# INTERPLAY OF CHEMICAL NEUROTRANSMITTERS REGULATES DEVELOPMENTAL INCREASE IN ELECTRICAL SYNAPSES

By



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# ABSTRACT

Coupling of neurons by electrical synapses (gap junctions) transiently increases during embryonic and/or early postnatal development of the mammalian central nervous system and plays an important role in a number of developmental events. A previous study revealed the mechanisms that control the developmental uncoupling of neuronal gap junctions, however, developmental regulation of neuronal gap junction coupling is largely unknown and is addressed in this dissertation. The current study revealed that the developmental increase in neuronal gap junction coupling is regulated by the interplay between the activity of group II metabotropic glutamate receptors (mGluR) and $GABA_A$ receptors ($GABA_AR$). Specifically, the experiments including dye coupling, electrotonic coupling, western blots and siRNA technology in the rat and mouse hypothalamus and cortex *in vivo* and *in vitro* demonstrated that activation of group II mGluRs augments, and inactivation prevents, the developmental increase in neuronal gap junction coupling and connexin36 (Cx36, neuronal gap junction protein) expression. In contrast, changes in $GABA_A$ receptor activity have the opposite effects. The regulation by group II mGluRs is through cyclic AMP/protein kinase A-dependent signaling, while the $GABA_AR$-dependent regulation is via influx of $Ca^{2+}$ through L-type voltage-gated $Ca^{2+}$ channels and activation of protein kinase C-dependent signaling. Further, the receptor mediated up-regulation of Cx36 requires a neuron-restrictive silencer element in the Cx36 gene promoter and the down-regulation involves the 3' untranslated region of the Cx36 mRNA, as shown using real-time quantitative polymerase chain reaction and luciferase reporter activity analysis. In addition, the methyl thiazolyl tetrazolium analysis indicates that mechanism for the developmental increase in neuronal gap junction coupling directly control the death/survival mechanisms in developing

neurons. Altogether, the results suggest a multi-tiered strategy for chemical synapses in developmental regulation of electrical synapses.

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I am also thankful to Janna V. Denisova and Dr. Youngfu Wang for allowing me to include their research data into my dissertation: MTT analysis data obtained by Janna and dye and electrotonic coupling data obtained by Youngfu. These data are shown in the Results section and are discussed; however, they are included only as an additional material supporting my own results obtained by Western blot, RT-qPCR, and luciferase reporter analysis experiments.

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| | |
|---|---|
| **AP5** | 2-Amino-5-phosphonopentanoic acid |
| **AMPA** | α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| **Bic** | Bicuculline |
| **CaMKII** | Calcium/calmodulin-dependent protein kinase II |
| **cAMP** | Cyclic AMP |
| **CNQX** | 6-Cyano-7-nitroquinoxaline-2,3-dione |
| **CNS** | Central nervous system |
| **CRE** | cAMP response element |
| **CREB** | cAMP responsive element binding protein |
| **Cx36** | Connexin36 |
| **DIV** | Day in vitro |
| **ER** | Endoplasmic reticulum |
| **GABA** | γ-Aminobutyric acid |
| **$GABA_AR$** | $GABA_A$ receptor |
| **GAPDH** | Glyceraldehyde-3-phosphate dehydrogenase |
| **Group II mGluR** | Group II metabotropic glutamate receptor |
| **$IP_3$** | Inositol trisphosphate |
| **IRES** | Internal ribosomal entry site |
| **MAPK** | Mitogen activated protein kinase |
| **mGluR2** | Metabotropic glutamate receptor subtype 2 |
| **mGluR3** | Metabotropic glutamate receptor subtype 3 |
| **miRNA** | MicroRNA |
| **NMDA** | N-methyl-D-aspartic acid |
| **NMDAR** | NMDA receptor |
| **NRSE** | Neuron-restrictive silencer element |
| **ORF** | Open reading frame |
| **P** | Postnatal |
| **PiTX** | Picrotoxin |

| | |
|---|---|
| **PKA** | Protein kinase A |
| **PKC** | Protein kinase C |
| **PVN** | Paraventricular nucleus |
| **REST** | RE1-silencing transcription factor |
| **RT-qPCR** | Real time quantitative polymerase chain reaction |
| **siRNA** | Small interfering RNA |
| **SON** | Supraoptic nucleus |
| **VGCC** | Voltage-gated calcium channel |
| **TTX** | Tetrodotoxin |
| **uAUG** | Upstream AUG |
| **uORF** | Upstream open reading frame |
| **3'UTR** | 3' Untranslated region |
| **5'UTR** | 5' Untranslated region |

# CHAPTER ONE

## General Introduction

## 1. Gap junctions – structure and function

### 1.1 Connexins

While, in the mature central nervous system (CNS), most neurons communicate with one another primarily by chemical synapses, a large subset of neurons also relies on electrical synapses for the purpose of fast and synchronous synaptic transmission (Benett, 1977). The structural correlates of electrical synapses are gap junctions which are illustrated in Fig. 1. Gap junctions are intercellular connections that contain two hemichannels (also known as connexons) composed of six transmembrane proteins called connexins (Kumar and Gilula, 1996). At least 20 isoforms encoding connexins in human and rodent genomes have been identified to date (Willecke et al., 2002; Mathias et al., 2010). This family of proteins has been generally named according to their approximate molecular weight (e.g., connexin36, Cx36 is ~36 kDa in mass). Since expression of connexins is tissue- and cell- specific, Cx26, Cx30, Cx32, Cx33, Cx36, Cx37, Cx40, Cx43, Cx45, Cx46, and Cx57 have all been reported to be present in the nervous system. Among them, Cx43 is the most abundantly expressed in astrocytes (Nagy and Rash, 2000). Cx36 is an exclusive component of neuronal gap junction, and Cx45 or Cx57 also can form neuronal gap junctions (Söhl et al., 2005). This dissertation primarily focuses on gap junctions in mammalian neurons, which are often synonymous with electrical synapses, with particular emphasis on Cx36 containing-gap junctions.

### 1.2 Gap junction channels

Connexins are integral membrane proteins containing four transmembrane-spanning domains, characterized by two extracellular loop domains, and cytoplasmic carboxy- and amino-terminal domains as well (Kumar and Gilula, 1996). The extracelluar regions are crucial for the

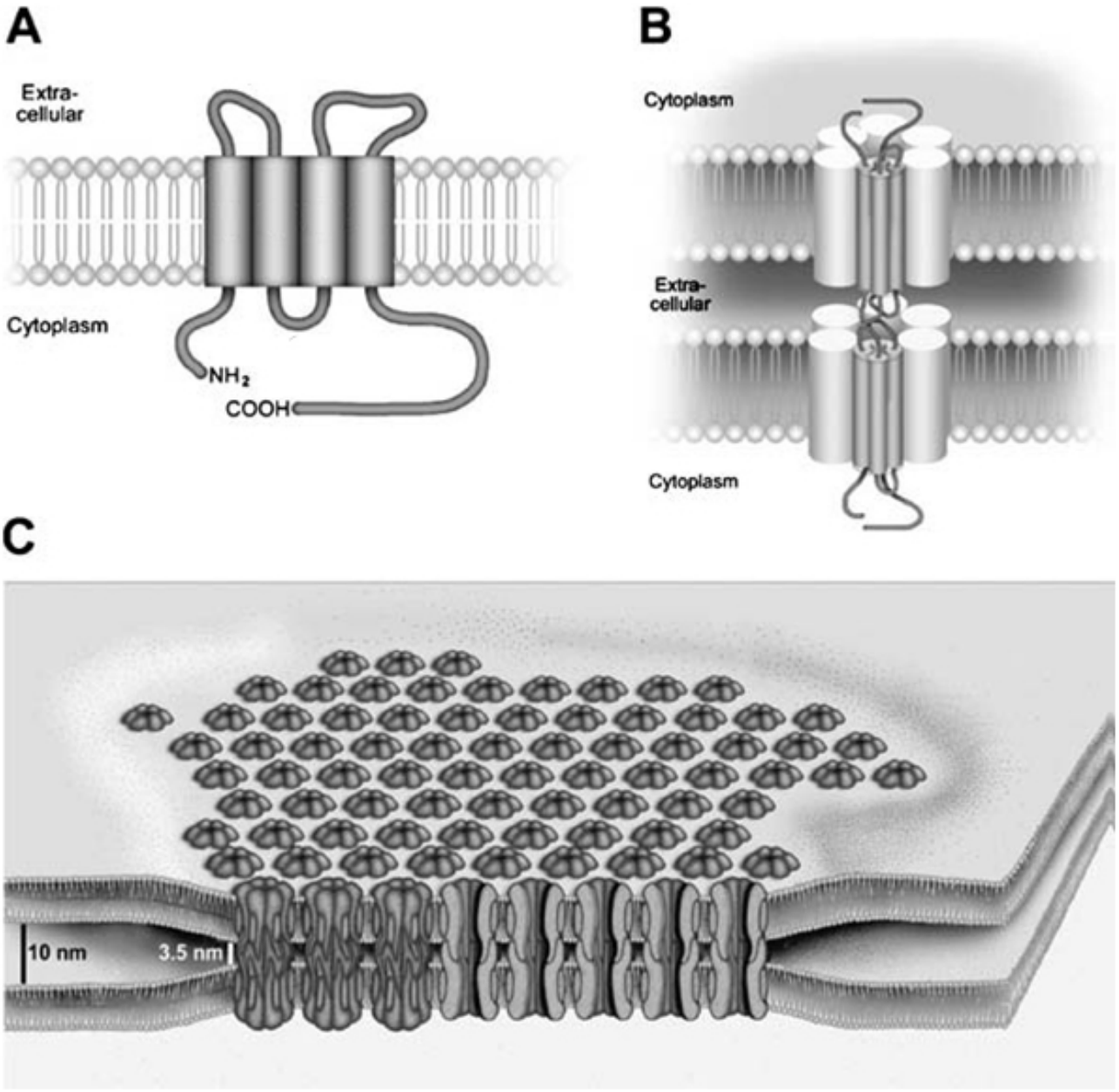
A
Extra-
cellular
Cytoplasm
NH2
COOH
B
Cytoplasm
Extra-
cellular
Cytoplasm
C
10 nm
3.5 nm

**Figure 1.** Diagram of gap junctions and their connexin constituents. (A) Connexin protein. (B) Single gap junction channel, formed by the apposition of two hemichannels also know as connexons. (C) Gap juncion plaque, assembled with tens to thousands of individual channel units. (Diagram is modified from Meier and Dermietzel, 2006).

docking between connexons on neighboring cells. The cytoplasmic domains have profound effects on characteristics of individual channel activity via association with a variety of partners, e.g., tight and adherens junctional proteins, protein kinases and phosphatases, and cytoskeletal elements (Dbouk et al., 2009). One connexon is located in the membrane of one cell and is attached to the connexon of the neighboring cell, forming a continuous aqueous pathway, a single gap junction channel, with an extremely low electrical resistance (Goodenough et al., 1996; Martin and Evans, 2002). In general, gap junction channels often cluster together to form gap junctional plaques containing tens to thousands of individual channel units (Söhl et al., 2005).

Connexons may have only one type of connexins (homomeric connexons), or more (heteromeric connexons), and the gap junction channels can be composed by two identical (homotypic channels) or different (heterotypic channels) types of connexons (Fig. 2). Most cells in the body and brain, thus, can have multiple connexins depending on their molecular compositions and selective interactions, resulting in diverse physiological characteristics. Cx36 has been reported to form homomeric/homotypic channels (Li et al., 2008).

### 1.3 Gap junctional intercellular communication

Gap junction channels can perform very unique functions in neuronal circuits by directly connecting the cytoplasmic compartments of two adjacent cells. In addition to the ability to pass electrical current, by $Na^+$ and $K^+$ ions, gap junction channels can also propagate various cytoplasmic molecules through the wide pores (12-14 Å). The intercellular exchange of $Ca^{2+}$, $IP_3$, cAMP, glucose, and small molecules (less than 1 kDa) through gap junction channels is mediated by passive diffusion. Nonbiological tracer molecules, such as Lucifer yellow, neurobiotin, ethidium bromide, and fluorescein derivatives, can also permeate. This trait makes

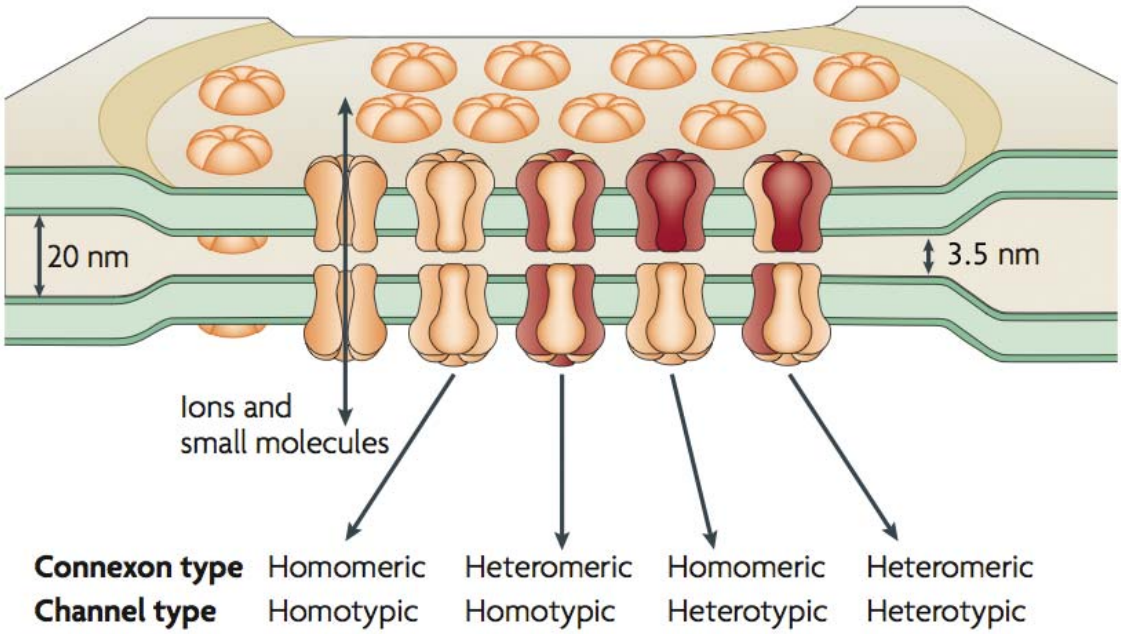
20 nm
3.5 nm
Ions and
small molecules
Connexon type Homomeric Heteromeric Homomeric Heteromeric
Channel type Homotypic Homotypic Heterotypic Heterotypic

**Figure 2.** Multiple types of connexons and gap junction channels. (Diagram is modified from Bloomfield and Völgyi, 2009).

the method of “dye coupling” a powerful tool to evaluate the incidences and extents of gap junction connection between neurons (Vaney, 2002; Arumugam et al., 2005). Gap junction channels display different selectivity and permeability to the passing molecules based upon connexin subtype compositions (Evans et al., 2006).

## 2. Regulation of gap junctional communication

Obviously, a variety of factors affect gap junctional intercellular communication at multiple and diverse levels. The effects of them can contribute to both short- and long-term regulation of gap junctional intercellular communication. Short-term regulation, which occurs rapidly within minutes to hours, mediates changes in the opening probability and/or unitary conductance of a single channel already in place at gap junctions. Long-term regulation (i.e. over hours or days or even longer periods) influences the number of junctional plaques and/or channels present in the membrane. It occurs at the transcriptional, translational, and post-translational levels by altering rates of synthesis, assembly, trafficking, and turnover for gap junction channels (Goodenough et al., 1996).

### 2.1 Regulation of the channel gating

The conductances of gap junction channels can be regulated by gating mechanisms, which can be affected by changes in transjunctional voltage, cytoplasmic pH, and intracellular $Ca^{2+}$ concentration (Spray et al., 1985). Many gap junction channels are voltage gated and can display multiple voltage-dependent conductance states (Goodenough and Paul, 2009). For most gap junction channels, conductance is maximal when the membrane voltage is equal between the interconnected cells and it declines symmetrically with deviations in either direction. Cx36

channels show the least voltage dependence of all connexin channels (Connors and Long, 2004).

The cytoplasmic pH is also involved in changes in gap junctional conductance. The conductance of many gap junction channels is sensitive to the pH of the cytoplasm and nearly insensitive to extracellular pH. Cytoplasmic acidification leads to closure of channels by protonation of amino acid side chains in the cytoplasmic domains of connexins (Duffy et al., 2002). However, Cx36 channels undergo unique regulation by cytoplasmic pH since their conductance is reduced by alkalosis rather than acidosis (Gonzalez-Nieto et al., 2008).

$Ca^{2+}$ plays a much less potent role in regulating gap junctional conductance than proton ion does. Intracellular $Ca^{2+}$ levels must rise to high levels, as seen under pathological conditions, in order to close gap junction channels (Connors and Long, 2004).

### 2.2 Regulation by chemical neurotransmitters

Chemical neurotransmitters are certainly important modulators of gap junction conductance. The responsible mechanism in most cases is via intracellular second messenger pathways rather than by a direct effect on channel properties. Phosphorylation in the intracellular domains of gap junction channels is the best characterized regulatory mechanism to alter the gating activity. In fact, most connexin subtypes have multiple phosphorylation sites for a broad range of protein kinases, such as protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and pp60 tyrosine kinase, each of which responds to different stimuli (Lampe and Lau, 2000; Solan and Lampe, 2005). The role of dopaminergic receptors in modulation of gap junctions has been well described in the neocortex. For example, domaminergic activations via $D_1$ and $D_2$ receptors reduced gap junction conductance by a PKA-dependant phosphorylation process in developing rat neocortical neurons (Rörig et al., 1995).

The effects of the other two monoaminergic neurotransmitters, noradrenaline and serotonin, on gap junction conductance have been also investigated in the mammalian brain. Both noradrenaline and serotonin produce a reduction of gap junction coupling (Rörig and Sutor, 1996; Zsiros and Maccaferri, 2008). Additionally, γ-aminobutyric acid (GABA) has been shown to reduce the extent and strength of gap junction coupling in the rat suprachiasmatic nucleus (Shinohara et al., 2000). On the contrary, GABAergic inactivation in the retina shows a decreased permeability of gap junctions connecting horizontal cells, implying a positive regulatory role of inhibitory neurotransmitter in gap junctional communication (Piccolino et al., 1982). Glutamate-mediated modulation of electrical synapses via different types of glutamate receptors has been also reported. For example, N–methyl–D–aspartate receptor (NMDAR) activations contribute to developmental gap junction uncoupling in the rat hypothalamus (Arumugam et al., 2005) and group I/II metabotropic glutamate receptor (mGluR) activation causes a long-lasting reduction of electrical synapse strength between the inhibitory neurons of the rat thalamic reticular nucleus (Landisman and Connors, 2005).

## 2.3 Regulation by molecular mechanisms

### 2.3.1 Transcriptional regulation

Like other genes, the expression of connexin genes is intricately regulated at many different levels involving transcription, post-transcriptional RNA processing, transcript stability and localization, and translation. The first level of connexin gene regulation is at the level of transcription where genes are differentially transcribed during development or in response to (patho) physiological signals. Connexins are encoded by single gene copies, which are found in many different chromosomes. The general genomic structure of connexin genes is well

conserved. Most connexin genes contain two exons separated by a large intron. The first exon has 5' untranslated region (5'UTR) and the second exon includes the complete open reading frame (ORF) of connexin gene and the subsequent 3' untranslated region (3'UTR). Unlike the vast majority of mammalian connexin genes, the coding region of Cx36 gene has been shown to be interrupted by a single intron featuring a small part (the first 71 bp starting with ATG) of the reading frame on exon 1 and the rest on exon 2 (Fig. 3). Having suggested the coding region has to be spliced correctly to undergo proper translation, alternative splicing may lead to expression of different forms and/or properties of Cx36 via a robust modification in the Cx36 coding region (Teubner et al., 2000).

