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Glial Connexins and Gap Junctions in CNS inflammation and disease

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Abstract

Gap junctions facilitate direct cytoplasmic communication between neighboring cells, facilitating the transfer of small molecular weight molecules involved in cell signaling and metabolism. Gap junction channels are formed by the joining of two hemichannels from adjacent cells, each composed of six oligomeric protein subunits called connexins (Cx). Of paramount importance to CNS homeostasis are astrocyte networks formed by gap junctions, which play a critical role in maintaining the homeostatic regulation of extracellular pH, K^+, and glutamate levels. Inflammation is a hallmark of several diseases afflicting the CNS. Within the past several years, the number of publications reporting effects of cytokines and pathogenic stimuli on glial gap junction communication has increased dramatically. The purpose of this review is to discuss recent observations characterizing the consequences of inflammatory stimuli on homocellular gap junction coupling in astrocytes and microglia as well as changes in connexin expression during various CNS inflammatory conditions.

Keywords

gap junction; connexin 43; astrocyte; microglia; cytokine; hemichannel

Introduction

A review dedicated solely to the topic of whether inflammation might affect cell-cell communication between glia facilitated by gap junctions was first published in 2004 (Kielian and Esen 2004). At the time, only a handful of reports had described the actions of select proinflammatory cytokines on glial gap junction communication (GJC). However, within the past several years, the number of publications reporting effects of cytokines and pathogenic stimuli on glial GJC has increased dramatically. Therefore, this review serves as an update to discuss these new findings and their potential involvement in the context of neuroinflammatory diseases of the CNS. To limit the scope of this review, only the effects of inflammation on GJC and Cx hemichannels in glia and alterations in CNS inflammatory diseases will be discussed. The reader is referred to other comprehensive reviews pertaining to the roles of GJC on other aspects of CNS homeostasis and pathology (Giaume and McCarthy 1996; Rozental et al. 2000; Rouach et al. 2002b; Theis et al. 2005).

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Connexins and gap junctions in brain glial cells: General overview

Gap junctions represent conduits that permit the direct trafficking of small molecular weight molecules between adjacent cells without contacting the extracellular milieu. Originally thought to facilitate the rather non-selective passage of molecules less than 1000 Da, recent evidence indicates that other properties such as charge and shape also dictate the ease of molecule transfer via gap junctions (Goldberg et al. 2002; Goldberg et al. 2004; Yeager and Harris 2007). During channel assembly, six connexin (Cx) isoforms oligomerize in the endoplasmic reticulum and Golgi compartments to form a hexameric hemichannel (or connexon), which is then transported to the plasma membrane (Musil and Goodenough 1993; Laird 2006). Once two hemichannels come into contact from opposing cells, a functional gap junction channel can be formed. Gap junction channels typically localize to discrete microdomains of the plasma membrane referred to as gap junction plaques (Sáez et al. 2003; Laird 2006). A total of 20 Cx family members have been identified in the mammalian genome (Willecke et al. 2002; Laird 2006) and during the 2007 International Gap Junction Conference (Elsinore, Denmark) experts in the field agreed to implement a new classification scheme for Cx gene nomenclature that was originally proposed at the same venue at an earlier date (Bruzzone 2001)(see http://www.genenames.org/genefamily/gj.php and http://www.informatics.jax.org/ for approved gene names for human and mouse, respectively). The naming of Cx proteins remains unchanged and is based on their molecular weight (Sohl and Willecke 2004). In order for gap junctions to form between adjacent cells, they must be in close enough apposition (i.e. within 4 nm), which is facilitated by adhesive events by molecules such as cadherins (Meyer et al. 1992; Giepmans 2004; Wei et al. 2005). Functionally, gap junctions enable the direct intercellular propagation of second messengers (such as Ca^{2+}, IP_3), metabolites (i.e. glutamate, 2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose (2-NBDG)), as well as energy molecules (i.e. ATP, ADP) between coupled cells (Sáez et al. 2003; Laird 2006) and these relationships have been well established in astrocytes (Giaume et al. 1997; Tabernero et al. 2006). Gap junction communication can be regulated at several levels including alterations in Cx transcription, translation, stability, post-translational processing (i.e. phosphorylation) (Solan and Lampe 2007), or insertion/removal from the cell membrane (Leithe and Rivedal 2007), as well as channel gating (i.e. influenced by intracellular pH) (Sáez et al. 2003; Laird 2006) and voltage changes. The kinetics of these changes can range anywhere from minutes to several hours. Recent evidence indicates that under the appropriate conditions, such as during inflammation, hemichannels are opened and facilitate two-way communication between the intracellular and extracellular milieus (De Vuyst et al. 2007; Retamal et al. 2007).

