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Regulation of connexin 36 expression during development

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Abstract

In the mammalian CNS, the expression of neuronal gap junction protein, connexin 36 (Cx36), increases during the first two weeks of postnatal development and then decreases during the following two weeks. Recently we showed that the developmental increase in Cx36 expression is augmented by chronic (2 week) activation of group II metabotropic glutamate receptors (mGluR), prevented by chronic receptor inactivation, and the receptor-dependent increase in Cx36 expression is regulated via transcriptional control of the Cx36 gene activity. We demonstrate here that acute (60 min) activation of group II mGluRs in developing cortical neuronal cultures causes transient increase in Cx36 protein expression with decrease during the following 24 hours. However, there is no change in Cx36 mRNA expression. In addition, the data indicate that transient increase in Cx36 expression is due to new protein synthesis. The results suggest that, during development, acute activation of group II mGluRs causes up-regulation of Cx36 via post-transcriptional mechanisms. However, if the receptor activation is sustained, transcriptional activation of the Cx36 gene occurs.

Keywords

Gap junctions; connexin 36; development; transcription; translation

Introduction

In the mammalian central nervous system (CNS), direct intercellular communication between neighboring cells occurs through electrical synapses (gap junctions). The coupling of neurons by gap junctions and the expression of the neuron-specific gap junction protein, connexin 36 (Cx36), increase during the first two weeks of postnatal development and play critical role in death/survival mechanisms in developing neurons [2,4]. The coupling and Cx36 expression subsequently decrease [1]. However, they increase in the mature CNS within two hours after different types of neuronal injury and play a critical role in injury-mediated neuronal death [10]. We showed recently that both prolonged (developmental) and acute (injury-mediated) increases in Cx36 protein expression are regulated by activity of

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group II metabotropic glutamate receptors (mGluR) [4,10]. However, molecular mechanisms in these two conditions are different: during development the regulation involves transcriptional mechanisms [4] while during neuronal injury the regulation is via post-transcriptional control of C×36 expression [10].

In the developmental study [4], the transcriptional control of C×36 protein expression was characterized using reverse transcription-quantitative real time polymerase chain reaction (RT-qPCR) and luciferase reporter analysis in neuronal cultures. These studies revealed a developmental increase, between day *in vitro* 3 (DIV3) and DIV15, in C×36 mRNA and C×36 gene promoter activity. In addition, we demonstrated a critical role in these mechanisms for a neuron-restrictive silencer element in the C×36 gene promoter. In the study in mature neurons subjected to injury [10], including ischemic injury, the utilization of post-transcriptional control to increase C×36 protein levels was suggested by the fact that mRNA levels did not change during the time-frame where protein levels rose. In the present study, we tested the hypothesis that, in developing neurons, acute activation of group II mGluRs induces transient up-regulation of C×36 expression via post-transcriptional mechanisms (i.e., it does not involve changes in C×36 mRNA expression) and only later, if the increased receptor activity is sustained, does the regulation of C×36 becomes transcriptional.

Material and methods

The experiments were carried out using neuronal cultures obtained from wild-type (C57bl/6 strain) mice in accordance with the National Institute of Health guidelines. The use of animal subjects was approved by the University of Kansas Medical Center Animal Care and Use Committee. Cultures were prepared and maintained as reported previously [4,10] and were obtained from the somatosensory cortex of day 16–17 mouse embryos. Cultures were raised in Neurobasal medium (Invitrogen, Carlsbad, CA, USA), in which the percentage of neurons reaches ~95%. Oxygen-glucose deprivation (OGD) was used as an ischemic injury model *in vitro* and was induced by transferring cultures for 30 min from normal conditions (95% air + 5% CO₂; 10 mM glucose) to OGD conditions (95% N₂ + 5% CO₂; no glucose). Sham OGD included change in the culture medium (with or without cycloheximide in it; see below), but no OGD. Western blot and RT-qPCR experiments were conducted using the approaches and antibodies or primers as described previously [4,10]. In western blot tests, optical density signals were normalized relative to tubulin and normalized values were compared to controls (set at 1.0). In RT-qPCR experiments, C×36 mRNA signals were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA signals, and normalized values were compared to controls (set at 1.0). Data were analyzed using the two-tailed paired Student's *t*-test or ANOVA with post hoc Tukey and InStat software (GraphPad Software, USA). Data are reported as mean ± S.E.M. for the number of samples indicated.

Results and discussion

Previous studies [4] showed that chronic (on DIV3–DIV15) administration of group II mGluR agonist (LY379268, 2 μM) in rat hypothalamic and mouse cortical neuronal cultures augments normal developmental increases in the expression C×36 protein and mRNA (as measured on DIV15). Here we tested changes in C×36 protein and mRNA expression after a short-term administration of the group II mGluR agonist in developing cortical neuronal cultures on DIV3 and DIV15. LY379268 (2 μM; Sigma-Aldrich) was added to cultures for 60 min and then washed out. The analysis was done at several time points after the drug wash out. On both DIV3 and DIV15, the expression of C×36 protein transiently increased (peaking at 3 hours post-treatment) and decreased by 24 hours post-treatment (Fig. 1a,b). However, there was no change in the expression of C×36 mRNA also for either culture (Fig.