In principle, the total transcriptional expression profile of genes is controlled by the interaction of transcription factors with their target elements present in the regulatory region of a gene. For most connexin genes, the basal promoter is located within 300 bp upstream of the transcriptional initiation site. This region contains binding sites for numerous transcription factors which help initiate a program of enhanced or suppressed gene transcription. In addition to the basal expression of connexin genes by the ubiquitous transcription factors such as TATA box binding proteins, Sp1, and AP-1, the presence of additional elements within the promoter region is also crucial for the exclusive regulation of connexin gene expression in a cell type-specific manner.

For example, the promoter region of Cx36 comprises a neuron-restrictive silencer element (NRSE) that binds the transcriptional repressor, RE1-silencing transcription factor (REST) in order to control neuron-specific expression of Cx36. Repressing role in Cx36 transcription by REST is mediated by the recruitment of histone deacetylase, a key regulator of transcription, to the promoter (Martin et al., 2003). In additional to NRSE, the presence of a highly conserved cAMP responsive element (CRE) within Cx36 promoter is also functionally

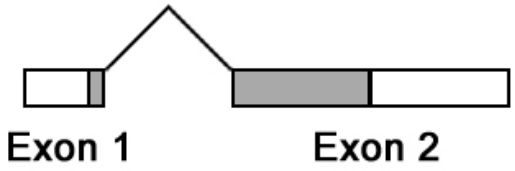
Exon 1
Exon 2

**Figure 3.** Structure of connexin36 gene. The coding region of Cx36 is interrupted by an intron. Untranslated and translated regions are depicted as boxes in white and gray, respectively.

related to glucose-induced Cx36 downregulation. CREs are targets of a large family of PKA-activated basic leucine-zipper CRE-binding factors including cAMP responsive element modulator and cAMP responsive element binding protein (CREB) which may function as transcription suppressors of Cx36 gene according to extracellular stimuli (Allagnat et al., 2005; Arumugam et al., 2005).

### 2.3.2 Post-transcriptional regulation

Although transcriptional control is expected to play a major role in determining the specific expression pattern of connexin genes, many other mechanisms can also contribute to gene expression regulation. After being transcribed, the abundance of transcripts can be changed by promoting either rapid mRNA decay or mRNA stabilization. Thus, the interactions between regulatory elements within mRNA and RNA-binding proteins or small, non-coding RNAs determine proper processes, such as RNA processing, translation, and decay. The 5' untranslated region (5'UTR) of Cx43 mRNA contains a strong internal ribosome entry site (IRES) which is sufficiently potent to permit connexin translation under stressful conditions (Schiavi et al., 1999). Cx32 mRNA also includes IRES which is important for the selective expression of this gene in cells of the nervous system, such as Schwann cells. The loss of IRES-mediated translation regulation has been implicated in Charcot-Marie-Tooth Disease, hereditary motor and sensory neuropathy (Hudder and Werner, 2000).

Another type of translational control is mediated by one or more short upstream open reading frames (uORFs) that appear in the 5'UTR of the mRNA preceding the main ORF. They are usually shorter than the main ORF and inhibit translation initiation of the downstream gene mainly by stalling a scanning ribosome initiation complex. For example, the 5'UTR of Cx41 mRNA contains three uORFs, playing an important role in the regulation of Cx41 translation

(Meijer and Thomas, 2003). The first uORF strongly inhibits translation, because mutation of the upstream AUG (uAUG) 1 enhanced translation, suggesting a ribosome scanning an mRNA with multiple uORFs translates a short peptide and dissociates prematurely after termination at uORF1. Therefore, translation of the main ORF depends on the availability of ribosomes that bypassed the uAUGs to reach the downstream main ORF (Meijer and Thomas, 2003). In the case of Cx43, there are various transcripts which exhibit different translational efficiencies. The 5'UTRs with higher translation efficiencies lack uAUGs, whereas those with lowest translational activities contain uAUGs with adequate Kozak consensus sequences (Pfeifer et al., 2004). In addition, the presence of uORFs in a sbubset of the transcripts for Cx31, Cx45, Cx46, and Cx47 has also been reported (Anderson et al., 2005). Curiously, the 5'UTR nucleotide sequence and arrangement of the uAUG in Cx36 genes are remarkably conserved across multiple species, implying functional roles in translation regulation.

Lastly, a class of small and non-coding transcripts called microRNAs (miRNAs) plays a major role in post-transcriptional gene regulation. miRNAs are important negative regulators of target gene expression by translational repression or mRNA cleavage by base pairing to complementary mRNA sequences that are frequently located in the 3' untranslated region (3'UTR). They are particularly enriched in the brain, where they play key roles in development as well as in adulthood (Vo et al., 2010; Hébert et al., 2010). Genomic analysis has revealed multiple miRNA binding sequences in 3'UTRs of Cx36, Cx43, and Cx45 genes, consistent with cell type-specific post-transcriptional repression of connexin synthesis (Rash et al., 2005). This report can be reconciled with previous data about the discrepancy between detection of mRNAs and the lack of detection of the corresponding proteins for several connexins in different brain regions, suggesting the possibility of inhibition of connexin translation by miRNAs (Rash et al., 2005). However, the functional study of miRNAs and their regulatory mechanisms in the

regulation of connexin expression during neural network development remains to be conducted in the future. miRNA-mediated translational regulation of Cx43 during muscle differentiation has been well studied. Two miRNAs, miR-206 and miR-1, have been implicated to inhibit translation without targeting the message for degradation (Anderson et al. 2006).

### 2.3.3 Post-translational regulation

A connexin protein after its translation undergoes a chemical modification called post-translational modification, chiefly phosphorylation. Many connexins (e.g., Cx31, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, Cx46, Cx50, and Cx56) have been shown to be phosphoproteins. Connexins do not seem to be glycosylated, but a few reports suggest other alterations, such as prenylation and acylation of Cx32 (Locke et al., 2006). In many connexins, the carboxy-terminal domain that is located in the cytoplasm seems to be the major region for phosphorylation. Cx36 and Cx56 can be additionally phosphorylated at the cytoplasmic loop domain (Solan and Lampe, 2009). Cx36 has been known to be phosphorylated by PKA (Urschel et al., 2006), PKC (Yevseyenkov et al., 2005), and calcium/calmodulin-dependent kinase II (CaMKII) (Alev et al, 2008). However, Cx26 with a relatively short carboxy-terminal domain is the only connexin that has been reported not to be phosphorylated (Traub et al., 1989). Since Cx26 can form functional channels, connexin phosphorylation is not exclusively required for the formation of gap junctions. Nevertheless, connexin phosphorylation by several kinase pathways clearly has been implicated in the regulation of gap junctional communication at several stages throughout its life cycle, not only channel gating, but also the rates of connexin trafficking, gap junction assembly, and turnover (Laird, 2005).

## 2.4 Gap junction assembly and degradation

The dynamic regulation of gap junction formation and degradation, like other membrane proteins, starts in the endoplasmic reticulum (ER). The ER is not only the site for connexin protein synthesis and processing but also functions as quality control center for newly synthesized proteins. When connexin proteins fail to fold correctly, degradation of misfolded connexins at the ER (i.e., ER-associated degradation) occurs via the ubiquitin/proteosomal system (Kelly et al., 2007). Connexins are post-translationally assembled into hemichannels in an intracellular compartment prior to transport to the plasma membrane. The location of connexin oligomerization depends on the type of connexin involved. For example, Cx32 assembles either in the ER or between the ER and Golgi, while Cx43 assembles in the trans-Golgi network. Once at the cell surface, a successfully assembled hemichannel in the plasma membrane of one cell aligns and docks with another from a neighboring cell to form an intercellular gap junction channel. After formation of a single gap junction is completed, the two hemichannels do not separate under physiological conditions. This gap junction may be laterally mobile through lipid rafts where it is clustered into a gap junction plaque. Growth of gap junctions occur by incorporation of additional gap junction channels to the plasma membrane followed by their incorporation to the periphery of existing gap junction plaques. Thus, small gap junction plaques can coalesce into larger plaques to function as electrical synapses (Saez et al., 2003).

Connexins turn over in the plasma membrane with a half-life of 1.5-5 h, which is much faster than most other membrane proteins (Musil et al, 2000). Gap junctions can be removed from the plasma membrane by the invagination, restriction, pinching off, and transport of entire junctions into the cytoplasm of one of the two adjacent cells. Once internalized, further degradation of gap junction channels occurs through lysosomal as well as proteosomal

pathways. Degradation may be regulated by ubiquininylation and phosphorylation (Segretain and Falk, 2004).

## 3. Connexin hemichannels and non-connexin gap junction channels

### 3.1 Connexin hemichannels

Recent studies have shown that hemichannels can be also active in an unopposed form on the single plasma membrane, presumably to have alternative functional roles to gap junctional communication (Goodenough and Paul, 2003). Although hemichannels can be opened to experimental manipulation, in general they are primarily closed to prevent metabolic stress and death caused by the collapse of ionic gradients, loss of small metabolites, and influx of $Ca^{2+}$ (Contreras et al., 2002). However, it has been demonstrated that activation of Cx43 hemichannels in the astrocyte plasma membrane causes the release of ATP into extracellular space (Goodenough and Paul, 2003). The subsequent activation of purinergic receptors on adjacent cells initiates and propagates $Ca^{2+}$ wave (Kang et al., 2008). In addition, hemichannels are also involved in release of signaling molecules other than ATP, i.e. nicotinamide adenine dinucleotide (Bruzzone et al., 2001), MAPK signaling in anti-apoptotic protection (Plotkin et al., 2002) and ephaptic neuronal communication in retina (Kamermans et al., 2001). Cx36 is unlikely to form functional hemichannels, but Cx35, the fish ortholog of the mammalian Cx36, has been shown to display voltage-gated hemichannel activity (Valiunas et al., 2004).

### 3.2 Non-connexin gap junction channels

Besides the connexin proteins, pannexins represent another family of gap junction

proteins in vertebrates. Pannexins form large-pore non-selective channels in the plasma membrane and appear to form gap junctions only rarely. Pannexin channel properties are more suited for transmembrane transport of $Ca^{2+}$ and ATP in response to physiological and pathological stimuli (Shestopalov and Panchin, 2008). Among identified 3 pannexins, pannexin1 and 2 constitute neuronal gap junctions mainly in pyramidal neurons in the cortex and hippocampus (Bruzzone et al., 2003). The channels function by releasing large signaling molecules, such as ATP and arachidonic acid derivatives. Pannexin1 may contribute to ischemic neuronal death, while pannexin coupling with purinergic receptors triggers initiation of inflammatory response (MacVicar and Thompson, 2010).

## 4. Distribution of gap junctions in the mature brain

### 4.1 Hippocampus

Dendrodentritic gap junctions between subpopulation of GABAergic interneurons are found in the various layers of CA1, CA3, and dentate gyrus of the hippocampus (Kosaka and Hama, 1985). Functional and molecular evidence suggested that electrically coupled pairs of interneurons express Cx36 mRNA (Venance et al, 2000). The role of electrical synapses between hippocampal interneurons is likely in generation of synchronized oscillatory activity (Bartos et al., 2002). Pyramidal neurons also express Cx36 in CA3 region and may be electrotonically coupled through axoaxonal gap junctions to mediate the generation of oscillation (Connors and Long, 2004). However, the degree of coupling between pyramidal neurons is believed to be much lower than that between interneurons (Venance et al, 2000; Traub et al., 2003). The role of electrical synapses within the dorsal hippocampus is recently suggested to be

linked to context-dependent fear learning and memory associated with theta rhythms (Bissiere et al, 2011).

### 4.2 Inferior olive

The inferior olive, which forms the sole source of climbing fiber input to Purkinje cell in the cerebellum, has shown strong electrical coupling (Long et al., 2002). The abundant gap junctions between olivary neurons express Cx36 (Rash et al., 2000). Cx36 deficient animals show a robust reduction in both the prevalence and the strength of electrical synapses, thus they represent no synchrony among knock-out cells. However, spontaneous subthreshold rhythmic activity is sustained in the Cx36 knock-out mouse, implying that electrical synapses are required for the synchronization of olivary rhythms rather than the generation (De Zeeuw et al., 2003).

### 4.3 Hypothalamus

The vast majority of neuronal groups in the hypothalamus express Cx36, which is observed in the paraventricular nucleus (PVN), anterior hypothalamic nucleus, supraoptic nucleus (SON), arcuate nucleus, and mammaillary region (Condorelli et al., 2000; Arumugam et al., 2005). The presence and functional significance of gap junction coupling in the SON and PVN have been well demonstrated: the gap junction coupling between neuroendocrine cells increases in response to dehydration, gestation and lactation suggesting an important role in the secretion of oxytocin and vasopressin via synchronization of neuroendocrine cells to the blood (Hatton, 1998). Moreover, many neurons in the suprachiasmatic nucleus communicate via

Cx36-containing electrical synapses to synchronize spiking activity for the purposes of regulation of normal circadian behavior (Long et al., 2005).

### 4.4 Neocortex

Similar to the hippocampus, in the mature neocortex, electrical synapses between interneurons are more prevalent than between excitatory pyramidal neurons (Galarreta and Hestrin, 2001). Both morphological and physiological studies have established the exclusive presence of electrical synapses between GABAergic interneurons of the same type. There are at least five different classes of electrically coupled networks of GABAergic neurons in the neocortex. These networks are extensive and nearly independent of each other type of coupled interneuron network suggesting distinct roles in coordinating cortical activity (Hestrin and Galarreta, 2005). The possible role of electrical synapses is to allow signaling among inhibitory neurons and promote their synchronous spiking within networks of inhibitory neurons (Galarreta and Hestrin, 1999). Cx36 is necessary for the network of dendritic (dendro-dendritic or dendro-somatic) gap junctions between cortical GABAergic interneurons (Fukuda et al., 2006). Cx36-containing electrical synapses in inhibitory neurons are responsible for the synchrony of oscillatory rhythmic activity in the theta and gamma frequency in the neocortex (Deans et al., 2001). Excitatory neurons (i.e., pyramidal neurons or spiny stellate neurons), on the other hand, do not use electrical synapses for communication except during the early postnatal period (Fukuda et al., 2007). Excitatory and inhibitory neurons do not show electrical synapses between each other, although Cx36 is involved in the rare electrical coupling between interneuron and pyramidal neuron in the immature cortex (Galarreta et al, 1999; Venance et al., 2000).

## 5. Function of gap junctional communication

### 5.1 Role of gap junctions during CNS development

The embryonic development of the mammalian brain and the subsequent maturation of the specific brain areas are complex processes. They involve a wide diversity of molecular and cellular mechanisms including gap junction-dependent communication between developing neurons as well as developing glial cells. In general, gap junction coupling contributes to the generation of spontaneous electrical activity and the passage of small signaling molecules for biochemical interactions between cells. It serves prominent roles during the developmental process of almost all regions of the brain, including neurogenesis, neuronal migration, cellular differentiation, and synapse formation (Elias and Kriegstein, 2008).

In the neocortex, gap junction coupling is particularly extensive during embryonic and early postnatal development (Lo Turco and Kreigstein, 1991; Peinado et al., 1993). Cx26, Cx36, Cx37, Cx43, and Cx45 have been found in the rodent neocortex during embryonic development. The expression of these connexins appears to be differentially regulated in a spatial and temporal fashion, which might have functional significance because each connexin subtype has different permeability and regulation properties (Cina et al., 2007). Gap junctions are expressed in almost every cell type of the neocortex, such as astrocytes, oligodendrocytes, pyramidal neurons, inhibitory interneurons, and progenitor cells. An early study suggested that gap junction-coupled neuroblasts with clusters of up to 90 cells in the ventricular zone can act as precursors for the functional columns found in the matured neocortex (Lo Turco and Kreigstein, 1991). Gap junction coupling can also influence the proliferation of neuronal progenitor cells by the synchronization of the cell cycle of closely located cells, which may play an important role in controlling the pattern of cortical neurogenesis (Bittman et al., 1997). Furthermore, gap junctions

contribute to the formation of neuronal cortical circuits in neonatal rodents by mediating synchronized oscillatory network activity (Khazipov and Luhmann, 2006).

On the other hand, the formation of gap junctions between adjacent cells can serve as dynamic adhesive contact points rather than intercellular channels for the direct communication. Gap junctions composed of Cx26 and Cx43 mediate glial-guided radial migration of newborn neurons in the cortex due to the adhesive property of connexins, perhaps interacting with the internal cytoskeletal elements (Elias et al., 2007). Hemichannel-mediated spread of $Ca^{2+}$ waves across progenitor cell populations, associated with purinergic receptors, also helps to fine-tune the establishment of neural networks during late neurogenesis (Weissman et al., 2004). Another important role of gap junctions in cell death communication during development has been also suggested. Gap junctions contribute to neuronal cell death that is caused by either increased or decreased NMDA receptor (NMDAR) activity in the developing rat hypothalamic neurons (de Rivero Vaccari et al., 2007). Taken together, connexin-composed gap junction channels, hemichannels, and adhesion molecules act in distinct ways to mediate radial glial division, neuronal migration, neuronal differentiation, and apoptosis.

The expression of connexins shows a highly dynamic pattern during neural development. The expression of Cx36 in the developing rodent brain shows two peaks; one at the end of embryogenesis followed by a decrease at birth and another at about postnatal day 14 (Gulisano et al., 2000). Almost all brain areas express Cx36 in the early postnatal life. The first peak of Cx36 expression coincides with the time of the greatest neurogenesis of the cortical cells, suggesting the regulatory role in both defining properties of morphogenetic boundaries (Gulisano et al., 2000) and influencing neural differentiation positively (Hartfiled et al., 2010). The role of the second, postnatal peak, which corresponds to the time of highest incidence of

neuronal coupling, has been suggested to be involved in the experience-dependent maturation of neuronal circuits (Maher et al., 2009).

### 5.2 Role of gap junctions during CNS injury

In addition to the crucial role in developing nervous system, many studies also have suggested the role of gap junctions in many neurological disorders, including epilepsy (Rouach et al., 2002), ischemia (Talhouk et al., 2008), brain trauma (Frantseva et al., 2002), demyelinating neuropathies (Herrmann, 2008), and hearing loss (Hoang et al., 2009). Several pathological conditions change the pattern of connexin expression, which leads to changes in cellular response to CNS injury.

Of 20 connexins, changes in both spatial and temporal Cx43 protein expression have been observed following various types of CNS injuries such as ischemia and traumatic brain injury (Rouach et al., 2002). Cx43 has been long considered as an important mediator of CNS injury since astrocytic Cx43-containing gap junctions are the most ubiquitously present and involved in maintaining the homeostasis of the extracellular milieu of neurons (Cronin et al., 2008). The response of Cx43 varies with severity of injury. For instance mild to moderate ischemic injury seems to induce Cx43 over-expression in injured region while severe injury appears to reduce Cx43 expression within the insulted site probably due to cell death in that region (Orellana et al., 2009). In animal models of ischemia, altered Cx43 protein levels have been shown in many brain areas, particularly in the hippocampus with an increased Cx43 expression level after transient forebrain ischemia (Rami et al., 2001). An elevated Cx43 expression can be also detected in striatum after mild to moderate global cerebral ischemia induced by bilateral carotid artery occlusion in rats (Hossain et al., 1994). Another model of focal cerebral ischemia using photothrombosis shows enhanced level of Cx43 expression in cortex as

well (Haupt et al., 2007). A similar increase in Cx43 expression is found in the ischemic penumbra in post-mortem human brain samples following focal brain ischemia (Nakase et al., 2006). In a model of *in vitro* ischemia, astrocytic Cx43 gap junctions and hemichannels remain functionally open enabling intercellular transfer of ions and metabolites between damaged and healthy cells (Cotrina et al., 1998). The increased probability of Cx43 hemichannel opening may involve cytoplasmic $Ca^{2+}$ changes, reactive oxygen species, and reductions in the concentration of extracellular $Ca^{2+}$ associated with global ischemia (Retamal et al., 2007; Decrock et al., 2009). In addition to astrocytic hemichannel opening, ischemic conditions also open neuronal gap junction hemichannels composed of pannexin1 (Thompson et al, 2006).