Astrocytes represent the largest gap junction coupled cell network within the CNS, where this mode of direct intercellular communication plays a role in the homeostatic regulation of extracellular pH, K^+, and glutamate levels (Anderson and Swanson 2000; Ransom 2000; Ransom et al. 2003). Astrocytes also influence CNS vascular tone and neuronal synapses, which are facilitated, in part, via GJC (Haydon 2001; Mulligan and MacVicar 2004; Volterra and Meldolesi 2005; Takano et al. 2006). The major Cx isoform comprising astrocytic gap junction channels is Cx43, with additional contributions from Cx30 and Cx26 (Dermietzel et al. 2000; Nagy et al. 2001; Rash et al. 2001; Altevogt and Paul 2004). However, *in vivo* Cx30 expression accounts for approximately 50% of astrocytic coupling in the hippocampus and in certain brain regions Cx30 expression is equal or higher than Cx43 (Dahl et al. 1996; Blomstrand et al. 2004). Functionally, the astrocyte network serves to effectively dilute substances cleared from the extracellular environment through the transfer of mediators from one astrocyte to many others which communicate over distances. In addition, astrocyte gap junctions facilitate the trafficking of glucose and its metabolites to provide a link between the cerebral vascular endothelium and neurons (Giaume et al. 1997; Goldberg et al. 1999; Tabernero et al. 2006) and are considered to form a molecular association for the long-distance

propagation of signals across astrocytic networks (Charles et al. 1991; Bezzi and Volterra 2001; Haydon 2001). Studies using primary astrocytes cultured from embryonic Cx43 homozygous (−/−) null mice have revealed an important role for Cx43 in the regulation of intracellular free calcium $[Ca^{2+}]_i$ signaling and functional dye coupling (Naus et al. 1997); however, other reports support a compensatory role of the minor Cx forms (Cx26, Cx30, Cx40, Cx45, and Cx46) in astrocyte junctional conductance (Scemes et al. 1998; Dermietzel et al. 2000). In addition to maintaining the homeostatic environment of the CNS, astrocytes are important in initiating and regulating immune responses through the release of numerous proinflammatory cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), to mention only a few (Dong and Benveniste 2001; Esen et al. 2004; Farina et al. 2007). As a result, astrocytes are now being considered as important players in the CNS response to both infectious as well as neurodegenerative diseases (Dong and Benveniste 2001; Farina et al. 2007).

Microglia represent an important innate immune effector cell population in the CNS parenchyma and under resting conditions, do not form gap junctions (Eugenín et al. 2001; Eugenín et al. 2003; Garg et al. 2005). However, recent work has demonstrated that when a critical threshold of cell activation is achieved (as dictated by the intensity/combination of the stimulus) microglia exhibit a homotypic adhesion event and homocellular GJC is established, albeit to a much more limited extent compared to astrocytes (Eugenín et al. 2001; Eugenín et al. 2003; Garg et al. 2005). In addition, a recent study has described Cx36 expression in cultured microglia that enables heterocellular GJC with neurons (Dobrenis et al. 2005).

In the context of neurodegenerative or infectious diseases of the CNS, inflammatory products released in response to noxious stimuli may have dramatic effects on the way that astrocytes and microglia singularly interact via gap junctions. Intriguingly, new evidence indicates that inflammatory stimuli can facilitate the opening of glial hemichannels to allow the bi-directional exchange of small molecules from the cytoplasmic to extracellular milieus (De Vuyst et al. 2007; Retamal et al. 2007). This concept has been supported by several previous as well as recent reports and remains an exciting area for continued research efforts. A more challenging issue will be to demonstrate the functional impact of alterations in GJC or hemichannel activity in living tissues or animal models to fully appreciate the complexities of the inflammatory milieu in regulating the properties of these channels.

Another intriguing development that will further shape definitions of channel communication was made by the recent discovery of pannexins. Pannexins are a novel family of proteins that share some sequence similarity to invertebrate innexins and have been proposed as a second group of mammalian gap junction proteins (Panchin et al. 2000; Barbe et al. 2006; Shestopalov and Panchin 2008). Pannexins are expressed in the CNS (Bruzzone et al. 2003; Ray et al. 2005) and recent studies have implicated pannexin 1 in regulating the proliferation and tumorgenicity of gliomas (Lai et al. 2007). Currently, no information is available regarding the effects of inflammation on pannexin expression; however, it has been recently demonstrated that pannexin 1 can influence cell death mediated by the purinergic receptor P2X7 (Locovei et al. 2007). Insights into this new area of investigation have been the topic of several recent reviews (Barbe et al. 2006; Shestopalov and Panchin 2008).

