examined the surface and intracellular expression of GluA1 and GluA2 in surface cross-linked cortical and striatal slices. Surface GluA1 levels in the cerebral cortex and striatum of WT and *Cln3*^{-/-} mice were similar (Figs. 2A and 3A). While intracellular GluA1 levels in the WT and *Cln3*^{-/-} cortex were statistically not different (Fig. 2A), the intracellular expression of GluA1 in the *Cln3*^{-/-} striatum was decreased to 56% of the WT level (Fig. 3A). Unlike GluA1, GluA2 surface expression in the cortex and striatum was significantly higher in *Cln3*^{-/-} than in WT mice, reaching 139% and 156% of the WT GluA2 level (Figs. 2B and 3B). Since the expression level of the GluA4 AMPA receptor subunit is low in the cortex and striatum [38], surface and intracellular levels of GluA4 were not examined in these brain regions.

In conclusion, our results show a significant increase in AMPA receptor surface expression in the cerebellum, cortex and striatum of *Cln3*^{-/-} mice, a mouse model of the childhood neurodegenerative disorder, juvenile CLN3 disease. These findings provide an explanation for the previously observed therapeutic effect of AMPA receptor inhibition in this mouse model [20] and suggest that CLN3 is involved in the regulation of AMPA receptor surface expression.

4. Discussion

In the present study we showed that the surface expression of AMPA receptors is abnormally increased in the brain of 1-month old *Cln3*^{-/-} mice. This increased surface expression of AMPA receptors is obviously not neurotoxic, since neurodegeneration in *Cln3*^{-/-} mice is first detected at the age of 6 months. The elevated surface level of AMPA receptors, however, is certainly able to dysregulate normal glutamatergic neurotransmission, resulting in the pronounced early motor coordination deficit of *Cln3*^{-/-} mice [40]. This notion is supported by our previous results demonstrating that attenuation of AMPA receptor activity in 1-month old *Cln3*^{-/-} mice by a single intraperitoneal injection of a specific AMPA antagonist immediately improves their motor coordination [20].

The surface expression level of AMPA receptors, which primarily determines their activity, is regulated by receptor cycling: the process of receptor endocytosis followed by either reinsertion into the cell membrane or lysosomal degradation [34]. Considering that CLN3 is an endocytic/lysosomal membrane protein, and several studies suggested a role for CLN3 in

endocytosis and endosomal trafficking [15, 26, 39], lack of CLN3 may easily disrupt AMPA receptor recycling. Our findings, that the surface expression of both GluA1 and GluA2 (but not GluA4) AMPA receptor subunits in the cerebellum and only of GluA2 in the cortex and striatum was increased in *Cln3*^{-/-} mice, indicate a region- and subunit-specific influence of CLN3 in AMPA receptor surface expression. This region- and subunit-specific effect of CLN3 deficiency is most likely due to the inherent, regionally different regulation of AMPA receptor surface expression. Specific cytosolic and membrane proteins interacting with AMPA receptor subunits control the surface expression of AMPA receptors [2, 35]. It has been shown that many of these AMPA receptor interacting proteins display differential regional expression [3, 12, 19, 35, 37], strongly suggesting that regional differences exist in the mode how the number and subunit composition of AMPA receptors on the cell surface are regulated. Supporting this notion, remarkable differences were found in the regulation of surface expression of GluA2-containing AMPA receptors in hippocampal pyramidal neurons, cerebellar stellate interneurons and Purkinje cells [16, 24, 36, 42]. A connection between CLN3 and AMPA receptor interacting proteins is suggested by our previous Affymetrix gene expression study, in which a significant upregulation of Protein 4.1G, an AMPA receptor interacting protein that facilitates AMPA receptor surface expression [9, 33], was found in the cerebellum of *Cln3*^{-/-} mice [7]. Further studies are required to identify the exact role of CLN3 in AMPA receptor cycling.

The selectively increased surface expression of the GluA2 subunit in the cortex and striatum of *Cln3*^{-/-} mice and the most pronounced upregulation of GluA2 compared to the other subunits in the *Cln3*^{-/-} cerebellum indicate a significant decrease in the number of Ca²⁺-permeable (GluA2-lacking) AMPA receptors. Interneurons in the cortex, cholinergic interneurons in the striatum, and stellate cells and Bergman glia in the cerebellum express GluA2-lacking, Ca²⁺-permeable AMPA receptors, which play important roles in short- and long-term synaptic plasticity [25]. The decreased surface expression of GluA2-lacking, Ca²⁺-permeable AMPA receptors in the brain of *Cln3*^{-/-} mice may cause disturbances in synaptic plasticity, contributing to the manifestation of neurological deficits.

An interesting result of our study is that while the surface expression of the GluA1 subunit was similar in the striatum of WT and *Cln3*^{-/-} mice, intracellular GluA1 expression in the *Cln3*^{-/-} striatum was decreased to 56% of the WT level (Fig. 3A). This decrease may contribute to the motor learning deficit of *Cln3*^{-/-} mice [20]. The striatum has an important role in motor learning [28, 43], and the increased synaptic strength during learning is achieved by inserting new GluA1-containing AMPA receptors into the postsynaptic membrane [18]. If less intracellular GluA1 is available for synaptic insertion in the striatum that can result in a reduced capacity of motor learning.