Similar changes are detected after the traumatic insult. Acute compression injury to the spinal cord leads to decrease in Cx43 expression in the white and gray matter area of spinal cord (Theriault et al., 1997). Enhanced Cx43 expression also can be seen in activated microglia in a brain stab wound injury model, which might be triggered by inflammatory mediators (Eugenin at al., 2001). Another animal model of traumatic brain injury using lateral fluid percussion shows an increase in Cx43 expression in the cortex and hippocampus (Ohsumi et al., 2006).

In addition to glial connexins, neuronal connexins also undergo spatiotemporal changes after specific insults. Global ischemia induced by temporary bilateral occlusion of common carotid arteries in adult male mice leads to a selective increase in Cx32 and Cx36 but not Cx43 proteins in the CA1 hippocampal region before the onset of neuronal death (Oguro et al., 2001). The induction of Cx36 immunoreactivity is also found in CA3 pyramidal neurons 1 hour after traumatic brain injury, and then Cx36 gradually decreases to control levels (Ohsumi et al., 2006). In addition, another traumatic injury model with the use of laser lesioning in the mouse retina shows an increased expression of Cx36 in the inner and outer plexiform layers in the penumbra

area without changes in mRNA expression (Striedinger et al., 2005). However, Cx36 mRNA and protein levels progressively decrease in the hippocampus, but not in other brain regions, of the epileptic model induced by systemic administration of 4-aminopyridine (Zappala et al., 2006).

Although the contribution of gap junctions to CNS injuries is likely, it is still surrounded by some controversy depending on the severity of the insult. For example, Cx36 may have dual roles, either beneficial or detrimental, since the presence of Cx36 can promote either survival or damage after injury (Striedinger et al., 2005; Wang et al., 2010). The spread of apoptotic or necrotic signals through gap junctions has been suggested to propagate cell death from stressed to neighboring cells thus the blockade of gap junctions can be potently neuroprotective (Thompson et al., 2006). However, a 'good samaritan' role by passing cell survival promoting factors such as metabolites or genetic materials between cells is also possible (Jäderstad et al., 2010). Gap junction channels may help to buffer harmful levels of substances by allowing diffusion of ions and molecules to less injured cells, which in turn may counteract cytotoxicity and contribute to cell survival in the initially affected cells (Nakase et al., 2006).

### 5.3 Cx36 knockout animal studies

Recently, the mechanisms underlying the behavioral phenotypes of Cx36-deficient animals have been addressed. The Cx36 knock-out mice have the relatively benign behavioral phenotype in spite of a loss of electrotonic coupling and changes in subthreshold activities in many areas including retina, neocortex, hippocampus, thalamic reticular nucleus, and inferior olivary nucleus (Connors and Long, 2004; Frisch et al., 2005; van der Giessen et al., 2008). They experience impaired function in retina and reproductive system (Bennett and Zukin, 2004). The retina of Cx36 knock-out mouse shows the normally developed cellular organization of the rod pathway, but the functional coupling between AII amacrine cells and bipolar cells is

disrupted leading to impaired scotopic vision (Guldenagel et al., 2001). Electrical coupling between suprachiasmatic nucleus neurons is also lost in Cx36 knock-out mice showing abnormal circadian behavior (Long et al., 2005). In addition, Cx36 knock-out mice exhibit a remarkable resistance to neuronal death caused by NMDAR dysfunction (de Rivero Vaccari et al., 2007) or focal cerebral ischemia (Wang et al., 2010). A number of Cx36 mutant studies support the significant physiological roles of neuronal gap junction coupling in learning and memory, although Cx36 deficient animals show the mild relatively normal behavioral phenotypes (Frisch et al., 2005; van der Giessen et al., 2008). However, to compensate for Cx36 loss, individual neurons presumably undergo changes in cytological and electroresponsive properties rather than upregulation of other connexins (De Zeeuw, et al., 2003).

## 6. Chemical synaptic transmission

### 6.1 GABA-dependent synaptic transmission

GABA is the major inhibitory neurotransmitter in the mammalian CNS, which generates hyperpolarizing response. GABA activates three pharmacologically distinct receptor families, classified as ionotropic $GABA_A$ receptor ($GABA_AR$) and $GABA_C$ receptor and metabotropic $GABA_B$ receptors. These receptors have different characteristics, each of which is linked to a different signaling pathway. The $GABA_AR$ and $GABA_C$ receptors are transmitter-gated $Cl^-$ channels that mediate fast synaptic transmission. Upon binding of GABA, $GABA_AR$ and $GABA_C$ receptor undergo conformational changes to allow $Cl^-$ to pass through its pore which inhibits the firing of new action potentials. By contrast, metabotropic $GABA_B$ receptors are coupled to G-proteins to influence $K^+$ and $Ca^{2+}$ channels via second messenger systems, thus producing slow and prolonged inhibitory signals to modulate neurotransmitter release (Luján et al., 2004).

During early neuronal development, GABA acting via $GABA_A$Rs initially functions as an excitatory neurotransmitter in a wide range of brain structures as a result of a high intracellular concentration of $Cl^-$. It leads to generation of sodium action potentials, elevation of intracellular $Ca^{2+}$ concentration, and interaction with NMDAR by removing the voltage-dependent magnesium block in immature neurons (Ben-Ari et al., 2007). However, during later stages of development, excitatory GABAergic activity becomes inhibitory due to the shift in the $Cl^-$ equilibrium potential (Liu et al., 2006).

The activity of GABA receptors develops earlier than that of glutamate receptors, suggesting that GABA is the principal excitatory transmitter during early development (Ben-Ari et al., 2007). In addition, GABA-mediated signaling has been implicated in many developmental processes like neuronal proliferation, migration, differentiation, synapse maturation and network formation (Owens and Kriegstein, 2002).

### 6.2 Glutamate-dependent synaptic transmission

Glutamate is the major excitatory neurotransmitter in the CNS and acts on two classes of glutamate receptors: ionotropic and metabotropic receptors. Ionotropic glutamate receptors, including NMDA, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), and Kainate, are ligand-gated nonselective cation channels that mediate fast excitatory synaptic transmission via flow of $K^+$, $Na^+$, and sometimes $Ca^{2+}$. They mediate excitatory communication between neurons, but the speed and duration of the current is different for each type (Luján et al., 2004). Metabotropic glutamate receptors are divided into three groups, group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluR4, mGluR6, mGluR7, and mGluR8) based on receptor structure and physiological activity (Fig. 4). Unlike ionotropic receptors, they activate various biochemical cascades, leading to the modulation of a variety of target molecules. This

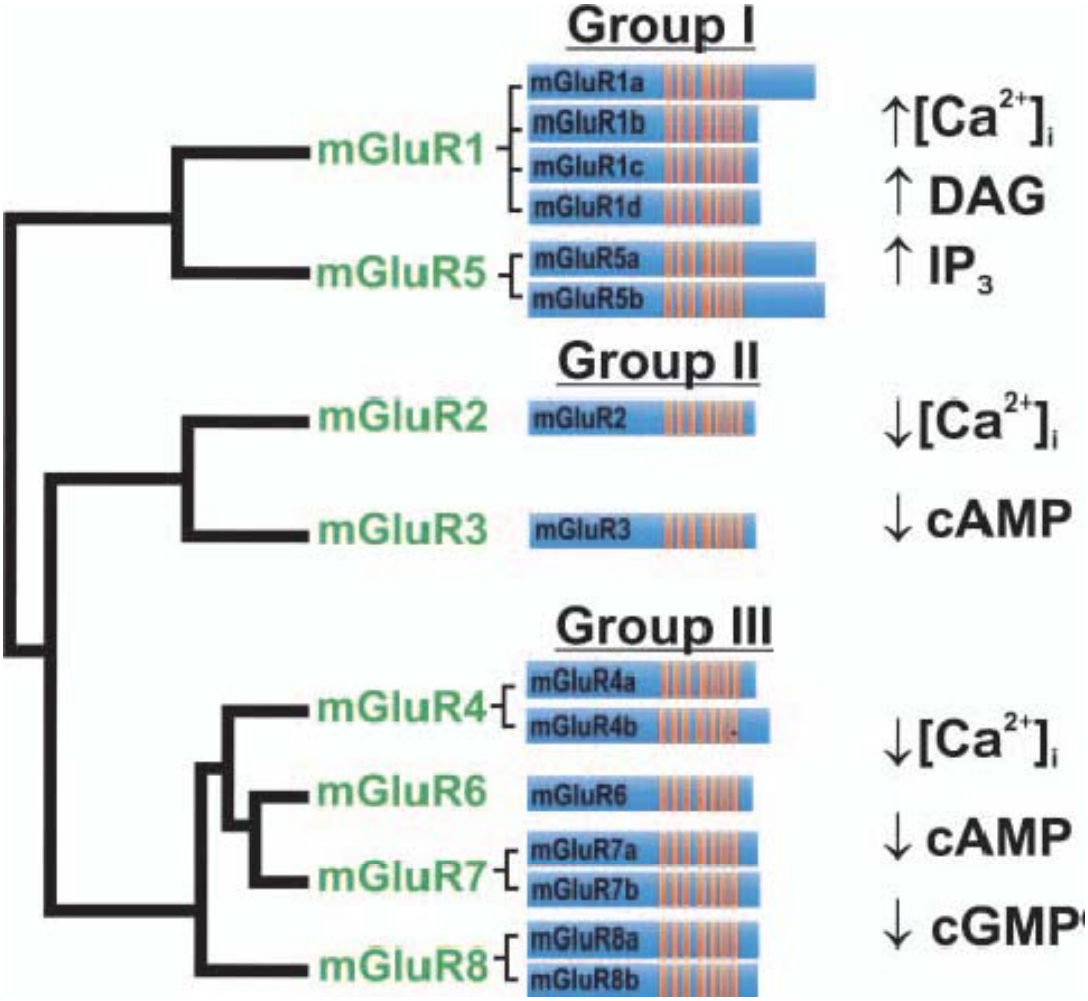
Group I
mGluR1a
mGluR1b
mGluR1c
mGluR1d
mGluR1
mGluR5a
mGluR5b
mGluR5
↑[Ca2+]i
↑ DAG
↑ IP3
Group II
mGluR2
mGluR2
mGluR3
mGluR3
↓[Ca2+]i
↓ cAMP
Group III
mGluR4a
mGluR4b
mGluR4
mGluR6
mGluR6
mGluR7a
mGluR7b
mGluR7
mGluR8a
mGluR8b
mGluR8
↓[Ca2+]i
↓ cAMP
↓ cGMP

**Figure 4.** Classification and chemical signaling pathways of mGluRs. The different splice variants are grouped by sequence homology and the vertically represented transmembrane domains separate the amino- and carboxy-termini. The right panel shows the changes in chemical signals upon the activation of the corresponding mGluR subtypes. (Diagram is from Coutinho and Knöpfel, 2002).

can lead to changes in the synapse's excitability, for example by presynaptic inhibition of neurotransmission, or modulation and even induction of postsynaptic responses (Luján et al., 2004). Group II mGluRs (mGluR2 and mGluR3) negatively regulate cAMP signaling. They have modulatory roles to influence other receptor function, for example, they are known to reduce the activity of NMDAR (Ghose et al, 2009).

Consistent with high sequence homology between mGluR2 and mGluR3, both receptors have similar signal transduction characteristics and agonist selectivity. However, separate mGluR2 and mGluR3 knockout mouse studies revealed the individual contributions of the mGluR2 versus mGluR3 in regulation of brain functions (Woolley et al., 2008; Pöschel et al., 2005). Furthermore, the postnatal expression of mGluR subtypes is differentially regulated in an age-dependent manner. In the brain, mGluR2 expression is low at birth and increases during postnatal development, whereas mGluR3 is highly expressed at birth and subsequently decreases during maturation to adult levels (Catania et al., 1994). The expression of mGluR2 and Cx36 shows parallel changes of the increasing pattern during early stages of postnatal period; they are low at birth and increase progressively later (Belluardo et al., 2000).

## 7. Aim of the study

Transient coupling of large groups of neurons by electrical synapses has been observed in a wide variety of regions in the developing mammalian nervous system (Bennet and Zukin, 2004). Gap junction-mediated interneuronal communication is prevalent during embryonic and/or early postnatal phase playing an important role in a number of developmental events; subsequently, it declines back to low level and persists only in restricted brain areas in adulthood. Apparently, during the early postnatal period, both gap junctions and developing chemical synaptic connections coexist, although gap junction expression seems to be

prerequisite for chemical synapses formation in specific spatiotemporal settings (Todd et al., 2010). Previous studies on the interactions between these two modes of communication focused mainly on the short time scale regulations such as gating and permeability of gap junction channels (Rörig et al., 1995; Rörig and Sutor, 1996). The long time scale mechanisms involved in changes in the level of Cx36-containing gap junction channel expression have not been investigated, and are of particular interest in this study. One recent study described that, during later stages of development, neuronal gap junction coupling decreases and this uncoupling event is regulated by the increasing glutamatergic synaptic transmission via ionotropic $Ca^{2+}$-permeable NMDAR and CREB signaling pathway (Arumugam et al., 2005). However, the mechanisms for regulation of the developmental increase in neuronal gap junction coupling at earlier stages have not been studied (Fig. 5). In addition, the issue whether gap junctions, during development, contribute to either cell death or survival still is not understood.

Therefore, the goal of this dissertation is to advance the understanding of the developmental regulation in Cx36-containing gap junction by exploring what and how certain physiological cues affect the developmental increase in electrical synapses and by further elucidating the functional implications of that developmental event. Specifically this study is designed to test the role of glutamate receptors and GABA receptors in the developmental increase in neuronal gap junction coupling and Cx36 expression as well as the developmental role of gap junctions in neuronal death/survival mechanisms. Evidently, some reports indicate that neuronal gap junction expression and communication may also increase in the adult nervous system after specific types of injuries or insults (Oguro et al., 2001; Chang et al., 2000). Furthermore, it is plausible to assume that developmental gap junction mechanisms are recapitulated during brain injury incidence. Therefore, the present studies will serve as a foundation for future studies to understand whether the developmental and injury-related

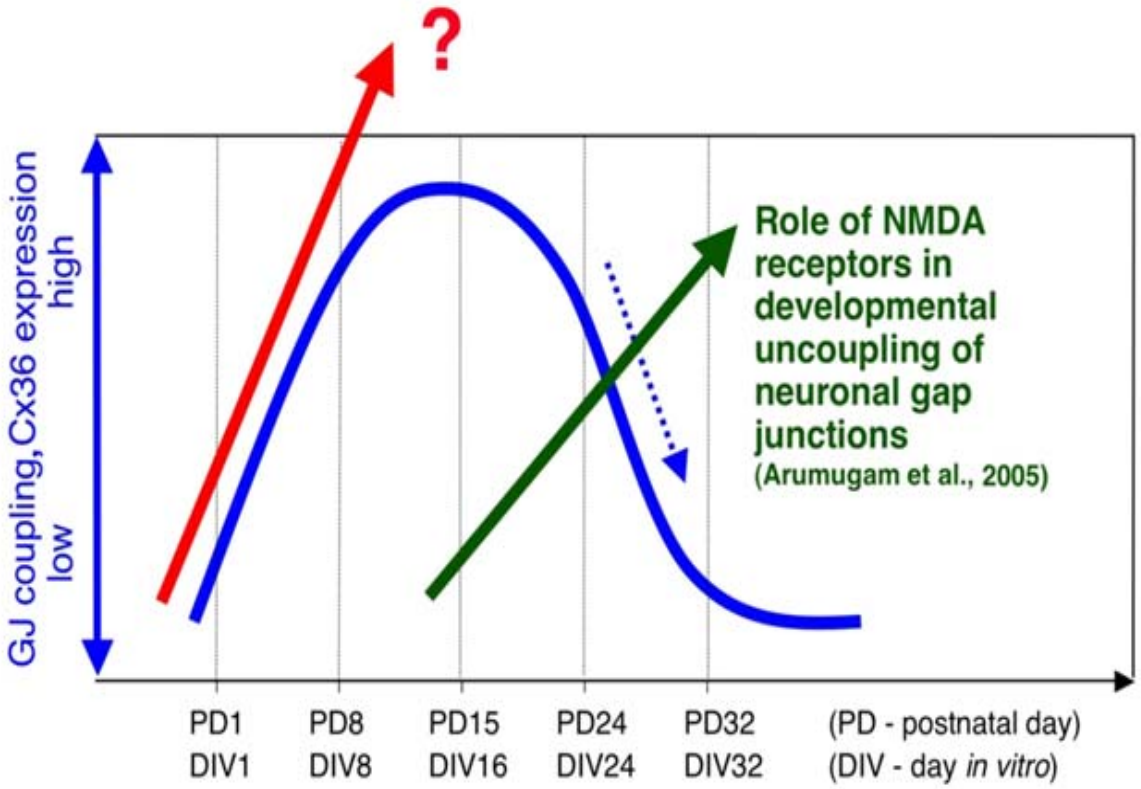
?
GJ coupling, Cx36 expression
high
low
Role of NMDA receptors in developmental uncoupling of neuronal gap junctions
(Arumugam et al., 2005)
PD1
DIV1
PD8
DIV8
PD15
DIV16
PD24
DIV24
PD32
DIV32
(PD - postnatal day)
(DIV - day *in vitro*)

**Figure 5**. Developmental changes in neuronal gap junction coupling and Cx36 expression in the rat hypothalamus. Cx36-mediated gap junction coupling is colored blue. The developmental increase in NMDAR-mediated glutamatergic synaptic activity is in green. The unknown developmental mechanism in neuronal gap junction coupling at an earlier postnatal age is colored red. GJ, gap junction.

increases in gap junction coupling employ the same cellular and molecular mechanisms. They will eventually benefit the novel strategies for gap junction-associated pharmaceutical development.

Experimental approaches included Western blot analysis, dye coupling, electrotonic coupling, real time quantitative polymerase chain reaction (RT-qPCR), small interfering RNA (siRNA) transfection, and luciferase reporter assay. The preparation used to study the developmental mechanisms in electrical synapses was the hypothalamus, which expresses gap junctions and is critical for homeostatic regulation and coordination of cardiovascular, nervous and endocrine functions (Hatton and Li, 1998). In particular, the hypothalamus plays an important variety of roles that include the regulation of heart rate, body temperature, feeding behavior, emotional behavior, circadian rhythms, and the endocrine system. Recent Cx36 knockout studies shed the light on the specific role of Cx36 containing-gap junctions in circadian behavior through hypothalamic intercellular synchronization (Long et al., 2005).

The dissertation consists of three parts in total: the general introduction (Chapter one), the research findings report entitled as “Interplay of Chemical Neurotransmitters Regulates Developmental Increase in Electrical Synapses” published in The Journal of Neuroscience (Park et al., 2011) (Chapter two), and conclusion and discussion of the further lines of research (Chapter three).