Candidate inflammatory mediators that impact glial GJC in CNS inflammatory diseases

When considering the preeminent inflammatory molecules conserved across many neurodegenerative and infections diseases of the CNS, candidates such as TNF-α, IL-1β, IL-6, and NO emerge (Nau and Bruck 2002; Kielian 2004; Imitola et al. 2005; Heneka and O'Banion 2007). Many of the biological actions of TNF-α and IL-1β are overlapping and include

activation of the endothelial cells comprising the blood-brain barrier (BBB), induction of adhesion molecule expression on cerebral microvascular endothelial cells, and subsequent activation of resident glia and infiltrating peripheral immune cells (Quagliarello et al. 1991; Wong and Dorovini-Zis 1992; Claudio et al. 1994; Wong and Dorovini-Zis 1996; Blamire et al. 2000). NO is a highly reactive free radical that can serve anti-microbial functions as well as cause significant damage to CNS tissue when expressed at high levels or when it combines with water to form the toxic metabolite peroxynitrite (MacMicking et al. 1997; Thoma-Uszynski S 2001; Winkler et al. 2001). IL-6 is a pleiotrophic cytokine produced primarily by activated astrocytes in the CNS, which promotes astrocyte proliferation and neuronal survival (Selmaj et al. 1990; Gruol and Nelson 1997; Van Wagoner and Benveniste 1999; Dong and Benveniste 2001). Although these proinflammatory molecules can exert beneficial roles in the correct context, if produced in excessive quantities IL-1β, TNF-α, IL-6, and NO may have detrimental effects on the integrity of surrounding normal brain tissue. Indeed, these cytokines have been implicated as playing a major role in the pathology of numerous CNS diseases including multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) and Alzheimer's disease (Meda et al. 1995; Renno et al. 1995; Benveniste 1997; Gruol and Nelson 1997; Tamatani et al. 1999; Combs et al. 2001; Jurewicz et al. 2003; Li et al. 2004; Hovelmeyer et al. 2005; McCoy et al. 2006; Nakazawa et al. 2006). To date, most studies investigating the effects of inflammation on glial GJC have focused on TNF-α and IL-1β (John et al. 1999; Duffy et al. 2000; Memê et al. 2004; Hinkerohe et al. 2005; Memê et al. 2006), with a more limited amount of information available regarding the role of NO (Bolanos and Medina 1996; Retamal et al. 2007). The effects of alternative proinflammatory molecules have not yet been extensively investigated. Therefore, for the purposes of this review, the discussion will be limited to the aforementioned mediators in addition to new reports using bacterial and viral stimuli.

Effects of inflammation on GJC in astrocytes

One of the first reports of cytokine-dependent changes in astrocyte GJC was provided by John et al. who demonstrated that IL-1β inhibited gap junction coupling in primary human fetal astrocytes (John et al. 1999). This change was associated with a concomitant reduction in Cx43 mRNA and protein expression (John et al. 1999; Duffy et al. 2000), the main Cx isoform comprising gap junctions in astrocytes. In addition, IL-1β was found to reduce the mean junctional conductance of astrocytic gap junction channels (John et al. 1999). These changes were observed when cells were treated with IL-1β for a 24 h period; however, a study by another group reported a transient effect of IL-1β on primary rodent astrocytes, where the cytokine induced an immediate blockade of astrocytic GJC via a p38-dependent signaling pathway, which dissipated by 24 h following IL-1β stimulation (Zvalova et al. 2004). Studies by Memê et al. demonstrated no effect of IL-1β alone on astrocytic Cx43 expression or GJC, although IL-1β in combination with alternative cytokines (i.e. TNF-α) was capable of reducing both responses (Memê et al. 2004; Memê et al. 2006). Two modes of gap junction regulation are commonly distinguished; a fast-acting mechanism that is commonly attributed to immediate changes in channel gating, or a long-term regulation, which occurs at the level of alterations in Cx43 mRNA and/or protein expression (Giaume and McCarthy 1996). Therefore, based on the differential timing effects reported for IL-1β on astrocytic Cx43 and GJC it appears that in a given context, both mechanisms of action may be plausible.