In summary, our results show a prominent increase in AMPA receptor surface expression in the brain of *Cln3*^{-/-} mice, providing an explanation for the therapeutic effect of AMPA receptor inhibition in this mouse model of juvenile CLN3 disease. Our findings also suggest that CLN3 is involved in the regulation of AMPA receptor surface expression.

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Abbreviations

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate
NMDA	N-methyl-D-aspartate
BS³	bis[sulfosuccinimidyl] suberate
ACSF	artificial cerebrospinal fluid

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Highlights

- We examined AMPA receptor surface expression in a mouse model of CLN3 disease.
- Surface levels of GluA1 and GluA2 were elevated in the cerebellum of *Cln3*^{-/-} mice.
- Surface level of GluA2 was elevated in the cortex and striatum of *Cln3*^{-/-} mice.
- These data suggest that CLN3 is involved in AMPA receptor cycling.

Fig. 1. AMPA receptor surface expression in the cerebellum of *Cln3*^{-/-} mice

The surface and intracellular expression levels of the GluA1, GluA2 and GluA4 AMPA receptor subunits in acutely isolated cerebellar slices from 1-month-old WT and *Cln3*^{-/-} male mice were determined by surface cross-linking and Western blotting. **(A)** GluA1 surface and intracellular expression in the cerebellum. The representative Western blot shows separation of surface GluA1 (in cross-linked, high-molecular-weight complexes) and intracellular GluA1. Notice the lack of surface bands in the cerebellar sample without surface cross-linking. The graph shows quantification of surface, intracellular, and total (surface + intracellular) GluA1 expression levels in surface cross-linked cerebellar samples from 7 WT and 8 *Cln3*^{-/-} mice. Columns and bars represent mean ± SEM. **(B)** GluA2 surface and intracellular expression in the cerebellum. A representative Western blot and quantification of surface, intracellular, and total GluA2 expression levels are shown. Columns and bars represent mean ± SEM (5 WT and 5 *Cln3*^{-/-} mice). **(C)** GluA4 surface and intracellular expression levels in the cerebellum of WT and *Cln3*^{-/-} mice are similar. A representative Western blot and quantification of surface, intracellular, and total GluA4 expression levels are shown. Columns and bars represent mean ± SEM (5 WT and 5 *Cln3*^{-/-} mice). The expression levels of GluA1, GluA2 and GluA4 were normalized to the total protein levels determined by Ponceau S staining. Statistical significance was determined by 2-way ANOVA with Bonferroni's post-test for pairwise multiple comparisons: ****p*<0.001, **p*<0.05.

Fig. 2. AMPA receptor surface expression in the cerebral cortex of *Cln3*^{-/-} mice

The surface and intracellular expression levels of the GluA1 and GluA2 AMPA receptor subunits in acutely isolated cortical slices from 1-month-old WT and *Cln3*^{-/-} male mice were determined by surface cross-linking and Western blotting. **(A)** GluA1 surface and intracellular expression levels in the cortex of WT and *Cln3*^{-/-} mice are similar. The representative Western blot shows separation of surface GluA1 (cross-linked, high-molecular-weight complexes) and intracellular GluA1. The graph shows quantification of surface, intracellular, and total (surface + intracellular) GluA1 expression levels in surface cross-linked cortical samples from 4 WT and 4 *Cln3*^{-/-} mice. Columns and bars represent mean $\pm$ SEM. **(B)** GluA2 surface and intracellular expression in the cortex. A representative Western blot and quantification of surface, intracellular, and total GluA2 expression levels are shown. Columns and bars represent mean $\pm$ SEM (5 WT and 5 *Cln3*^{-/-} mice). The expression levels of GluA1 and GluA2 were normalized to the total protein levels determined by Ponceau S staining. Statistical significance was determined by 2-way ANOVA with Bonferroni's post-test for pairwise multiple comparisons: * $p < 0.05$.

Fig. 3. AMPA receptor surface expression in the striatum of *Cln3*^{-/-} mice

The surface and intracellular expression levels of the GluA1 and GluA2 AMPA receptor subunits in acutely isolated striatal slices from 1-month-old WT and *Cln3*^{-/-} male mice were determined by surface cross-linking and Western blotting. **(A)** GluA1 surface and intracellular expression in the striatum. Surface expression is similar in WT and *Cln3*^{-/-} mice but the intracellular expression is decreased in the *Cln3*^{-/-} striatum. The representative Western blot shows separation of surface GluA1 (cross-linked, high-molecular-weight complexes) and intracellular GluA1. The graph shows quantification of surface, intracellular, and total (surface + intracellular) GluA1 expression levels in surface cross-linked striatal samples from 5 WT and 5 *Cln3*^{-/-} mice. Columns and bars represent mean $\pm$ SEM. **(B)** GluA2 surface and intracellular expression in the striatum. A representative Western blot and quantification of surface, intracellular, and total GluA2 expression levels are shown. Columns and bars represent mean $\pm$ SEM (5 WT and 5 *Cln3*^{-/-} mice). The expression levels of GluA1 and GluA2 were normalized to the total protein levels determined by Ponceau S staining. Statistical significance was determined by 2-way ANOVA with Bonferroni's post-test for pairwise multiple comparisons: * $p < 0.05$.