# CHAPTER TWO

## Interplay of Chemical Neurotransmitters Regulates Developmental Increase in Electrical Synapses

## 1. Introduction

Transient coupling of large groups of neurons by electrical synapses (gap junctions) is a general phenomenon in the developing mammalian CNS: it has been documented in different regions of the CNS (neocortex, hippocampus, hypothalamus, striatum, locus coeruleus, spinal cord, etc.) and in different species (rat, mouse, ferret, opossum, etc.) (Bennett and Zukin, 2004). Neuronal gap junction coupling increases during embryonic and/or early postnatal development and plays an important role in a number of developmental events, including neuronal differentiation, cell death, cell migration, synaptogenesis, and neural circuit formation (Allen and Warner, 1991; Walton and Navarrete, 1991; Peinado et al., 1993; Lin et al., 1998; Bani-Yaghoub et al., 1999; Personius et al., 2001; de Rivero Vaccari et al., 2007). It is believed that the contributions of gap junctions are via the passage of $Ca^{2+}$, $IP_3$, cAMP, and small molecules between the cells and coordination of metabolic and transcriptional activities in developing neurons (Kandler and Katz, 1998). In addition, gap junctions contribute to the generation of the highly synchronized excitatory electrical activity that is a hallmark of the developing brain (Feller et al., 1996; Ben-Ari, 2001). During later stages of development, neuronal gap junction coupling decreases (Arumugam et al., 2005). However, it increases in the mature CNS during neuronal injuries, such as ischemia, traumatic brain injury, inflammation, and epilepsy (Chang et al., 2000; Frantseva et al., 2002; de Pina-Benabou et al., 2005; Nemani and Binder, 2005; Thalakoti et al., 2007).

The developmental uncoupling of neuronal gap junctions occurs in response to increasing chemical synaptic (glutamatergic) transmission and activation of NMDARs (Arumugam et al., 2005). In addition, acute modulation of neuronal gap junction coupling by a number of neurotransmitter receptors in the developing and mature CNS has been reported (Hatton, 1998). However, whether chemical neurotransmitter receptors also regulate the

increases in neuronal gap junction coupling that occur during development and injuries is not known. Here we studied the cellular and molecular mechanisms for the developmental increase in neuronal gap junction coupling. The model system for the present study is the hypothalamus, which expresses gap junctions and is critical for homeostatic regulation and coordination of cardiovascular, nervous, and endocrine functions (Saper, 2004). We demonstrate that the developmental increase in neuronal gap junction coupling and expression of Cx36, neuronal gap junction protein are regulated by an interplay between the activity of group II mGluRs and $GABA_A$Rs. We also show that this regulation is via NRSE in the Cx36 gene promoter and post-transcriptional control of Cx36 mRNA. Finally, our data suggest that the mechanisms for the developmental increase in neuronal gap junction coupling contribute to the regulation of neuronal death/survival mechanisms in developing neurons.

## 2. Materials and Methods

### Animal care

The use of animal subjects in these experiments was approved by the University of Kansas Medical Center Animal Care and Use Committee. The experiments were conducted in accordance with NIH guidelines. Sprague-Dawley rats, Cx36 knockout mice (C57bl/6 background strain) and wild-type mice (C57bl/6) of either sex were used. The Cx36 knockout was originally created by Dr. David Paul (Harvard Medical School). Mice were genotyped as described (de Rivero Vaccari et al., 2007).

**Pharmacological treatments of animals**

Rat pups of either sex received daily subcutaneous injections of drugs in 3 increasing sets of concentrations: LY379268 - 3, 5, and 7 mg/kg; LY341495 - 1.5, 2, and 2.5 mg/kg; muscimol - 0.25, 0.5, and 0.75 mg/kg; bicuculline - 1, 1.25, and 1.5 mg/kg; administered, respectively, on postnatal days 1-5 (P1-5; dissolved in 20 µl of sterile saline), P6-10 (40 µl), and P11-15 (60 µl). Control animals received the corresponding volumes of sterile saline. The forebrain was dissected and weighted on P15 and only animals that received muscimol administrations demonstrated slightly reduced forebrain weight (not shown). However, because the loading of protein in all western blots was normalized (i.e., 50 µg of protein per lane) and the expression of tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the hypothalamus was not different between the control and muscimol-treated animals (not shown), we concluded that a decrease in Cx36 expression in muscimol-treated rats was rather specific, i.e., it reflected the $GABA_AR$ dependent down-regulation of Cx36 and was not due to a nonspecific decrease in the amount of protein (e.g., because of decrease in the size of the forebrain).

**Brain slice and culture preparations and culture treatments**

To prepare brain slices, rats were anesthetized (nembutal; 70 mg/kg; intraperitoneal injection, i.p.), sacrificed, the brain was removed, and 400 µm-thick coronal hypothalamic slices were prepared (at 2-4°C) and kept (at 20-22°C) in artificial cerebrospinal fluid as described (Belousov and van den Pol, 1997). Cultures were prepared as reported previously (Belousov et al., 2001) from the medial hypothalamus or somatosensory cortex obtained from day 18-19 (rat) or 16-17 (mouse) embryos. Pregnant animals were anesthetized (nembutal; 70 mg/kg; i.p.) before embryos were removed. After disaggregation using papain, neurons were plated on

glass coverslips and raised in Neurobasal medium (Invitrogen, Carlsbad, CA, USA, cat.# 21103), in which the percentage of neurons reaches ~95% (Wang et al., 2008). The medium was supplemented with B-27 (Invitrogen, cat.# 17504) and 0.5 mM L-glutamine. The culture medium was changed twice a week. Drug and siRNA treatments and luciferase construct transfections were performed using sister cultures. Cell survival was estimated by analyzing the number of live neurons as described (Belousov et al., 2001) and none of the chronic treatments reduced neuronal survival as compared to the control (only luciferase construct transfections and siRNA treatments induced neurodegeneration in cultures after, respectively, 5 and 7 days; therefore, in those experiments cells were exposed to the agents for not more than 4 and 6 days, respectively). Dendritic processes were analyzed using calcein AM staining and were only affected by activation of $GABA_A$Rs (slight increase) and inactivation of $GABA_A$Rs (slight decrease). However, because these changes were opposite to those in Cx36 expression and dye coupling, we concluded that the changes in dendrites were not responsible for alterations in the expression of gap junctions. For chronic $GABA_A$R blockade *in vitro*, we followed a previously proposed protocol (Muller et al., 1993), where for maximal effect the use of both bicuculline and picrotoxin was proposed.

**Dye coupling**

Dye coupling in slices and cultures was performed as described in detail (Arumugam et al., 2005). The pipette solution contained (mM): 145 $KMeSO_4$, 10 HEPES, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 2 Na-ATP, and 0.3 Na-GTP, 0.2% neurobiotin (Vector Laboratories, Burlingame, CA, USA, cat.# SP-1120; MW323; gap junction permeable dye), and 0.04% dextran Alexa Fluor 594 (Invitrogen, cat.# D22913; MW10,000; gap junction-impermeable dye) (pH 7.2, 3-7 MΩ electrode resistance). Cells were patched using Multiclamp 700-B amplifier and pCLAMP10

software (Molecular Devices, Sunnyvale, CA, USA). *In slices:* On the day of preparation, slices were randomly numbered and the number and condition (a drug treatment) were documented in a database (Access) for later identification. Magnocellular neurons in the hypothalamic PVN and SON were labeled after initial identification based on their location, size, and electrophysiological properties (Arumugam et al., 2005). Only one neuron per slice was labeled as reported (Arumugam et al., 2005). After labeling, the slices were fixed, processed, and stained with fluorescein avidin D (FITC, 1:200, Vector Laboratories). Alexa 594 fluorescence and neurobiotin staining were visualized using, respectively, Texas Red and FITC filters in Nikon 80i fluorescent microscope, PHOTOMETRICS ES2 camera and OpenLab Software (Improvision, Lexington, MA, USA). The incidence of gap junction coupling was analyzed blindly for experimental groups as described (Arumugam et al., 2005). *In cultures:* Because different cell types are morphologically indistinguishable and electrophysiological characterization of cell types is not elaborated, neurons in cultures were chosen randomly. Neurons were labeled, stained, and analyzed as described above for slices. This experiment was conducted by Dr. Youngfu Wang.

**Electrotonic coupling**

To determine electrotonic coupling, dual whole-cell current clamp recordings were conducted in cultures from pairs of randomly-chosen neurons. Test current steps (500 ms, -100 pA) were applied to cell 2 (injected cell) and electrotonic responses were detected in cell 1 (non-injected cell). Recordings were done at a holding potential of -65 mV. Data were monitored using an electrophysiology approach (see above) and analyzed off-line with Clampfit 10 (Molecular Devices). The coupling coefficient was calculated as the response amplitude in the non-injected cell (cell 1) divided by the amplitude in the injected cell (cell 2). Cells were

considered as coupled if the coupling coefficient was above 1.6%. This experiment was conducted by Dr. Youngfu Wang.

**Western blots**

Experiments were performed as reported in detail (Arumugam et al., 2005). Hypothalamic tissue or cultured cells were homogenized in a lysis buffer and total protein was determined using the Bio-Rad DC protein assay method. Fifty µg of protein was loaded in each lane, transferred to 0.45 µm Polyvinylidene difluoride membrane and processed with a blocking solution and antibodies. Rabbit anti-Cx36 (0.5µg/ml; Zymed, San Francisco CA, USA, cat.# 51-6300), rabbit anti-Cx43 (0.2 µg/ml; Zymed, cat.# 71-0700), rabbit anti-mGluR2 (0.5µg/ml; Millipore, Billerica, MA, USA, cat.# AB9209), rabbit anti-mGluR3 (0.5µg/ml; Sigma, St. Louis, MO, USA, cat.# G1545), mouse anti-Tubulin (1:10,000; Sigma, cat.# T6793), and rabbit anti-GAPDH (1:10,000; Cell Signaling Technology, Danvers, MA, USA, cat.# 2118) were used as the primary antibodies and they were visualized with horseradish peroxidase conjugated anti-rabbit (1:10,000, Zymed, cat.# G21234) or anti-mouse (1:10,000, Zymed, cat.# G21040) antibodies. Signals were enhanced using ECL detection reagents (Amersham Biosciences, Piscataway, NJ, USA). Band optical density was determined by using Quantity One quantification analysis software 4.5.2 (Bio-Rad, Hercules, CA, USA). All optical density signals were normalized relative to tubulin or GAPDH, and experimental samples were compared to controls (set at 1.0). Tubulin and GAPDH levels per unit of total protein did not vary significantly among samples used in this study.

**siRNA**

The mGluR2, mGluR3, and Cx36 siRNAs were purchased from Dharmacon RNAi Technologies (Lafayette, CO, USA; cat.# M-080176-00, L-094437-01 and L-090683-00, respectively). Each siRNA consisted of four pooled 19-nucleotide duplexes and was used in a final concentration of 50 nM. mGluR2 and mGluR3 siRNA transfections were performed on day *in vitro* 3 (DIV3) by using Lipofectamine 2000 (Invitrogen) and cells were processed on DIV7 for western blot analysis. Cx36 siRNA transfections were conducted on DIV10 and dye coupling was analyzed on DIV15. All transfections effectively reduced protein levels. Scrambled siRNAs were used as controls and were ineffective.

**Real time-quantitative polymerase chain reaction (RT-qPCR)**

Experiments were performed as reported (Al-Kandari et al., 2007). Total RNA was isolated from cultures using Trizol method (Invitrogen). Total RNA (1 µg) was reverse transcribed with oligo-dT primers and the SuperScript II kit (Invitrogen) according to the manufacturer's instructions. One µl of the reverse transcription reaction material was used as template for RT-qPCR using a Bio-Rad iCycler in a total volume of 20 µl with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and amplified for 40 cycles for 15 sec at 95°C and for 20 sec at 60°C. The following primer pairs were used: rat Cx36, 5'-CTATGTGTGAAAGGGCAGGTT-3' (sense) and 5'-AGCACTACGCAAATGAGGGCAA-3' (antisense); rat GAPDH, 5'CAAGGCTGTGGGCAAGGTCAT-3' (sense) and 5'-GCAGGTTTCTCCAGGCGGCAT-3' (antisense). RT-qPCR was performed using at least 4 separately prepared sets of culture samples. For each sample set, Cx36 mRNA signals were normalized to GAPDH mRNA signals, and normalized values were compared to controls (set at 1.0).

**Luciferase reporter activity analysis**

Rat Cx36 gene promoter fragments were PCR amplified from -984 to +115 relative to the transcription start site of the Cx36 gene (GeneID 50564, official name Gjd2*)* from a rat bacterial artificial chromosome containing the gene. Primers used were as follows: 5'-GCGAGATCTCGCTGTGCATCCGGAGGCAGC-3' for the antisense primer, 5'-GCGGCTAGCCCCTGGTTCGCTGCTAGGCAC-3' for the sense primer. The PCR products digested with NheI and BglII were cloned into the luciferase reporter plasmid pGL3basic (Promega, Madison, WI, USA). Site-directed mutagenesis was performed to produce an NRSE deleted plasmid using the Transformer Site Directed Mutagenesis Kit (Clontech Inc. Palo Alto, CA) and the following primers: 5'- TAAAATCGATAAGGGTCCGTCGACCGATGC-3' for selection primer and 5'- GAGACTGCGGGAGTCCGAGGTGCTGTCCAG-3' for mutagenic primer (the mutated nucleotide is underlined). The full length 3'UTR of the rat Cx36 mRNA (accession number NM 019281) was PCR-amplified using sense primer, 5'-TCGAGGATCCAAAGGGCAGGTTTGGGGAAG-3' and antisense primer, 5'-GTTAGTCGACCAGGCCAAATGTCTGTCCAG-3'. The BamHI-SalI-digested products were cloned into the Cx36 promoter-containing pGL3basic vector replacing the SV40 poly A signal. All plasmid constructs were verified by sequencing. Cells were transfected on DIV3 using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions. Some cultures were incubated (DIV4-DIV7) in the constant presence of receptor agonists and antagonists; cultures incubated in the absence of drugs served as a control. On DIV7, cell lysates were harvested and luciferase assay was performed with the DLR-Dual Luciferase kit (Promega) and Turner TD-20/20 Luminometer as described previously (Al- Kandari et al., 2007). All transfections for luciferase assay included an expression plasmid for *Renilla*

luciferase. The firefly luciferase activity was normalized to *Renilla* luciferase activity, to account for variation in transfection efficiency.

**Methyl thiazolyl tetrazolium (MTT) assay**

Neuronal viability in cultures was quantitatively evaluated by MTT assay. Cultures were raised in 24-well plates. In all NMDA tests, NMDA was added to the culture medium on DIV14 (100 μM for 60 min in hypothalamic cultures or 10 μM for 30 min in cortical cultures) and then washed-out. LY341495- and LY379268-treated cultures were chronically (on DIV3-DIV15) incubated in the presence of these agents. Carbenoxolone was added to the culture medium on DIV14 (alone or together with NMDA) and remained in the medium until the end of the experiment (on DIV15). On DIV15, 24 hrs after NMDA wash-out, neurons were incubated with MTT (MTT cell Viability Assay Kit, Biotium, Inc.; Hayward, CA, USA; 40 μM, 400 μl per well) at 37°C for 4 hrs. Then the medium was carefully aspirated and 400 μl of DMSO per well was added to dissolve the blue formazan product. To measure the absorbance, 200 μl of the medium from each well in 24-well plate were transferred into an independent well in a 96- well plate. The values of absorbance at 570 nm were measured using a microplate reader (μQuant, BioTek, Winooski, VT, USA). Further, as indicated above, cultures that are raised in Neurobasal medium contain mostly neurons (up to 95%). However, to control specifically for neuronal cell death, a separate group of cultures ($n$ = 6) was subjected to a high concentration of glutamate (500 μM) starting from DIV14 for 24 hrs that killed all neurons, but did not affect glial cell survival. The absorbance in these purified glial cultures was measured, averaged, and the result was subtracted from the individual absorbance data in neuronal culture groups so that the final result would represent only neuronal death/survival. Finally, the absorbance results in

experimental groups were normalized to control groups. This experiment was conducted by Janna V. Denisova.

### Drugs and reagents

All drugs were obtained from Sigma or Tocris (Ellisville, MO, USA) unless otherwise specified.

### Statistical analysis

Data were analyzed using the two-tailed Student's *t*-test (paired, when possible), ANOVA or Fisher's exact probability test and InStat software (GraphPad Software, San Diego, CA, USA). Data are reported as mean ± SEM for the number of samples indicated. Each date point represents indicated day ± 1, except for siRNA and luciferase reporter activity experiments, where all dates are as indicated.

## 3. Results

### Developmental increase in neuronal gap junction coupling *in vivo*

Developmental changes in neuronal gap junction coupling were determined in magnocellular neurons in acute slices of the PVN and SON of the rat hypothalamus. We utilized a dye coupling method (Arumugam et al., 2005) that included the use of two dyes: neurobiotin, which passes through gap junctions (coupling tracer), and dextran Alexa Fluor 594, which is gap junction impermeable (Fig. 6*A-C*). We also performed western analysis on dissected

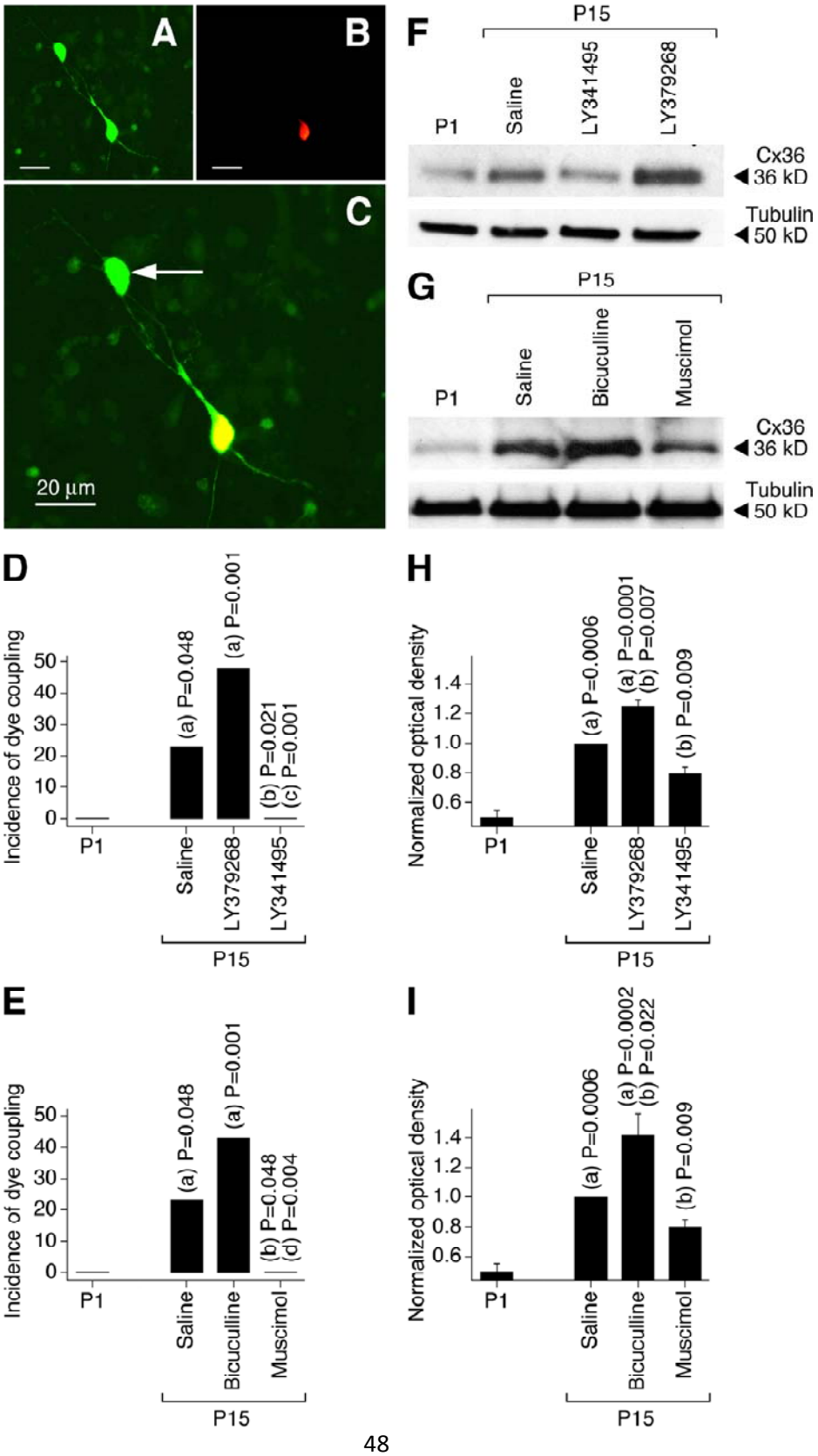
A
B
C
20 µm
F
P15
P1
Saline
LY341495
LY379268
Cx36
36 kD
Tubulin
50 kD
G
P15
P1
Saline
Bicuculline
Muscimol
Cx36
36 kD
Tubulin
50 kD
D
Incidence of dye coupling
50
40
30
20
10
0
P1
Saline
LY379268
LY341495
(a) P=0.048
(a) P=0.001
(b) P=0.021
(c) P=0.001
P15
H
Normalized optical density
1.4
1.2
1.0
0.8
0.6
P1
Saline
LY379268
LY341495
(a) P=0.0006
(a) P=0.0001
(b) P=0.007
(b) P=0.009
P15
E
Incidence of dye coupling
50
40
30
20
10
0
P1
Saline
Bicuculline
Muscimol
(a) P=0.048
(a) P=0.001
(b) P=0.048
(d) P=0.004
P15
I
Normalized optical density
1.4
1.2
1.0
0.8
0.6
P1
Saline
Bicuculline
Muscimol
(a) P=0.0006
(a) P=0.0002
(b) P=0.022
(b) P=0.009
P15