Another interesting study has shown that treatment of human fetal astrocytes with IL-1β leads to a reduction in Cx43 expression; however, a reciprocal increase in the tight junction protein claudin-1 was observed (Duffy et al. 2000). Closer examination of IL-1β-treated astrocytes revealed the presence of rudimentary tight junctions between cells. Because some constituents of the macromolecular scaffolding complexes that form tight junctions and gap junctions are conserved (by virtue of physical interactions with Cx43), these findings suggest that

inflammation may not only lead to alterations in GJC but also the remodeling of such macromolecular associations that may impact cell shape, division, and/or migration (Toyofuku et al. 1998; Duffy et al. 2000; Giepmans 2004). The authors proposed that the opposing actions of IL-1β on Cx43 and claudin-1 expression in astrocytes may effectively reduce GJC and decrease bulk fluid movement in the inflamed CNS (Duffy et al. 2000).

In contrast to IL-1β, fewer reports are available documenting the effects of TNF-α and NO on homocellular GJC in purified astrocyte cultures. A discussion on the effects of IL-1β, TNF-α, and other cytokines on astrocyte GJC in the presence of microglia will be presented in a later section. In purified astrocytes, TNF-α has been shown to depolarize cells; however, similar to the rapid effects of IL-1β, the ability of TNF-α to alter astrocytic resting membrane potential was transient and within 20 h following cytokine exposure membrane potentials had returned to baseline (Koller et al. 1998). This TNF-α-induced depolarization of astrocytes was PKC-dependent and led to a concomitant reduction in inward rectifying K^+ currents (Koller et al. 1998). A link between astrocyte depolarization and GJC is made because the former affects the ability of astrocytes to control local ion homeostasis and glutamate uptake in the brain, effects that are also attributed to astrocyte syncytia (Ozog et al. 2002). Indeed, a recent study has demonstrated that exposure of primary rat astrocytes to TNF-α for as little as 2 h inhibited GJC coincident with an increase in Cx43 phosphorylation suggesting rapid alterations in channel gating (Haghikia et al. 2008). Similar to the study by Koller et al. described above (Koller et al. 1998), TNF-α treatment led to a depolarization of the astrocytic resting membrane potential (Haghikia et al. 2008). The rapid kinetics of inhibition of GJC and depolarization of astrocytes following TNF-α exposure suggests immediate alterations in channel activity rather than *de novo* changes in Cx mRNA and/or protein expression. However, the Cx43 gene is sensitive to TNF-α regulation, where expression is decreased upon cytokine treatment (Fernandez-Cobo et al. 1999). A recent study has reported that individually, neither IL-6, TNF-α, nor IFN-γ had a significant effect on astrocyte GJC, whereas IL-1β induced a modest decrease in coupling (Memê et al. 2006). However, cells were not analyzed until 24 h following cytokine treatment, which based on previous studies, suggests that changes may have occurred at earlier time points but were not detected. In contrast, co-administration of TNF-α + IL-1β led to a dramatic reduction in gap junction coupling and Cx43 expression indicating that a threshold of cell activation was required to elicit these changes (Memê et al. 2006). Similar to the effects of other proinflammatory mediators, astrocyte gap junction activity is attenuated in response to NO (Bolanos and Medina 1996; Retamal et al. 2007). Exposure of astrocytes to LPS resulted in a dose-dependent inhibition in GJC that was attributed to NO since blockade of its synthesis with the specific iNOS inhibitor N^G-methyl-L-arginine (NMMA) was capable of restoring gap junction activity (Bolanos and Medina 1996; Retamal et al. 2007). Since NO is known to be associated with several CNS pathological conditions including Alzheimer's and Parkinson's disease and MS (Calabrese et al. 2000; Law et al. 2001; Smith and Lassmann 2002; Acar et al. 2003; Hunot and Hirsch 2003), modulation of astrocyte GJC may have drastic implications on the well-being and cell survival of both neurons and glial networks.

Collectively, these studies have illuminated the fact that the effects of cytokines on astrocytic Cx43 expression and GJC may be influenced by numerous factors including the species of astrocyte origin, culture conditions, and/or whether fetal or neonatal cells are evaluated. As described above, IL-1 alone has been reported to have either dramatic (John et al. 1999), transient (Zvalova et al. 2004), or minimal effects (Memê et al. 2004; Memê et al. 2006) on astrocyte Cx43 levels and function. The consequences of TNF-α treatment on astrocyte GJC are more in agreement, where cytokine-induced reductions occur within a rapid time frame (i.e. several hours following cytokine exposure). One factor to consider is whether cytokine treatment elicits the release of additional proinflammatory molecules from cytokine-activated astrocytes. If correct, these molecules may exert redundant autocrine/paracrine effects, rendering changes in Cx43 protein expression difficult to detect or assign to one individual