1c,d), suggesting that the regulation of Cx36 expression likely is post-transcriptional. In addition, under OGD culture conditions on DIV15, we found that the treatment of cultures with cycloheximide (50 mg/ml; Sigma-Aldrich; a blocker of protein synthesis) prevents the ischemia-mediated increase in Cx36 (Fig. 2). This indicates that the increased Cx36 protein expression following ischemia is due to new protein synthesis, rather than changes in protein stability.

Based on the obtained results, we believe that two potential mechanisms may be responsible for the observed post-transcriptional up-regulation of Cx36 protein levels during early time points following group II mGluR agonist treatment or ischemia. First, a rapid decline in micro-RNA (miRNA)-dependent suppression of Cx36 translation may be occurring. Indeed, the 3'-untranslated region (3'UTR) of the Cx36 mRNA contains binding sites for a number of brain-specific microRNAs, including mir-9, mir-128a and mir-128b [5], that are involved in the regulation of neuronal development, differentiation and morphogenesis [7,9]. However, given the observed kinetics of the Cx36 increase, this mechanism does not seem likely to be operating.

The second possibility is activation of Cx36 mRNA translation. There are numerous mechanisms by which translation of specific mRNAs or groups of mRNAs can be increased rapidly. In particular, a number of specific mRNAs are activated under cellular stress conditions, which can be initiated by cellular signaling events. For example, translation of activating transcription factor 4 (ATF4) mRNA through a mechanism of "leaky scanning" by the small ribosomal subunit is increased under endoplasmic reticulum (ER) stress, whereas the bulk of global translation is inhibited [3]. Importantly, many different types of cellular signaling events can modulate, both positively and negatively, the ER stress pathways that result in translational activation of ATF4 [6]. We speculate that group II mGluR agonist activation falls in this category. The Cx36 mRNA contains, within its 5'UTR, sequences that are highly consistent with the utilization of "leaky scanning" as a regulatory mechanism for increasing translation. In addition, from published reports [8], we noted that the kinetics of ATF4 protein induction and silencing following ischemia is remarkably similar to what we observed for Cx36 following ischemia [10] and activation of group II mGluRs (Fig. 1a; also [10]). This is, of course, a highly speculative model that will require further studies to confirm.

As with our previous observations of Cx36 regulation during development [4] and neuronal injury [10], we find that in response to group II mGluR agonists, neurons employ multiple mechanisms to regulate the abundance of Cx36 protein and therefore the extent of gap junction coupling. The existence of multiple mechanisms facilitates changes in Cx36 protein levels that are appropriately timed and scaled and that match the strength and duration of the initiating signals. For example, as reported here and in the previous study [10], in response to short-term activation of group II mGluR receptors a rapid increase in Cx36 protein occurs most likely via translational mechanisms. It is unlikely that such a rapid increase in protein levels could be achieved solely through transcriptional mechanisms, nor is such a response necessarily desirable, given the acute nature of receptor activation. However, with chronic exposure to group II mGluR agonists, as investigated in the developmental study [4], a long-term adaptation in the form of transcriptional up-regulation of the Cx36 appears to occur. While the molecular bases for these two regulatory schemes are distinct, their utilization presumably allows for maximum flexibility in the control of gap junctional coupling between neurons.

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Research Highlights

- > Acute activation of group II mGluRs or ischemia both increase C×36 protein expression
- > The regulation likely is due to new protein synthesis
- > Thus, the regulation is via post-transcriptional mechanisms
- > If receptor activation sustains, transcriptional activation of the C×36 gene occurs

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Fig. 1.

Changes in Cx36 protein and mRNA expression following short-term treatment with the agonist of group II mGluRs. (a–d) Statistical data from western blot (a,b) and RT-qPCR (c,d) experiments in mouse neuronal somatosensory cortical cultures on DIV3 (a,c) and DIV15 (b,d) are shown. Representative western blots also are illustrated (a,b). In all graphs, statistical analysis: two-tailed paired Student's *t*-test; (i) $P=0.0005$, (ii) $P=0.0008$, (iii) $P=0.006$, shown relative to control (C); $n = 4-6$; mean $\pm$ S.E.M. LY379268 (2 μ M) was added to cultures for 60 min, then washed out, and the analysis was done at the time points after wash out as indicated in the figures.

Fig. 2.

Increased Cx36 protein expression following ischemia is due to synthesis of new protein. Western blot experiments were done in mouse neuronal somatosensory cortical cultures on DIV15. Statistical analysis of changes in the expression of Cx36 following treatment with cycloheximide (CHX; 50 mg/ml) and OGD is shown. CHX was present in the culture medium for 10 min before, 30 min during, and 10 min after OGD or sham OGD and then was washed out. The analysis was done two hrs after OGD or sham OGD. Statistical analysis: ANOVA with post hoc Tukey; statistical significance is shown relative to control (i) and non-treated plus OGD (ii); $n = 5-15$ per group; mean $\pm$ S.E.M.