**Figure 6.** Developmental increase in neuronal gap junction coupling in the rat hypothalamus *in vivo* is regulated by an interplay between the activity of group II mGluRs and $GABA_A$Rs. ***A-C,*** Images of neurobiotin (***A,*** green) and dextran Alexa 594 (***B,*** red) staining in an SON slice (P15; bicuculline-treated rat) are superimposed in ***C*** (shown at higher magnification). Yellow indicates dye colocalization in the primary-labeled neuron. Arrow indicates the secondary-labeled neuron. ***D, E,*** Incidence of dye coupling. Statistical significance was calculated using the Fisher's exact probability test (19-22 primary-labeled neurons per data point; data for SON and PVN are combined; also see Table 1). ***F-I,*** Expression of Cx36 protein in the hypothalamus. Optical density signals are normalized relative to tubulin and P15 saline-treated controls are set at 1.0. Statistical analysis (***H, I***): Student's *t*-test; mean ± SEM; *n* = 10 in each group. In all graphs, statistical difference is shown relative to: (a), P1; (b), P15 saline; (c), LY379268; (d) bicuculline. ***A-E,*** Data were obtained by Dr. Yougfu Wang.

hypothalamus to confirm the dye coupling observations by measuring developmental changes in the expression of Cx36, a gap junction protein that is neuron specific and is essential for functional gap junctions in the hypothalamus (Belluardo et al., 2000; Rash et al., 2000; Long et al., 2005) (Fig. 6*F,G*). The incidence of dye coupling, i.e. the percentage of primary-labeled neurons coupled to one or more secondary-labeled neurons, and the expression of Cx36 both increased between P1 and P15 (Fig. 6). This indicates a developmental increase in neuronal gap junction coupling in the hypothalamus. Daily (on P1-P15) subcutaneous administration of the group II mGluR agonist LY379268 augmented, and group II mGluR antagonist LY341495 prevented, these developmental increases in gap junction coupling and Cx36 expression (Fig. 6*D,F,H,* and Table 1). In contrast, daily administration of the $GABA_AR$ agonist muscimol prevented, and $GABA_AR$ antagonist bicuculline augmented, the developmental increases in gap junction coupling and expression of Cx36 (Fig. 6*E,G,I,* and Table 1) (see Materials and Methods for drug concentrations).

**Developmental increase in neuronal gap junction coupling *in vitro***

A previous study in primary rat hypothalamic cultures indicated (Arumugam et al., 2005) that a developmental increase in neuronal gap junction coupling occurs during the first two weeks of *in vitro* development. This increase was prevented by blockade of action potentials (with 2 μM tetrodotoxin, a voltage-gated sodium channel blocker), and was not affected by inactivation of NMDARs (with 100 μM AP5). These results suggested that the developmental increase in neuronal gap junction coupling is regulated via action potential-dependent (synaptic) release of neurotransmitters, but NMDARs are not involved in this regulation.

**Table 1.** Neuronal dye coupling in the hypothalamus *in vivo* and *in vitro*.

| Conditions | Total number of primary-labeled neurons | Number of dye-coupled primary-labeled neurons, (%) | Coupling index[i] 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|
| Slice, rat[ii] | | | | | | |
| P1 | 19 | 0 (0), P = 0.049 | - | - | - | - |
| P15, saline | 21 | 5 (23.8) | 3 | - | 1 | 1 |
| P15, bicuculline | 21 | 10 (47.6), P = 0.197 | 6 | 3 | 1 | - |
| P15, muscimol | 19 | 0 (0), P = 0.049 | - | - | - | - |
| P15, LY341495 | 22 | 0 (0), P = 0.021 | - | - | - | - |
| P15, LY379268 | 21 | 9 (42.9), P = 0.326 | 6 | 3 | - | - |
| Culture, rat[iii] | | | | | | |
| DIV3, control | 32 | 0 (0), P = 0.024 | - | - | - | - |
| DIV15, control | 32 | 6 (18.8) | 5 | 1 | - | - |
| DIV15, Bic/PiTX+A/C | 25 | 11 (44.0), P = 0.047 | 10 | 1 | - | - |
| DIV15, muscimol | 35 | 1 (2.9), P = 0.048 | 1 | - | - | - |
| DIV15, LY341495 | 24 | 0 (0), P = 0.032 | - | - | - | - |
| DIV15, LY379268 | 20 | 6 (33.3), P = 0.500 | 4 | 2 | - | - |
| DIV15, Cx36 siRNA | 25 | 0 (0), P = 0.029 | - | - | - | - |
| DIV15, SCR siRNA | 21 | 4 (19.0), P = 1.0[iii] P = 0.037[iv] | 2 | 1 | 1 | - |
| Culture, wild-type mouse[iii] | | | | | | |
| DIV15, control | 26 | 3 (11.5) | 2 | 1 | - | - |
| DIV15, Bic/PiTX+A/C | 21 | 8 (38.1), P = 0.043 | 5 | 2 | 1 | - |
| DIV15, LY379268 | 24 | 9 (37.5), P = 0.047 | 6 | 1 | 1 | 1 |
| Culture, Cx36 knockout mouse[iii] | | | | | | |
| DIV15, control | 20 | 0 (0) | - | - | - | - |
| DIV15, Bic/PiTX+A/C | 21 | 0 (0)[v] | - | - | - | - |
| DIV15, LY379268 | 21 | 1 (4.8), P = 1.0 | 1 | - | - | - |

[i] Coupling index is the number of secondary-labeled neurons coupled to the primary labeled neuron. [ii-iii] Statistical significance was calculated using the Fisher's exact probability test relative to P15 saline[ii] and the corresponding DIV15 controls[iii]. Statistical significance for SCR siRNA (scrambled siRNA) group is shown relative to the control[iii] and Cx36 siRNA[iv v]. P value cannot be calculated. Bic/PiTX+A/C, culture treated with bicuculline, picrotoxin, AP5, and CNQX. For drug concentrations, see the text. All data were obtained by Dr. Yougfu Wang.

In the present study, using neurobiotin with dextran Alexa Fluor 594 and western analysis we observed increases in neuronal dye coupling and Cx36 expression in developing rat hypothalamic neuronal cultures between DIV3 and DIV15 (Fig. 7 and Table1). In cultures, as in the hypothalamus *in vivo*, the developmental increases in dye coupling and Cx36 expression were augmented by the chronic (on DIV3-DIV15) activation of group II mGluRs with LY379268 (2 μM) or DCG-IV (10 μM; another group II mGluR agonist) and were prevented by inactivation of group II mGluRs with LY341495 (2 μM) or EGLU (100 μM; another group II mGluR antagonist) (Fig. 7*D,F,H,*and Table 1; also see below Fig. 10*A*).

Further, the developmental increases in gap junction coupling and the expression of Cx36 were prevented by activation of $GABA_A$Rs with muscimol (25 μM) or GABA (100 μM) and were augmented by $GABA_A$R inactivation with bicuculline plus picrotoxin (100 μM + 500 μM; in these tests, ionotropic glutamate receptor antagonists, 100 μM AP5 and 10 μM CNQX, were co-administered with the $GABA_A$R antagonists to prevent an increase in glutamate-dependent activity) (Fig. 7*E,G,I,* and Table 1; also see below Fig. 10*B*). Similar results were obtained in cultures using electrotonic coupling analysis (Fig. 8). However, no difference in Cx36 expression was observed between the controls and cultures that were chronically treated (on DIV3-DIV15) with AP5 plus CNQX (100 μM and 10 μM), DHPG (10 μM; group I mGluR agonist), AIDA (100 μM; group I mGluR antagonist), PPG (10 μM; group III mGluR agonist), MSOP (100 μM; group III mGluR antagonist), nicotine (100 μM; nicotinic acetylcholine receptor agonist), atropine plus mecamylamine (100 μM each; muscarinic and nicotinic acetylcholine receptor antagonists), baclofen (20 μM; $GABA_B$ receptor agonist) or phaclofen (100 μM; $GABA_B$ receptor antagonist) (Fig. 9).

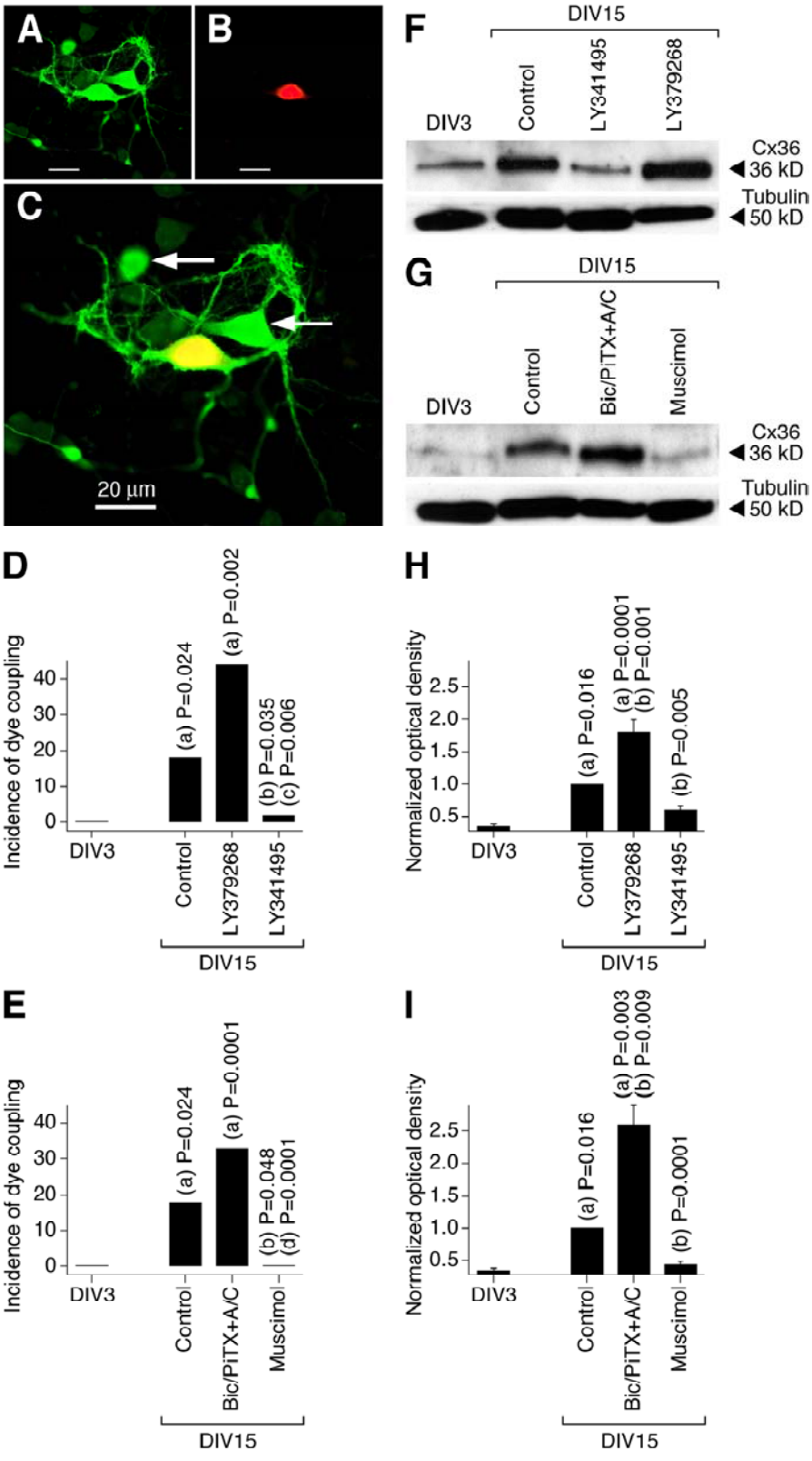
A
B
C
20 µm
F
DIV15
DIV3
Control
LY341495
LY379268
Cx36
36 kD
Tubulin
50 kD
G
DIV15
DIV3
Control
Bic/PiTX+A/C
Muscimol
Cx36
36 kD
Tubulin
50 kD
D
Incidence of dye coupling
40
30
20
10
0
(a) P=0.024
(a) P=0.002
(b) P=0.035
(c) P=0.006
DIV3
Control
LY379268
LY341495
DIV15
H
Normalized optical density
2.5
2.0
1.5
1.0
0.5
(a) P=0.016
(a) P=0.0001
(b) P=0.001
(b) P=0.005
DIV3
Control
LY379268
LY341495
DIV15
E
Incidence of dye coupling
40
30
20
10
0
(a) P=0.024
(a) P=0.0001
(b) P=0.048
(d) P=0.0001
DIV3
Control
Bic/PiTX+A/C
Muscimol
DIV15
I
Normalized optical density
2.5
2.0
1.5
1.0
0.5
(a) P=0.016
(a) P=0.003
(b) P=0.009
(b) P=0.0001
DIV3
Control
Bic/PiTX+A/C
Muscimol
DIV15

**Figure 7.** Developmental increase in neuronal gap junction coupling in the rat hypothalamus *in vitro* is regulated by an interplay between the activity of group II mGluRs and $GABA_A$Rs. ***A-C,*** Images of neurobiotin (***A***) and dextran Alexa 594 (***B***) staining and their overlap (***C***) are shown (DIV15; culture treated with bicuculline, picrotoxin, AP5, and CNQX; Bic/PiTX+A/C). Yellow indicates dye colocalization in the primary-labeled neuron. Arrows indicate the secondary-labeled neurons. ***D, E,*** Incidence of dye coupling. Statistical significance was calculated using the Fisher's exact probability test (20-35 primary-labeled neurons per data point, also see Table 1). ***F-I,*** Expression of Cx36 protein. Optical density signals are normalized relative to tubulin and DIV15 controls are set at 1.0. Statistical analysis (***H, I***): Student's *t*-test; mean ± SEM; $n = 6$ in each group. In all graphs, statistical difference is shown relative to: (a), DIV3; (b), DIV15 control; (c), LY379268; (d) Bic/PiTX+A/C. ***A-E,*** Data were obtained by Dr. Yougfu Wang.

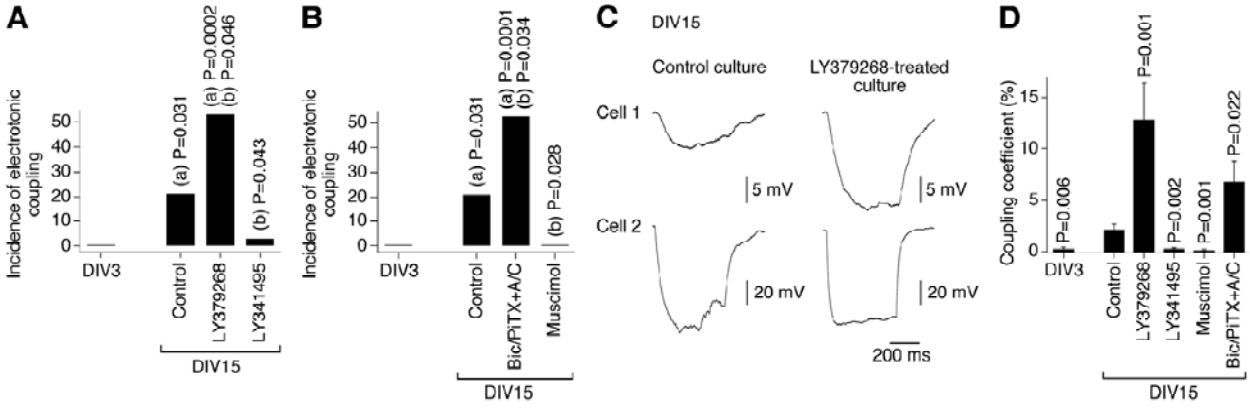
A
Incidence of electrotonic coupling
50
40
30
20
10
0
(a) P=0.031
(a) P=0.0002
(b) P=0.046
(b) P=0.043
DIV3
Control
LY379268
LY341495
DIV15
B
Incidence of electrotonic coupling
50
40
30
20
10
0
(a) P=0.031
(a) P=0.0001
(b) P=0.034
(b) P=0.028
DIV3
Control
Bic/PiTX+A/C
Muscimol
DIV15
C
DIV15
Control culture
LY379268-treated culture
Cell 1
5 mV
5 mV
Cell 2
20 mV
20 mV
200 ms
D
Coupling coefficient (%)
15
10
5
0
P=0.006
P=0.001
P=0.002
P=0.001
P=0.022
DIV3
Control
LY379268
LY341495
Muscimol
Bic/PiTX+A/C
DIV15

**Figure 8.** Developmental increase in neuronal gap junction coupling is regulated by an interplay between the activity of group II mGluRs and $GABA_A$Rs. Data from electrotonic coupling experiments in rat hypothalamic cultures are presented. The coupling was determined as described in Materials and Methods. The number of coupled pairs was as follows: DIV3, 0 of 21; DIV15: Control, 6 of 28; LY379268, 8 of 15; LY341495, 1 of 32; muscimol, 0 of 22; Bic/PiTX+A/C, 10 of 19. ***A, B,*** Incidence of electrotonic coupling represents the percentage of neuronal pairs that demonstrated the coupling. Statistical significance: Fisher's exact probability test; (a) relative to DIV3; (b) relative to DIV15 control. ***C,*** Representative traces of electrotonic responses are shown (each trace is the average voltage response from 5 sequential steps). ***D,*** Statistical analysis of the coupling coefficient (see Materials and Methods): Students *t*-test relative to DIV15 control; mean ± SEM (responses from all of the tested pairs are included in the analysis). Note, that on DIV15, the incidence of electrotonic coupling and the coupling coefficient both are higher (relative to the control) in cultures subjected to chronic activation of group II mGluRs and inactivation of $GABA_A$Rs and are lower in cultures subjected to inactivation of group II mGluRs and activation of $GABA_A$Rs. Data were obtained by Dr. Yougfu Wang.