cytokine. Alternatively, species differences may also influence changes in Cx43 expression associated with cytokine treatment. For example, John et al. demonstrated that IL-1β reduced Cx43 expression in human fetal astrocytes (John et al. 1999). Since Cx expression is under developmental control (Lee et al. 2005; Rochefort et al. 2005; Sutor and Hagerty 2005), the stage at which cells are procured may influence the nature of responses obtained. Another variable could be the length of time that astrocytes are cultured *in vitro* and whether cells are propagated as mixed glial cultures for extended periods or whether highly purified astrocytes are procured immediately *ex vivo*. For example, upon isolation, human fetal astrocytes contain relatively few microglia and thus do not experience the same co-culture environment as neonatal glial cultures from rodents that initially comprise a mixture of astrocytes and significant numbers of microglia (Farina et al. 2005). In fact, in order to obtain highly purified astrocyte cultures, cytotoxic agents are commonly used to deplete microglia (Thiele and Lipsky 1992; Hamby et al. 2006). However, as noted in recent studies, this approach does not necessarily eliminate microglia which are notorious for hiding beneath astrocyte monolayers (Hewett et al. 1999; Saura 2007). This is a very important point since previous experiments by others have shown that microglia can attenuate astrocyte GJC and Cx43 expression (Rouach et al. 2002a; Faustmann et al. 2003; Hinkerohe et al. 2005; Memê et al. 2006; Retamal et al. 2007) as described in a later section.

Consequences of pathogenic stimuli on GJC in astrocytes and microglia

Inflammation is a hallmark of CNS infections elicited by bacterial and viral pathogens (Garden 2002; Nau and Bruck 2002; Kielian 2004). A general consequence of inflammation in response to these infectious agents is reactive gliosis typified by astrocyte hypertrophy and proliferation of astrocytes and microglia. Although a few earlier reports had investigated the effects of pathogens on GJC, this topic has received increasing attention over the past few years with several recent studies describing the consequences of both bacterial and viral pathogens on glial gap junction coupling (Garg et al. 2005; Zhao et al. 2006; Esen et al. 2007; Eugenín and Berman 2007; Koster-Patzlaff et al. 2007). One of the first studies to examine the impact of pathogens on astrocyte gap junction activity was conducted with the parasites *Trypansoma cruzi* and *Toxoplasma gondii* (Campos de Carvalho et al. 1998). Following parasite exposure, functional dye coupling was reduced and accompanied with a re-distribution of connexin expression in infected astrocytes. Parasitized cells displayed reduced punctuate gap junctional staining; however, there were no alterations in the total levels of Cx43 protein or its phosphorylation state. These results were interpreted to suggest that parasitic infection induces alterations in the targeting of Cxs to the cell membrane and/or influences their assembly as subunits into functional channels (Campos de Carvalho et al. 1998). A recent study by the same group has also demonstrated that *Trypansoma cruzi* leads to a reduction in Cx43 expression in cardiac myocytes with prolonged parasite exposure (Adesse et al. 2007).

Recent studies have focused on the effects of the gram-positive bacterium, *Staphylococcus aureus* (*S. aureus*) on influencing GJC in astrocytes versus microglia (Garg et al. 2005; Esen et al. 2007). *S. aureus* is capable of CNS colonization and is a common etiologic agent of brain abscesses in humans (Mathisen and Johnson 1997; Townsend and Scheld 1998; Kielian 2004). Exposure of both astrocytes and microglia to *S. aureus* induces the production of a wide range of proinflammatory mediators including TNF-α, IL-1β, and NO (Kielian 2002; Esen et al. 2004). Recognition of *S. aureus* and its major cell wall product peptidoglycan (PGN) occurs in microglia and astrocytes via the pattern recognition receptor Toll-like receptor 2 (TLR2) and the central adaptor molecule MyD88 (Esen et al. 2004; Kielian et al. 2005; Esen and Kielian 2006). The interest in studying the effects of *S. aureus* and PGN on glial GJC is justified by several reasons. First, Cx43 expression and gap junction coupling are influenced by p38 MAPK signaling pathways (Warn-Cramer et al. 1996; Warn-Cramer et al. 1998) and TLR2 engagement by microbial stimuli leads to activation of the MAPK cascade (Akira 2006; O'Neill