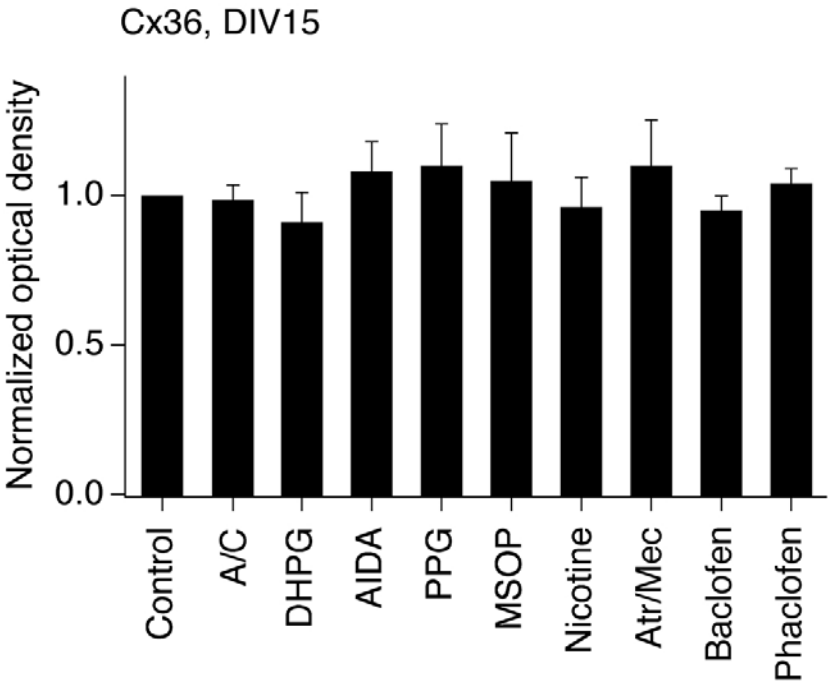
Cx36, DIV15
Normalized optical density
1.0
0.5
0.0
Control
A/C
DHPG
AIDA
PPG
MSOP
Nicotine
Atr/Mec
Baclofen
Phaclofen

**Figure 9.** Ionotropic glutamate receptors, group I mGluRs, group III mGluRs, acetylcholine receptors and $GABA_B$ receptors are not involved in the regulation of developmental increase in neuronal gap junction coupling. Pharmacological manipulations (see text for details and concentrations) were performed in rat hypothalamic cultures on DIV3-15 followed by assessment of Cx36 protein expression on DIV15. Optical density signals are normalized relative to tubulin, and normalized values are compared to the control (set at 1.0). Statistical data are presented: paired Student's *t*-test relative to control; mean ± SEM; *n* = 6 in each group. No significant difference between the control and indicated treatments was found. A/C, AP5 plus CNQX; Atr/Mec, atropine plus mecamylamine.

Taken together, our *in vivo* and *in vitro* data suggest that the developmental increase in gap junction coupling in hypothalamic neurons is regulated by an interplay between the activity of group II mGluRs and $GABA_A$Rs. In addition, group I mGluR, group III mGluR, NMDA, non-NMDA, acetylcholine and $GABA_B$ receptors are not involved in these regulatory mechanisms.

**Cellular mechanisms for developmental increase in gap junction coupling**

Signaling pathways for group II mGluRs and $GABA_A$Rs are well-characterized. Group II mGluRs (that include mGluR2 and mGluR3) negatively regulate cAMP/PKA (cAMP-dependent protein kinase)-dependent signaling (De Blasi et al., 2001; Conn et al., 2005). $GABA_A$R is a $Cl^-$-permeable ion channel. In mature neurons, an activation of $GABA_A$Rs causes $Cl^-$ influx and cell hyperpolarization; in developing neurons, an activation of $GABA_A$Rs causes an efflux of $Cl^-$ ions and cell depolarization that results in a $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels (VGCC) and activation of $Ca^{2+}$-dependent protein kinases (Stein and Nicoll, 2003). In primary rat hypothalamic cultures, $GABA_A$R-mediated responses are excitatory during the first 1-2 weeks of development and then the excitation is replaced by inhibitory responses (Obrietan and van den Pol, 1995).

To determine whether group II mGluRs and $GABA_A$Rs regulate the developmental increase in neuronal gap junction coupling via, respectively, cAMP/PKA- and $Ca^{2+}$- dependent signaling pathways, we performed additional pharmacological manipulations in rat hypothalamic cultures on DIV3-DIV15 and detected Cx36 expression by western analysis (Fig. 10). An increase in cytoplasmic levels of cAMP (by 8-Br-cAMP plus IBMX; 100 μM + 50 μM; a cell-permeable analog of cAMP that activates PKA and a non specific phosphodiesterase inhibitor

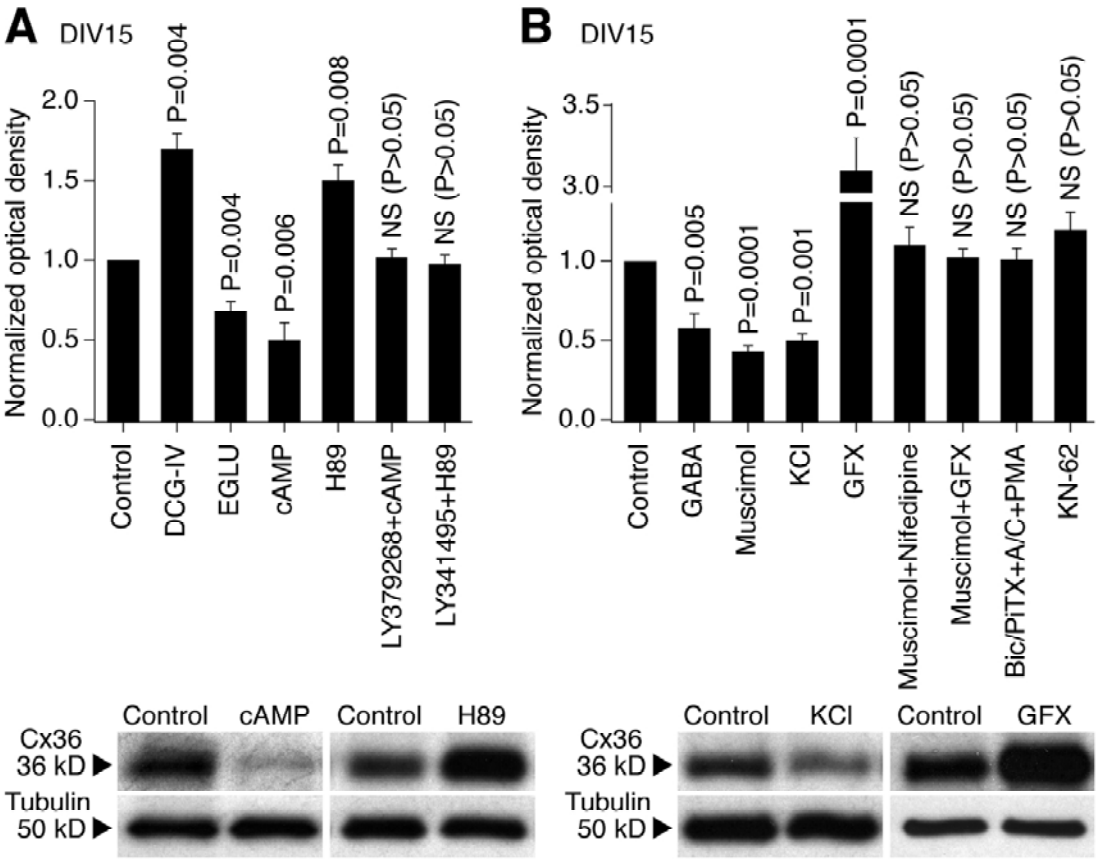
A
DIV15
Normalized optical density
2.0
1.5
1.0
0.5
0.0
P=0.004
P=0.004
P=0.006
P=0.008
NS (P>0.05)
NS (P>0.05)
Control
DCG-IV
EGLU
cAMP
H89
LY379268+cAMP
LY341495+H89
B
DIV15
Normalized optical density
3.5
3.0
1.0
0.5
0.0
P=0.005
P=0.0001
P=0.001
P=0.0001
NS (P>0.05)
NS (P>0.05)
NS (P>0.05)
NS (P>0.05)
Control
GABA
Muscimol
KCl
GFX
Muscimol+Nifedipine
Muscimol+GFX
Bic/PiTX+A/C+PMA
KN-62
Control
cAMP
Control
H89
Cx36
36 kD
Tubulin
50 kD
Control
KCl
Control
GFX
Cx36
36 kD
Tubulin
50 kD

**Figure 10.** Signal transduction pathways. Pharmacological manipulations (see text for details) were performed in rat hypothalamic cultures on DIV3-DIV15 followed by assessment of Cx36 protein expression on DIV15. ***A,*** The regulation of Cx36 by group II mGluRs is through cAMP/PKA-dependent signaling. ***B,*** The regulation of Cx36 by $GABA_A$Rs is through $Ca^{2+}$/L-type VGCC/PKC-dependent signaling. CaMKII is not involved in the developmental regulation of Cx36. In both figures: above, statistical data; below, representative blots for cAMP/PKA- (***A***) and $Ca^{2+}$/PKC-dependent (***B***) signaling are shown. Statistical analysis: paired Student's *t*-test; mean ± SEM; *n* = 6 in each group. Optical density values are normalized to tubulin and compared to controls (set at 1.0). cAMP, 8-BrcAMP plus IBMX; GFX, GF 109203X; NS, non-significant.

that increases intracellular cAMP levels) reduced, and the blockade of PKA (by H89; 1 μM; PKA antagonist) augmented, the developmental increase in Cx36 expression (Fig. 10*A*). Further, the effects of group II mGluR agonist and antagonist in the developmental regulation of Cx36 (see Fig. 7) were prevented, respectively, by the increase in cAMP levels and inactivation of PKA (Fig. 10*A*).

The developmental increase in Cx36 expression also was prevented by KCl {1-5 mM; that causes cell depolarization and $Ca^{2+}$ influx through L-type VGCCs (Bessho et al., 1994)} and was augmented by the blockade of PKC (with GF 109203X; 0.5-1 μM) (Fig. 10*B*). Further, the effect of the $GABA_AR$ agonist, muscimol (25 μM), was prevented by blockade of L-type VGCCs with nifedipine (10 μM) or PKC inactivation (Fig. 10*B*), while the effect of $GABA_AR$ antagonists (see Fig. 7) was prevented by activation of PKC (with phorbol 12-myristate 13-acetate, PMA; 1 μM) (Fig. 10*B*). However, the developmental increase in Cx36 expression was not affected by inactivation of another calcium-regulated protein kinase, CaMKII (with KN-62; 2.5 μM; Fig. 10*B*).

These data suggest that the developmental regulation of Cx36 by group II mGluRs is via cAMP/PKA-dependent signaling; by $GABA_ARs$ is via $Ca^{2+}$/L-type VGCC/PKC-dependent signaling; and that CaMKII is not involved in control of the developmental increase in Cx36 expression.

**The roles of mGluR2 vs. mGluR3 and group II mGluRs vs. $GABA_ARs$**

We used an siRNA approach in rat hypothalamic cultures to determine the importance of mGluR2 vs. mGluR3 in the developmental increase in expression of Cx36 (Fig. 11*A,B*). The genetic suppression of both mGluR2 and mGluR3 (on DIV3-DIV7) decreased Cx36 protein levels, suggesting that both receptors are important for the developmental increase in neuronal gap junction coupling.

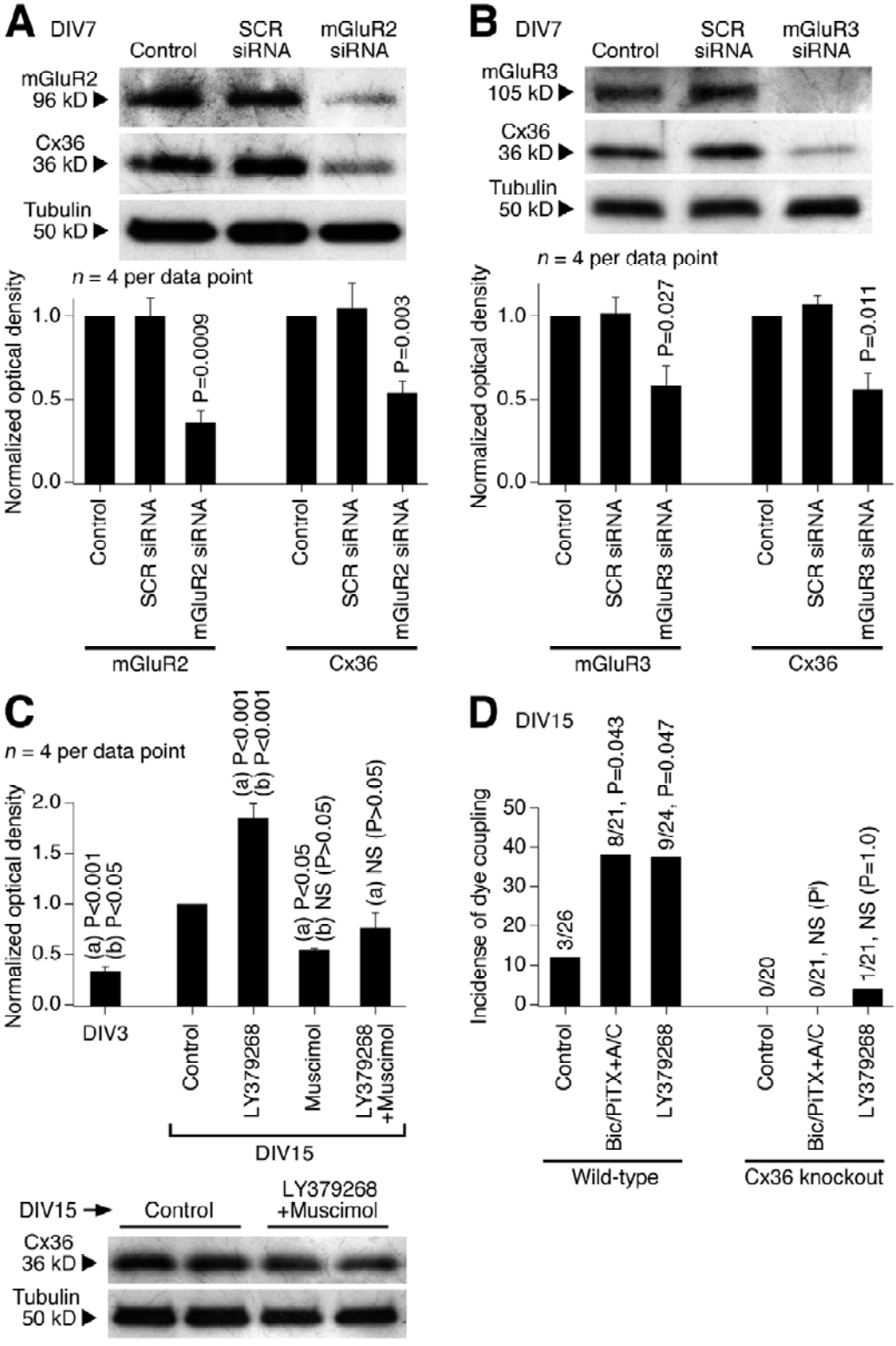
A
DIV7
Control
SCR siRNA
mGluR2 siRNA
mGluR2 96 kD
Cx36 36 kD
Tubulin 50 kD
n = 4 per data point
Normalized optical density
P=0.0009
P=0.003
Control
SCR siRNA
mGluR2 siRNA
mGluR2
Cx36
B
DIV7
Control
SCR siRNA
mGluR3 siRNA
mGluR3 105 kD
Cx36 36 kD
Tubulin 50 kD
n = 4 per data point
Normalized optical density
P=0.027
P=0.011
Control
SCR siRNA
mGluR3 siRNA
mGluR3
Cx36
C
n = 4 per data point
Normalized optical density
(a) P<0.001
(b) P<0.05
(a) P<0.001
(b) P<0.001
(a) P<0.05
(b) NS (P>0.05)
(a) NS (P>0.05)
DIV3
Control
LY379268
Muscimol
LY379268 +Muscimol
DIV15
DIV15
Control
LY379268 +Muscimol
Cx36 36 kD
Tubulin 50 kD
D
DIV15
Incidense of dye coupling
3/26
8/21, P=0.043
9/24, P=0.047
0/20
0/21, NS (P)
1/21, NS (P=1.0)
Control
Bic/PiTX+A/C
LY379268
Wild-type
Cx36 knockout

**Figure 11.** Characterization of the mechanisms for developmental increase in neuronal gap junction coupling. Western blot (***A-C***) and dye coupling (***D***) tests were performed in neuronal cultures prepared from the rat (***A-C***) and mouse (***D***) hypothalamus. ***A, B,*** siRNA suppression (on DIV3-DIV7) of mGluR2 (***A***) and mGluR3 (***B***) decreases both the receptor and Cx36 protein levels. Representative images (above) and statistical data (below; paired Student's *t*-test relative to control; mean ± SEM) are shown. Stainings were done sequentially on one membrane. SCR, scrambled siRNA. ***C,*** A combined activation (on DIV3-DIV15) of group II mGluRs and $GABA_A$Rs (with LY379268 plus muscimol) does not affect significantly the developmental up-regulation of Cx36 expression. Statistical analysis: ANOVA with post hoc Tukey; (a), relative to DIV15 control; (b), relative to LY379268 plus muscimol; mean ± SEM. ***D,*** In wild-type cultures, dye coupling increases between DIV3 (not shown) and DIV15 (Control) and this increase is augmented by inactivation of $GABA_A$Rs (Bic/PiTX+A/C) and by activation of group II mGluRs (LY379268). In Cx36 knockout cultures, neither the developmental nor the treatment-mediated increases occur. The number of dye-coupled primary-labeled neurons of the total number of primary-labeled neurons and statistical significance (Fisher's exact probability test; relative to the corresponding control) on DIV15 are shown. $P^i$, P value cannot be calculated. ***D,*** Data were obtained by Dr. Youngfu Wang.

We also tested the importance of group II mGluRs vs. $GABA_A$Rs (Fig. 11*C*). Rat hypothalamic cultures were chronically treated (on DIV3-DIV15) with LY379268 (2 μM) and muscimol (25 μM). A combined activation of group II mGluRs and $GABA_A$Rs did not affect significantly the developmental up-regulation of Cx36. However, the effect of activating $GABA_A$Rs dominated the effect of activating group II mGluRs: on DIV15, the expression of Cx36 was not different between muscimol-treated and LY379268 plus muscimol-treated cultures, but it was significantly different between LY379268-treated and LY379268 plus muscimol-treated cells (Fig. 11*C*). These data are in agreement with the observation that, in cell cultures on DIV3-DIV15, blockade of $GABA_A$Rs increases Cx36 expression to a higher level than activation of group II mGluRs (2.64±0.35 vs. 1.81+0.20 normalized optical density; P = 0.045; unpaired Student's *t*-test; *n* = 6 in each group; see Fig. 7*H,I*).

**Specificity of the mechanisms for increase in gap junction coupling**

We tested whether the regulation by group II mGluRs and $GABA_A$Rs is specific for neuronal Cx36-containing gap junctions. First, we found that the developmental increase in neuronal dye coupling in rat hypothalamic cultures is prevented by Cx36 siRNA (transfected on DIV10 and tested on DIV15; Table 1). Second, neuronal dye coupling was measured in hypothalamic cultures prepared from wild-type and Cx36 knockout mice. As expected, in wild-type cultures, the coupling was observed on DIV15 and was increased by both LY379268 and bicuculline plus picrotoxin (in the presence of AP5 and CNQX; in concentrations as in rat cultures) (Fig. 11*D*; Table 1). However, the coupling was not observed in Cx36 knock-out cultures, whether untreated or treated (Fig. 11*D*; Table 1). Moreover, in the rat hypothalamus *in vivo* and *in vitro*, the expression of a presumptive astrocytic connexin, Cx43 (Rash et al., 2000), was not different between the control and the treatment conditions, including the treatments with group II

mGluR and $GABA_AR$ agonists and antagonists (Fig. 12). The data suggest that the group II mGluR/$GABA_AR$-dependent regulation is specific for neuronal Cx36-containing, but not glial Cx43-containing gap junctions. Moreover, this regulation is exclusive for Cx36 as no increase in gap junction coupling occurs in Cx36-deficient neurons.

We also examined whether these developmental mechanisms operate in other CNS regions and species and whether they are active in mature neurons. The activity of group II mGluRs and $GABA_ARs$ was pharmacologically modulated (on DIV3-DIV15) in developing neuronal cultures prepared from the mouse somatosensory cortex, and western analysis of Cx36 expression revealed changes that were similar to those in rat hypothalamic cultures (Fig. 13). This suggests that the same regulatory mechanisms are employed in the developing mouse cortex and rat and mouse hypothalamus. Further, we tested mature cultures prepared from the rat hypothalamus. In these cultures, chronic (DIV30-DIV36) activation of group II mGluRs (with LY379268), but not inactivation of $GABA_ARs$ (with bicuculline plus picrotoxin, in the presence of AP5 and CNQX; in concentrations as above), increased the expression of Cx36 (Fig. 14). Similar results were obtained in mature cultures prepared from the mouse cortex (not shown). These data suggest that the group II mGluRs may contribute to the up-regulation of neuronal gap junction coupling in mature neurons, though $GABA_ARs$, that are inhibitory in the mature CNS (Obrietan and van den Pol, 1995; Stein and Nicoll, 2003), presumably do not regulate the coupling after neuronal maturation. {The receptor-mediated inhibition of Cx36 expression was not tested as the expression of Cx36 is low in mature neuronal cultures (Arumugam et al., 2005)}.

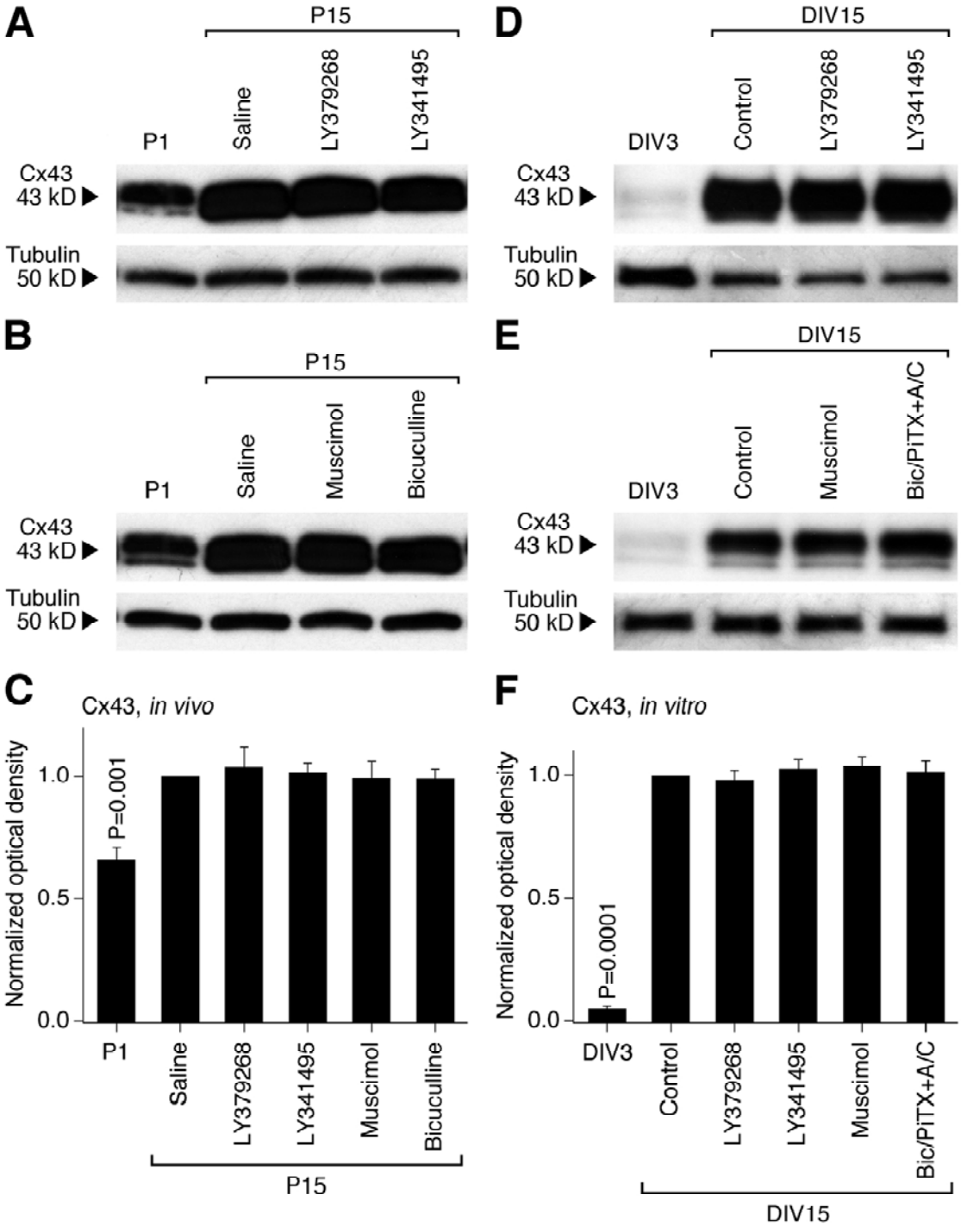
A
P15
P1
Saline
LY379268
LY341495
Cx43
43 kD
Tubulin
50 kD
D
DIV15
DIV3
Control
LY379268
LY341495
B
P15
P1
Saline
Muscimol
Bicuculline
E
DIV15
DIV3
Control
Muscimol
Bic/PiTX+A/C
C
Cx43, in vivo
Normalized optical density
1.0
0.5
0.0
P=0.001
P1
Saline
LY379268
LY341495
Muscimol
Bicuculline
P15
F
Cx43, in vitro
P=0.0001
DIV3
Control
LY379268
LY341495
Muscimol
Bic/PiTX+A/C
DIV15

**Figure 12.** Group II mGluRs and $GABA_A$Rs are not involved in the developmental regulation of hypothalamic expression of glial connexin43. Western blots for Cx43 (***A, B, D, E***) and statistical data (***C, F***) in the developing rat hypothalamus (***A-C***) and hypothalamic cultures (***D-F***) are presented. In ***C*** and ***F,*** statistical analysis is done using the paired Student's *t*-test relative to P15 saline or DIV15 control; mean ± SEM; *n* = 6 in each group. Data are normalized and analyzed as described in Fig. 6 and 7 for Cx36. Cx43 expression increases during development both *in vivo* and *in vitro*, however, the increase is not affected by group II mGluR and $GABA_A$R agonists or antagonists (i.e., no statistical significance between the saline/control and experimental groups on P15 or DIV15 is detected).

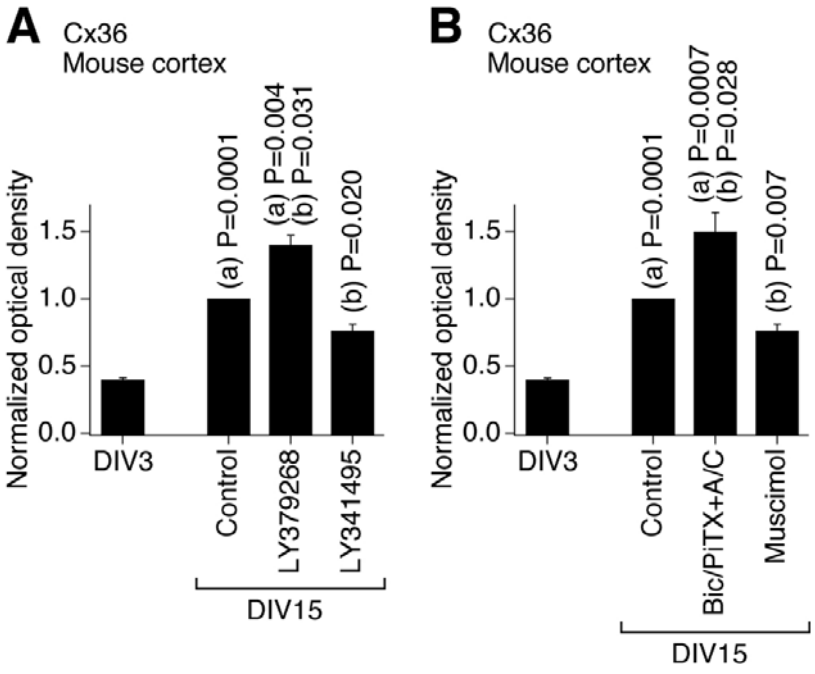
A
Cx36
Mouse cortex
Normalized optical density
1.5
1.0
0.5
0.0
(a) P=0.0001
(a) P=0.004
(b) P=0.031
(b) P=0.020
DIV3
Control
LY379268
LY341495
DIV15
B
Cx36
Mouse cortex
Normalized optical density
1.5
1.0
0.5
0.0
(a) P=0.0001
(a) P=0.0007
(b) P=0.028
(b) P=0.007
DIV3
Control
Bic/PiTX+A/C
Muscimol
DIV15

**Figure 13.** Developmental increase in neuronal gap junction coupling in the mouse cortex *in vitro* is regulated by an interplay between the activity of group II mGluRs and $GABA_A$Rs. ***A, B,*** Expression of Cx36 protein. Data are normalized and analyzed as described in Fig. 7. Statistical analysis: Student's *t*-test; (a), relative to DIV3; (b), relative to DIV15 control; mean ± SEM; *n* = 6 in each group. Drug concentrations are as in experiments in rat hypothalamic cultures shown in Fig. 7.

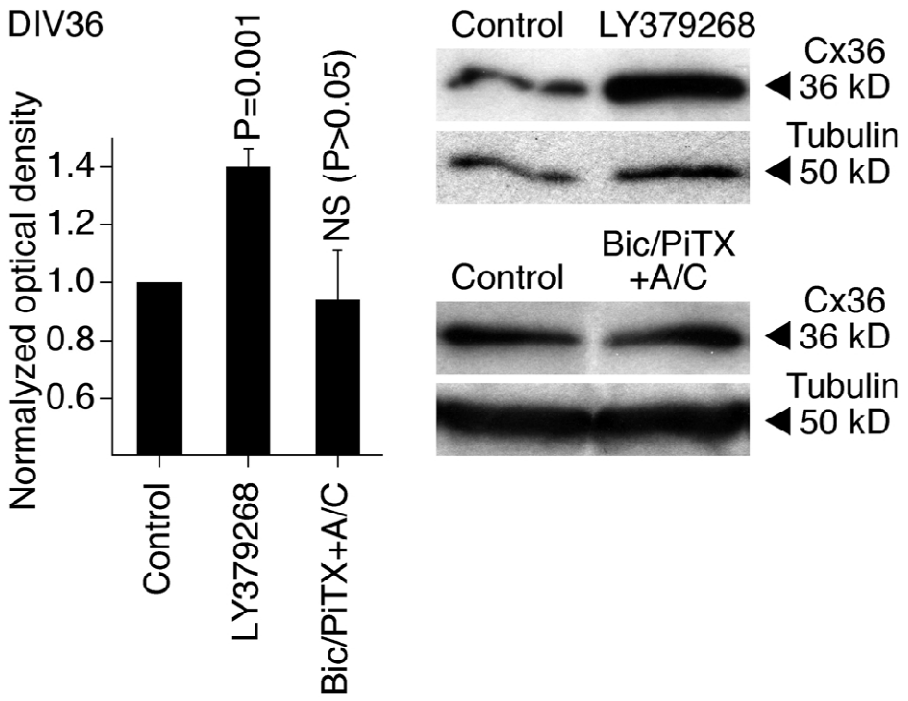
DIV36
Normalyzed optical density
1.4
1.2
1.0
0.8
0.6
P=0.001
NS (P>0.05)
Control
LY379268
Bic/PiTX+A/C
Control
LY379268
Cx36
36 kD
Tubulin
50 kD
Control
Bic/PiTX
+A/C
Cx36
36 kD
Tubulin
50 kD

**Figure 14.** Regulation of Cx36 expression in mature neurons. Pharmacological treatments were conducted in rat hypothalamic neuronal cultures on DIV30-DIV36 followed by assessment of Cx36 protein expression on DIV36. Statistical analysis: paired Student's *t*-test relative to control; mean ± SEM; *n* = 7, LY3792688; *n* = 5, Bic/PiTX+A/C. Data are normalized and analyzed as described in Fig. 7.

**Molecular mechanisms for developmental increase in gap junction coupling**

In response to either group II mGluR activation or $GABA_AR$ inactivation Cx36 protein levels increase during development and we tested whether this is associated with increased Cx36 mRNA. The expression of Cx36 mRNA was evaluated in rat hypothalamic cultures by RT-qPCR (Fig. 15*A*). We observed an increase in Cx36 mRNA levels between DIV3 and DIV15. Further, this increase was augmented by both activation of group II mGluRs (with LY379268) and inactivation of $GABA_ARs$ (with bicuculline plus picrotoxin in the presence of AP5 and CNQX; in concentrations as above). However, the increase was not affected by both inactivation of group II mGluRs (with LY341495) and activation of $GABA_ARs$ (with muscimol). The data suggest that the receptor-regulated increase, but not the decrease, in Cx36 expression during development may be controlled by transcriptional mechanisms.

Based on RT-qPCR experiments, we set to determine if an element within the Cx36 proximal promoter is responsible for the developmental increase in Cx36 mRNA levels (Fig. 15*B*). Cultures were transfected (on DIV3) with a plasmid containing the rat Cx36 promoter linked to the firefly luciferase gene and treated (on DIV4-DIV7) with the group II mGluR and $GABA_AR$ agonists and antagonists. On DIV7, cells were harvested and luciferase assay was performed. The Cx36 proximal promoter (-984/+115) supported robust transcription. As in RT-qPCR experiments, the Cx36 promoter activity was augmented by activation of group II mGluRs and inactivation of $GABA_ARs$, and was not affected by LY341495 and muscimol. The increases in Cx36 promoter activity were completely abolished by deletion of an NRSE located at nucleotide position -164/-144 (Martin et al., 2003). In addition, NRSE deletion increased the basal expression of Cx36 promoter (Fig. 15*B*). The data suggest that NRSE has a repressive effect on Cx36 promoter activity and this repression is removed by activation of group II mGluRs or inactivation of $GABA_ARs$.

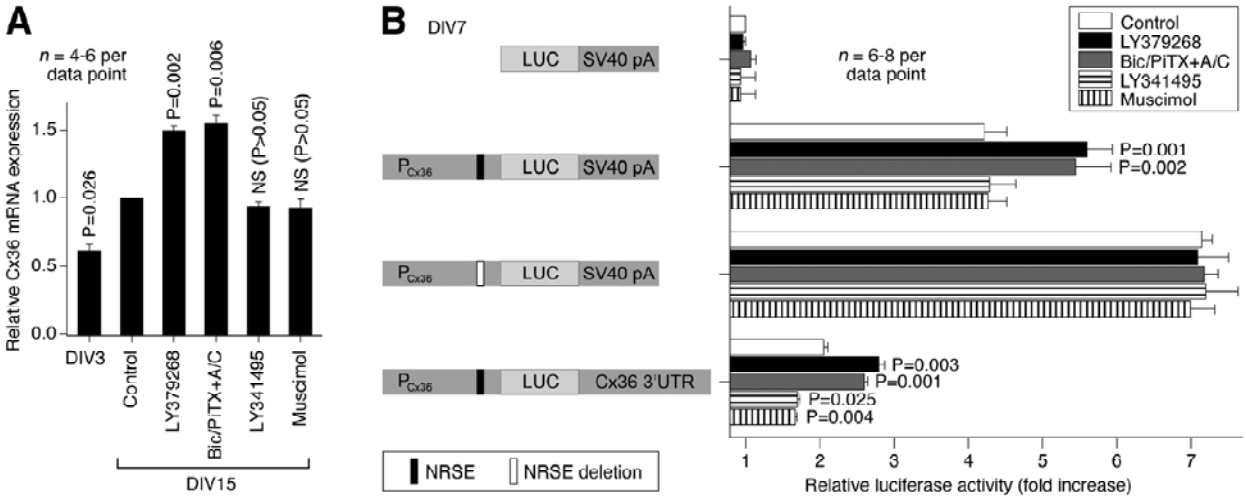
A
n = 4-6 per data point
Relative Cx36 mRNA expression
1.5
1.0
0.5
0.0
P=0.026
P=0.002
P=0.006
NS (P>0.05)
NS (P>0.05)
DIV3
Control
LY379268
Bic/PiTX+A/C
LY341495
Muscimol
DIV15
B
DIV7
LUC
SV40 pA
P Cx36
LUC
SV40 pA
P Cx36
LUC
SV40 pA
P Cx36
LUC
Cx36 3'UTR
NRSE
NRSE deletion
n = 6-8 per data point
Control
LY379268
Bic/PiTX+A/C
LY341495
Muscimol
P=0.001
P=0.002
P=0.003
P=0.001
P=0.025
P=0.004
1
2
3
4
5
6
7
Relative luciferase activity (fold increase)

**Figure 15.** Molecular mechanisms for the developmental regulation of connexin36. ***A,*** RT-qPCR analysis in rat hypothalamic cultures demonstrates that Cx36 mRNA expression increases during development and this increase is augmented by activation of group II mGluRs (LY379268) and inactivation of $GABA_A$Rs (Bic/PiTX+A/C). However, it is not affected by inactivation of group II mGluRs (LY341495) and activation of $GABA_A$Rs (muscimol). Relative Cx36 transcript levels are normalized to GAPDH and normalized values are compared to DIV15 controls (set to 1.0). Statistical analysis: paired Student's *t*-test relative to control; mean ± SEM. ***B,*** Dual-luciferase reporter assay. Cells were transfected on DIV3 with the luciferase (LUC) reporter plasmids driven by the rat Cx36 promoter (PCx36), incubated in the absence or in the presence of indicated agents and then assayed on DIV7. NRSE deleted plasmid was produced using site-directed mutagenesis. The plasmid containing Cx36 3'UTR was constructed by replacing the original SV40 poly A signal (SV40 pA) in the plasmid containing PCx36 with the full length rat Cx36 3'UTR. Firefly luciferase values are normalized relative to Renilla luciferase values to control for transfection efficiency and the results are presented as relative activity of the promoter constructs compared to the pGL3basic vector (set to 1.0). Statistical analysis: paired Student's *t*-test relative to the corresponding non-treated control; mean ± SEM.