and Bowie 2007). Second, there was a precedent established for pathogenic stimuli (parasites and the gram-negative antigen LPS) to modulate GJC in glia (Campos de Carvalho et al. 1998). However, prior to our work we were unaware of any studies examining the ability of intact bacteria to influence coupling in any CNS cell type. Interestingly, *S. aureus* and PGN were found to differentially regulate Cx43 expression and gap junction coupling in astrocytes versus microglia, a phenomenon we coined a "syncytial switch" (Kielian and Esen 2004) (Figure 1). Specifically, in astrocytes *S. aureus* and PGN led to a significant reduction in Cx43 mRNA and protein expression concomitant with a nearly complete loss of homocellular gap junction coupling (Esen et al. 2007). The effects of bacteria on astrocyte coupling were found to be mediated, in part, via MAPK-dependent signal(s) since the p38 MAPK inhibitor SB202190 was partially effective at rescuing the defect in astrocyte coupling observed following *S. aureus* exposure. In contrast, a dramatic induction in Cx43 mRNA and protein expression was detected in primary microglia that coincided with the establishment of functional coupling that was shown to be gap junction-mediated since it was effectively prevented with 18-α-glycyrrhetinic acid (AGA) (Garg et al. 2005). Interestingly, although we observed a rather robust induction of Cx43 protein expression in microglia in response to PGN, the majority of Cx43 immunoreactivity was intracellular and the number of gap junction coupled microglia was rather low (i.e. 2–4 cells displayed Lucifer yellow dye transfer)(Garg et al. 2005). This finding suggests that Cx43 may play a role in other, as of yet unidentified, processes in microglia. A discussion of gap junction-independent roles for Cxs is presented in a later section of this review. Our results were in agreement with Eugenín et al. who reported the formation of a small microglial syncytium in response to LPS + IFN-γ (Eugenín et al. 2001). However, other laboratories have failed to detect Cx43 expression in microglia with LPS alone (Dobrenis et al. 2005; Memê et al. 2006). We propose that collectively the evidence suggests that in order to detect Cx43 expression and GJC in microglia a threshold level of cell activation must be achieved. This is supported by the finding that a single stimulus is generally less effective at inducing Cx43 and consequent GJC, whereas combinations of cytokines or complex bacterial antigens are effective inducers of both events in microglia. In our studies, it is important to acknowledge the complicated biochemical nature of PGN compared to other well studied microbial antigens such as LPS. Biochemically, PGN is an insoluble heterogeneous molecule with extensive cross-linkages facilitated by a large percentage of O-acetylated resides (Lowy 1998; Dziarski 2003). Once phagocytized, PGN is degraded into muramyl dipeptide, a ligand for the intracellular pattern recognition receptor NOD2 (Murray 2005; Strober et al. 2006). Therefore, it is likely that PGN is sufficient to induce Cx43 expression and subsequent GJC in microglia because of its structural complexity and potent proinflammatory activity (Dziarski 2003; Garg et al. 2005; Kielian et al. 2005). In addition, studies that failed to demonstrate Cx43 expression in microglia were either performed with co-cultures of astrocytes (Rouach et al. 2002a; Faustmann et al. 2003; Hinkerohe et al. 2005) or neurons (Dobrenis et al. 2005). It is possible that in order for Cx43 to be induced to detectable levels, microglia must be in close enough proximity to trigger protein expression and in large numbers. Therefore, the proximity and numbers of microglia in co-culture experiments may be one reason for the inability to detect Cx43 in these cells. Indeed, upon activation with cytokines or microbial stimuli, microglia exhibit homotypic adhesion events facilitated by an increase in the expression of adhesion molecules such as ICAM-1 (Shrikant et al. 1995; Lee and Benveniste 1999). In addition, since it is known that gap junction channel formation requires that coupled cells be within close proximity and is a cell adhesion-dependent event, these possibilities remain plausible (Meyer et al. 1992; Giepmans 2004; Wei et al. 2005).

Recent studies have investigated the effects of viruses (Eugenín and Berman 2007; Koster-Patzlaff et al. 2007) or viral immunostimulatory motifs (Zhao et al. 2006) on astrocytic GJC. Interestingly, unlike bacterial stimuli or proinflammatory cytokines that limit gap junction coupling, infection of astrocytes with either HIV-1 or Borna Disease Virus (BDV) resulted in either minimal changes or heightened GJC in astrocytes (Eugenín and Berman 2007; Koster-

Patzlaff et al. 2007). These findings speak to the importance of evaluating distinct classes of pathogens for their ability to affect glial networks rather than assuming that diverse antigens exert similar biological effects. In a recent study, Eugenín and Berman demonstrated that uninfected astrocytes surrounding foci of HIV-infected cells underwent apoptosis which could be prevented by treating astrocytes with gap junction blockers (Eugenín and Berman 2007). Although the authors found that HIV-1 infection led to a slight reduction in astrocytic gap junction transfer (i.e. 20–40%), the residual coupling was sufficient to propagate toxic signal (s) to uninfected neighboring gap junction coupled cells. It is interesting that HIV-1 infected astrocytes were protected from apoptotic death and the authors speculate that this could be mediated by the anti-apoptotic actions of viral proteins and/or the release of CCL2 from infected cells (Eugenín and Berman 2007). One interesting finding in this study was that the gap junction blockers AGA and octanol exacerbated glutamate release from HIV-1 infected astrocyte cultures. A potential mechanism responsible for this phenomenon was not discussed, but could potentially be ascribed to gap junction-independent effects since these compounds can also influence other ion channels (Rozental 2000). Similar to HIV-1, BDV augments astrocytic Cx43 expression and dye coupling as well as region-specific alterations in Cx43 expression in a rat model of infection (Koster-Patzlaff et al. 2007). In contrast to infection with live virus, exposure of astrocytes to polyI:C, a synthetic RNA molecule that mimics dsRNA viruses and virus replication intermediates, resulted in a reduction in Cx43 expression and GJC in primary human fetal astrocytes (Zhao et al. 2006). These effects were found to be mediated by a NF-κB and PI3K-dependent mechanism since pharmacological inhibitors of both pathway (s) were capable of partially restoring the polyI:C-induced changes in astrocytic gap junction coupling and Cx43 expression (Zhao et al. 2006). Recently, we have expanded our analysis to investigate the effects of numerous ligands of the TLR family on GJC in astrocytes and microglia including Pam2Cys (TLR2), polyI:C (TLR3), CpG DNA (TLR9) as well as the NOD2 agonist MDP (Syed et al, manuscript in preparation). Our results indicate complex and differential effects of these microbial ligands on glial coupling. For example, in microglia ODN and polyI:C were most effective at inducing GJC; however, these stimuli led to only slight increases in Cx43 expression. In contrast, polyI:C and Pam2Cys were both potent inducers of Cx43 protein in microglia, whereas they were not as effective in their ability to establish microglial coupling (Syed et al., manuscript in preparation). In astrocytes, all four pathogen motifs attenuated gap junction coupling, whereas differential effects were observed with regard to the ability of each stimulus to modulate Cx43 expression (Syed et al., manuscript in preparation). These findings suggest that a wide range of pathogens, both viral and bacterial, are likely capable of influencing glial homocellular coupling in the CNS. In turn, this could significantly impact the extent of neuron survival during infection, even at distant sites from the primary nidus of infection based on the extensive network of the astrocyte syncytium. However, further studies are required to delineate the effects of intact pathogens versus antigenic motifs based on new emerging evidence suggesting that the two may not necessarily produce the same outcome in terms of effects on glial coupling (Zhao et al. 2006; Eugenín and Berman 2007; Koster-Patzlaff et al. 2007).

Effects of inflammation on GJC in astrocyte-microglia co-cultures

The consequences of microglial-derived cytokines and on astrocytic GJC have been the focus of several recent studies (Rouach et al. 2002a; Faustmann et al. 2003; Hinkerohe et al. 2005; Mem et al. 2006; Retamal et al. 2007). The first reports examining the effects of microglia on astrocyte coupling revealed that the mere presence of microglia, in the absence of any exogenous stimulation, led to a reduction in astrocytic GJC and Cx43 expression (Rouach et al. 2002a; Faustmann et al. 2003). The inhibitory effects of microglia on astrocyte coupling were observed in a dose-dependent manner with higher numbers of microglia exerting maximal reductions in coupling (Rouach et al. 2002a). At face value this finding appears somewhat unexpected; however, it may be explained, in part, by the fact that numerous studies have