Next, we conducted experiments to determine whether the mechanism for the receptor-mediated decrease in Cx36 expression during development involves posttranscriptional regulation (Fig. 15*B*). We constructed the plasmid containing the full length Cx36 3'UTR (~1.5kb) and the luciferase reporter driven by the Cx36 promoter (-984/+115). Whereas this construct demonstrated reduced luciferase activity (as compared to the plasmid containing the Cx36 promoter alone), it showed not only significant increases, but also decreases in response to the treatments that, respectively, increase (group II mGluR agonists and $GABA_AR$ antagonists) or decrease (group II mGluR antagonists and $GABA_AR$ agonists) Cx36 protein expression (Fig. 15*B*). Together, the results indicate that the mechanisms that orchestrate the developmental changes in Cx36 expression involve both the Cx36 promoter-mediated transcriptional regulation through NRSE and regulatory events mediated via the 3'UTR, perhaps involving post-transcriptional mechanisms.

**Functional implications**

Our previous study demonstrated (de Rivero Vaccari et al., 2007) that neuronal gap junctions play a critical role in the mechanisms of neuronal death/survival during development. Specifically, in rat and wild-type mouse hypothalamic neuronal cultures, we found that hyperactivation or inactivation of NMDARs both induced neuronal death exclusively during the peak of developmental gap junction coupling (i.e., on DIV14-17). Further, both types of NMDAR-dependent neuronal death were completely prevented by pharmacological inactivation of gap junctions (with carbenoxolone and 18-α-glycyrrhetinic acid) or genetic knockout of Cx36. In the present study, we set to determine whether manipulation of the mechanisms regulating the developmental increase in neuronal gap junction coupling affects the death/survival mechanisms in developing neurons. We used a model of NMDAR-dependent excitotoxicity.

Experiments were conducted in rat hypothalamic cultures, non-treated or chronically treated (on DIV3-15) with the group II mGluR agonist (LY379268) or antagonist (LY341495; in concentrations as above). Administration of NMDA (100 μM; on DIV14 for 60 min) induced significant neuronal death in non-treated cultures that was detected 24 hrs later (on DIV15) using MTT assay (Fig. 16*A*). NMDA-mediated neuronal death was more pronounced in LY379268-treated cultures, i.e., in the cultures with high levels of neuronal gap junction coupling and Cx36 expression. Further, NMDA-mediated neuronal death was not observed in LY341495-treated cultures, i.e., in the cultures that lack neuronal gap junction coupling due to low levels of Cx36. NMDA-mediated neuronal death also was prevented by the gap junction blocker carbenoxolone (25 μM; Fig. 16*B*), supporting the notion that gap junctions participate in NMDAR-dependent death in developing neurons. Similar results were obtained in wild-type mouse cortical neuronal cultures (not shown). The results indicate that the mechanisms for the developmental increase in neuronal gap junction coupling play a role in regulation of neuronal death/survival during development.

## 4. Discussion

We demonstrated here that the developmental increase in neuronal gap junction coupling in the rat and mouse hypothalamus and cortex is regulated by an interplay between the activity of group II mGluRs and $GABA_A$Rs. The regulation by group II mGluRs is via cAMP/PKA-dependent signaling. The regulation by $GABA_A$Rs, which cause neuronal excitation during development (Obrietan and van den Pol, 1995; Stein and Nicoll, 2003), is via $Ca^{2+}$ influx through VGCCs and activation of PKC. We also showed that other glutamate receptors, acetylcholine receptors, $GABA_B$ receptors and CaMKII are not involved in these regulatory mechanisms. A previous study in the rat hypothalamus indicated (Arumugam et al., 2005) that blockade of

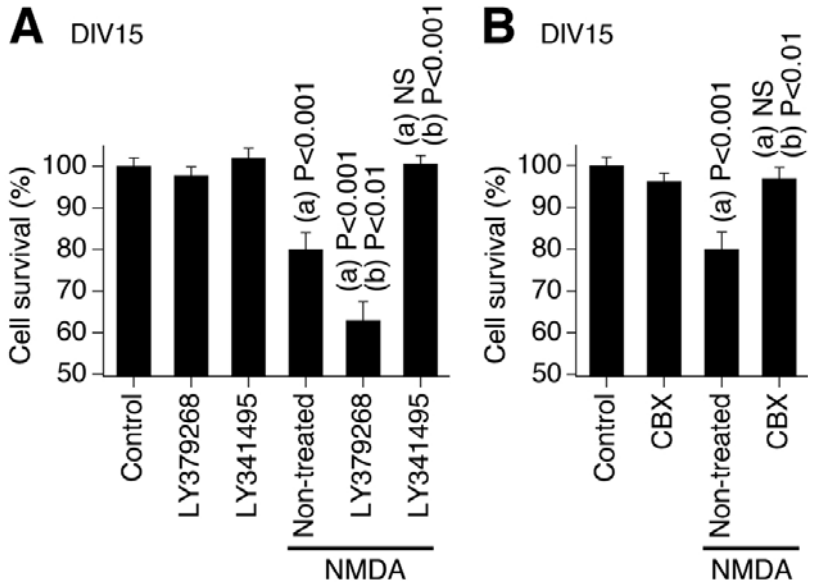
A
DIV15
Cell survival (%)
100
90
80
70
60
50
(a) P<0.001
(a) P<0.001
(b) P<0.01
(a) NS
(b) P<0.001
Control
LY379268
LY341495
Non-treated
LY379268
LY341495
NMDA
B
DIV15
Cell survival (%)
100
90
80
70
60
50
(a) P<0.001
(a) NS
(b) P<0.01
Control
CBX
Non-treated
CBX
NMDA

**Figure 16.** Mechanisms for the developmental increase in neuronal gap junction coupling play a role in regulation of neuronal death/survival during development. Experiments were conducted in rat hypothalamic neuronal cultures. NMDA (100 μM) was added to the culture medium on DIV14 for 60 min and then washed-out. The MTT analysis of neuronal death was conducted 24 hrs later (on DIV15). ***A,*** NMDA administration induces neuronal death in non-treated cultures. NMDA-mediated neuronal death is augmented in cultures chronically treated (on DIV3-DIV15) with the group II mGluR agonist (LY379268) and does not occur in cultures chronically treated with the group II mGluR antagonist (LY341495). The mGluR agents by themselves do not affect cell survival. ***B,*** NMDA-mediated neuronal death also is prevented by co-administration of the gap junctional blocker carbenoxolone (CBX). In both graphs, statistical data are shown. Statistical analysis: ANOVA with post hoc Tukey; (a), relative to control; (b), relative to non-treated plus NMDA condition; mean ± SEM; *n* = 6 in each group. All data were obtained by Janna V. Denisova.

action potentials prevents the developmental increase in neuronal gap junction coupling. Together with the results described here, this implicates a role for action potential-dependent synaptic release of glutamate and GABA in regulation of the developmental increase in neuronal gap junction coupling.

Group II mGluRs include mGluR2 and mGluR3, and our experiments suggested that both of them are important for the developmental increase in neuronal gap junction coupling. Data also indicated that the effects of $GABA_AR$ agents in modulation of gap junctions are stronger than those of group II mGluR agents. This supports a more important role for $GABA_ARs$ than group II mGluRs in these regulatory mechanisms, which may be explained by the fact that formation of GABAergic synapses in the CNS precedes the formation of glutamatergic synapses (Ben-Ari, 2002). It is possible, however, that the pattern of expression of particular neurotransmitter receptors and timing of the switch of $GABA_ARs$ from excitation to inhibition during development determine when the developmental increase in neuronal gap junction coupling occurs. Future experiments are needed to evaluate this prediction.

Cx43 is a presumptive glial connexin (Rash et al., 2000), that also is involved in a number of developmental events, including neuronal migration (Elias et al., 2007). Our study showed that the expression of Cx43, a presumptive glial connexin, also increases during development, however, the increase is not affected by the group II mGluR and $GABA_AR$ agents. This suggests that the regulation by group II mGluRs and $GABA_ARs$ is specific for Cx36-containing, but not Cx43-containing gap junctions. Moreover, this regulation is exclusive for Cx36 as no increase in gap junction coupling (either developmental or treatment-mediated) occurs in Cx36-deficient neurons (Fig. 11*D*). NRSE is a DNA sequence element in a promoter region of a number of neuronal genes that binds REST and regulates the transcriptional activity of these genes. REST activity is influenced by both $Ca^{2+}$- and cAMP-dependent signaling

(Nadeau and Lester, 2002; Somekawa et al., 2009). Rat and mouse Cx36 genes contain NRSEs (-164/-144 and +201/+221, respectively) (Cicirata et al., 2000; Martin et al., 2003) and occupancy of the Cx36 NRSE by REST is observed in mouse neuronal stem cells (Johnson et al., 2008). Further, REST/NRSE-dependent signaling negatively regulates Cx36 mRNA expression in insulin-producing cells (Martin et al., 2003). Our study in developing hypothalamic neurons agrees with those findings and demonstrates that deletion of the NRSE in Cx36 promoter region results in both higher basal transcription and loss of responsiveness to the group II mGluR agonist and $GABA_AR$ antagonists. Thus, the data suggest that the receptor-mediated increase in Cx36 expression during development is regulated by group II mGluRs and $GABA_ARs$ via removal of the NRSE-dependent repression of Cx36 promoter activity.

In contrast, the mechanisms for the receptor-mediated decrease in expression of Cx36 during development may not be due to new mRNA synthesis; rather involving posttranscriptional mechanisms dependent upon sequences within the 3'UTR. While we cannot totally rule out the presence of a transcriptional regulatory element in the region of 3'UTR, given that the Cx36 mRNA levels did not change in response to inactivation of group II mGluRs or activation of $GABA_ARs$, this possibility seems unlikely. Rather, we suspect that this regulation may be via miRNAs, small non-coding RNAs, which bind to complementary sites on 3'UTR of target mRNAs and reduce gene expression primarily through translational repression (Bartel, 2004). Many miRNAs are brain-specific (Sempere et al., 2004) and are involved in the regulation of neuronal development, differentiation, and morphogenesis (Smirnova et al., 2005; Vo et al., 2005). The 3'UTR of Cx36 mRNA contains binding sites for a number of brain-specific miRNAs, including miR-9, miR-128a, and miR-128b (Rash et al., 2005). A focus of future studies will be to identify which, if any, of these miRNAs target Cx36 and to determine the specific signals that regulate their action.

During pharmacological manipulations, changes occur not only in the incidence of dye and electrotonic coupling, but also in the coupling coefficient. Together with data demonstrating alterations in Cx36 protein expression, these results suggest the possibility of adding/removing Cx36 molecules to/from individual neurons.

We also postulate that group II mGluR and $GABA_AR$ agonists and antagonists exert specific, physiological effects on neuronal gap junction coupling. First, the agents modulate the developmental increases in the coupling and Cx36 expression in a similar way *in vivo* and *in vitro*. Second, the increases are modulated not only by receptor agonists and antagonists, but also by manipulating the corresponding receptor-coupled intracellular signaling. Third, it is unlikely that the effects are simply through the regulation of electrical activity as the developmental increases in coupling and/or Cx36 expression are prevented both by TTX (that reduces the action potential activity)(Arumugam et al., 2005) and by KCl (that depolarizes neurons and increases the action potential activity)(Fig. 10*B*). Finally, the developmental increase in Cx36 is affected not only by pharmacological treatments, but also by genetic manipulations for the expression of mGluR2 and mGluR3.

A previous study demonstrated a role for glutamate-dependent synaptic transmission in the developmental uncoupling of neuronal gap junctions (that occurs in the hypothalamus between days 15 and 30 *in vivo* and *in vitro*) (Arumugam et al., 2005). The data indicated that developmental uncoupling is due to activation of NMDARs and $Ca^{2+}$-, CaMKII-, PKC-, and CREB- dependent down-regulation of Cx36. Together with the present work, our studies strongly suggest that developing chemical synapses regulate electrical synapses. We postulate that during early postnatal development, $GABA_AR$-dependent excitation maintains the expression of Cx36 in neuronal circuits at a low level (via $Ca^{2+}$/PKC signaling and 3'UTR of the Cx36 mRNA). The subsequent transition from $GABA_AR$ excitation to inhibition, in combination

with increased activity of the group II mGluRs, result in the developmental up-regulation of Cx36 (via the NRSE in the Cx36 gene) and increased neuronal gap junction coupling. However, the developmental increase in the activity of NMDARs then causes down-regulation of Cx36 (via $Ca^{2+}$-dependent signaling, including CREB) and gap junction uncoupling. If mechanisms for the developmental regulation of gap junctions have universal character in the CNS (as we show here for the hypothalamus and cortex), the variations among different CNS regions in the timing of neuronal gap junction coupling and uncoupling (Bennett and Zukin, 2004) presumably can be explained by the interregional differences in the activity of these mechanisms (i.e., receptor and synaptic activity and timing of the excitation/inhibition switch for $GABA_A$Rs). Other additional, region-specific factors probably also contribute.

In the developing and mature CNS, neuronal gap junction coupling also may be modulated acutely by changes in the activity of neurotransmitter receptors (Hatton, 1998). We believe that acute and developmental alterations in the coupling represent different functional aspects of gap junction physiology. The acute modulation of gap junctions by neurotransmitter receptors likely involves gating mechanisms and plays a role in rapid modifications in neuronal connectivity and signaling in response to changes in chemical synaptic activity (Hatton, 1998). In contrast, the developmental changes in gap junctions likely are related to specific genetic programs and/or developmental pathways during the period when chemical synapses are still being established. It is possible, however, that the acute modulation of coupling may translate into a prolonged modification if the changed level of receptor activity sustains.

In the developing CNS, programmed cell death helps to establish the final number of neurons and contributes to distribution of various cell classes and neuronal circuit formation (Nijhawan et al., 2000). The activity of NMDARs also is the factor that plays a role in cell survival versus death decisions during neuronal development (Scheetz and Constantine- Paton,

1994; de Rivero Vaccari et al., 2006). It has been suggested that during development gap junctions are involved in the regulation of apoptosis and NMDAR dependent neuronal death (Cusato et al., 2003; de Rivero Vaccari et al., 2007). We showed here that the NMDAR-mediated excitotoxicity is eliminated or augmented if the amount of gap junctional coupling is reduced or increased, respectively. This suggests that mechanisms for the developmental increase in neuronal gap junction coupling directly regulate death/survival mechanisms in developing neurons. This also implies a role for gap junctions in formation of neuronal circuits via regulation of neuronal death/survival.

# CHAPTER THREE

## Conclusion and Future Perspectives

## 1. Conclusion

New knowledge regarding developmental mechanisms of neuronal gap junction-based electrical synapses provides a fundamental understanding of how complex neural networks operate during development. This thesis describes novel findings on the mechanism underlying the developmental increase in electrical synapses between neurons as well as role of these synapses during development.

1) We conclude that the developmental increase in neuronal gap junction coupling is regulated by the interplay between the activity of group II mGluRs and $GABA_A$Rs. An activation of group II mGluRs increases neuronal gap junction coupling via cAMP/PKA dependent signaling. In contrast, an activation of $GABA_A$Rs has the counteracting effects on the developmental increase in neuronal gap junction coupling via $Ca^{2+}$/PKC dependent signaling.

2) The receptor mediated increase in Cx36 expression occurs at the transcriptional level and depends on relief of the NRSE-mediated repression of Cx36 promoter activity. Additionally the regulatory sequences in the 3'UTR of the Cx36 mRNA is necessary for neurotransmitter receptor-mediated suppression of Cx36 expression.

3) Finally, the results indicate that the mechanisms for the developmental increase in neuronal gap junction coupling serves a critical role in the regulation of NMDAR-dependent death in developing neurons.

## 2. Future Perspectives

Our study showed that during development, GABA and glutamate regulate the decrease and increase in neuronal gap junction coupling by the activation of ionotropic ($GABA_AR$s) and metabotropic (group II mGluRs) receptors, respectively. In addition to the major functional difference between two types of receptors, the expression of each receptor is differentially regulated during the first two postnatal weeks with distinct spatial and temporal profiles. And furthermore, developmental changes in $GABA_AR$-mediated synaptic activity, from depolarizing to hyperpolarizing, occur during this time window in the rat hypothalamus (Obrietan and van den Pol, 1995). Therefore a more detailed study of how those two receptors work in concert in a shorter period of development may reveal that a complex synaptic response plays a critical role in the precise modulation of the developmental changes in gap junction based-electrical synapses.

The present study also showed that each receptor acts through the corresponding signaling pathway for the developmental regulation of neuronal gap junction coupling i.e., cAMP/PKA-dependent signaling is negatively coupled to group II mGluRs and $GABA_AR$s activation allows intracellular $Ca^{2+}$ increases and subsequent PKC activation. Although each receptor-mediated signaling transduction seems to be functionally preserved, why are two different pathways separately needed to regulate neuronal gap junction coupling? In-depth studies on the functional interplay of cAMP/PKA- and $Ca^{2+/}$PKC-dependent signaling mechanisms are necessary to better understand mechanisms for the developmental regulation of neuronal electrical synapses. Those studies should address the following questions: (i) are there central molecules where all complex intracellular signals converge?; (ii) are there other levels of regulations?; and (iii) are there the mechanisms of cross-regulation between two distinct signaling pathways leading to the final integrated cellular response?

Our study with the use of RT-qPCR analysis and luciferase reporter assay indicated that the developmental change in Cx36 expression requires both the Cx36 promoter-mediated transcriptional regulation through NRSE and post-transcriptional gene regulatory events involving the 3'UTR. Namely, the balance between the levels of NRSE-dependent transcriptional response and 3'UTR-mediated regulatory response seems to adjust the extent of Cx36 expression in the developing neurons. Specific receptor mediated-stimuli can generate changes in gene expression by activating intracellular protein kinase cascades that finally can carry the integrated signals to the nucleus. The functional contributions of two independent protein kinases, PKA and PKC, for Cx36 expression have been observed in the present study, but little is known about protein kinase substrates which lie downstream targeting to Cx36 gene expression. The leading candidates are transcription factors and/or miRNAs, which can be linked to the current findings on molecular mechanisms, suggesting transcriptional regulation through REST and post-transcriptional regulation by brain-specific miRANs. Moreover, both components have been already implicated in regulating neuronal gene expression and mediating neuronal identity (Wu and Xie, 2006). On the other hand, the regulation of Cx36 genes also can be at the level of translation and/or degradation rate of the protein.

What physiological roles might be played by mechanisms for the developmental increase in neuronal gap junction coupling? According to our study, it seems likely that neuronal gap junctions during development are tightly associated with the regulation of death in developing neurons (at least, NMDAR-dependent death). It would be interesting to investigate the correlation between Cx36-containing gap junctions and cell death related pathways, such as the tumor necrosis factor-induced path or the Fas-Fas ligand-mediated path. Consequently, the ultimate question can be to define the nature of cell death signals; what is the cell-killing message and how is it delivered through gap junctions?

The coupling also increases in the mature CNS during neuronal injuries (Chang et al., 2000; Frantseva et al., 2002; de Pina-Benabou et al., 2005; Nemani and Binder, 2005; Thalakoti et al., 2007), and selective blockade of Cx36- containing gap junctions is neuroprotective (Wang et al., 2010). Given that neuronal injuries are characterized by excessive release of glutamate (Arundine and Tymianski, 2004) and that activation of group II mGluRs increases Cx36 expression in mature neurons (present study), it will be interesting to see whether mechanisms for the injury-related increases in neuronal gap junction coupling are group II mGluR- dependent and whether inactivation of these mechanisms has a neuroprotective effect.

In closing, the mechanisms for developmental increase in neuronal gap junction coupling characterized herein offer key insights how developing chemical synapses influence electrical synapses. Elucidating the complex mechanisms in the development of neuronal gap junction coupling will be helpful to understand whether injury-related increases in neuronal gap junction coupling employ same mechanisms or not.

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