shown that cultured microglia assume a partially activated phenotype as evidenced by increased expression of major histocompatibility complex II (MHC class II) as well as co-stimulatory molecules (i.e. CD80 and CD86)(de Groot et al. 1992; Carson et al. 1998; Floden and Combs 2006). However, one must consider that in the normal CNS, it is unlikely that microglia exert such negative effects on astrocyte GJC since a tonic inhibitory environment would considerably impact the effectiveness of the astrocyte syncytium for maintaining ion homeostasis and glutamate clearance from the extracellular milieu. Faustmann et al. demonstrated that astrocyte GJC and Cx43 expression were reduced in co-cultures containing 30% unstimulated microglia but not at lower numbers (i.e. 5% microglia), the latter of which would be more reminiscent of the ratio of microglia to astrocytes in the normal CNS (Faustmann et al. 2003). In contrast, higher microglial numbers would be more reflective of neuroinflammatory conditions where mitogenic or chemotactic signals would be expected to increase the frequency of microglia. The same group went on to show that the addition of any single proinflammatory cytokine including TNF-α, IL-1β, IL-6, or IFN-γ to astrocyte co-cultures containing 5% microglia was sufficient to now attenuate astrocyte coupling and Cx43 expression, whereas without exogenous cytokines the inclusion of 5% microglia had no effect on these parameters (Hinkerohe et al. 2005). In addition, these same cytokines when added to co-cultures containing 5% microglia led to a depolarization of astrocytic resting membrane potential. As mentioned earlier, depolarization of astrocytes interferes with their homeotstatic functions including glutamate uptake and K^+ buffering, both of which have been shown to be voltage-dependent (Kimelberg et al. 1989; Flott and Seifert 1991). Therefore, the ability of proinflammatory cytokines to simultaneously inhibit astrocyte GJC and depolarize cells represents a “double whammy” since both negatively impact the astrocyte syncytium and maintenance of CNS homeostasis would be disrupted. An intriguing finding of the Hinkerohe et al. study was the ability of two cytokines with well-documented anti-inflammatory effects, TGF-β1 and IFN-β, to partially restore the cytokine-induced reductions in astrocyte GJC and Cx43 expression as well return astrocytic resting membrane potentials to normal values in astrocyte-microglia co-cultures (Hinkerohe et al. 2005). The ability of TGF-β1 and IFN-β to reverse these changes suggests they may be of benefit in the context of neuroinflammation. Indeed, IFN-β is one of the main treatments to attenuate inflammation in patients suffering from MS (Bermel and Rudick 2007; Markowitz 2007).

Separate studies by the Giaume laboratory demonstrated that in microglia-astrocyte co-cultures, LPS stimulation led to a significant reduction in astrocyte GJC concomitant with a decrease in Cx43 protein levels (Memê et al. 2006; Retamal et al. 2007). The inhibitory effects of microglia were attributed to a soluble factor(s) released from activated microglia since the transfer of microglial-conditioned supernatants from LPS-stimulated cells led to identical reductions in astrocytic coupling and Cx43 expression. These secreted factors were identified as TNF-α and IL-1β since blocking antibodies and soluble cytokine receptors specific for each mediator could partially reverse the inhibitory effects of microglial-conditioned medium on astrocyte coupling (Memê et al. 2006). However, it is important to note that additional, as of yet unidentified, molecules are also responsible for this phenomenon since inactivation of TNF-α and IL-1β activity did not fully restore the block in astrocyte GJC. Interestingly, a recent report from the same group has revealed that proinflammatory cytokines differentially regulate astrocyte GJC and Cx43 hemichannels (Retamal et al. 2007). Specifically, as mentioned above, the combination of IL-1β + TNF-α inhibited astrocytic gap junction coupling, whereas ethidium bromide (EtBr) and 2-NDBG uptake via Cx43 hemichannels was enhanced. Specificity of Cx43 hemichannels for dye entry was provided by the finding that EtBr uptake in response to cytokine stimulation was prevented by Cx43 mimetic peptides as well as astrocytes derived from Cx43-deficient mice prevented (Retamal et al. 2007). A separate study also demonstrated that LPS exerted differential effects on GJC and hemichannel function in Cx43-expressing C6 glioma cells by inhibiting and potentiating activity, respectively (De Vuyst et al. 2007). These findings suggest that during neuroinflammatory responses that attenuate astrocyte GJC, Cx43

hemichannels are opened providing a conduit for the bi-directional trafficking of small molecules from the astrocytic cytoplasm to extracellular milieu. However, the opening of Cx43 hemichannels during neuroinflammatory conditions *in vivo* remains to be demonstrated.

Relevant to another neuroinflammatory disorder, Memê et al. recently showed that a fragment of β-amyloid (Aβ25–35), one of the molecules attributed to the pathogenesis of Alzheimer's disease, potentiated the inhibitory effects of microglial-derived cytokines on astrocyte GJC (Memê et al. 2006). Specifically, although it had no effect alone, treatment of primary astrocytes with Aβ25–35 in conjunction with IL-1β + TNF-α potently inhibited astrocytic gap junction coupling and Cx43 expression. Collectively, these studies illuminate an important cross-talk pathway between astrocytes and microglia in regulating homocellular GJC of the former. Currently, the evidence suggests that the presence of low numbers of microglia, reminiscent of what would be encountered in the normal CNS, does not significantly impact the astrocyte syncytium unless microglia are activated by immunostimulatory compounds such as LPS. However, in response to inflammation as mimicked *in vitro* by the addition of numerous microglia or the presence of cytokines or other antigens, microglia provide signal(s) to attenuate astrocyte GJC concomitant with hemichannel opening. It remains to be determined whether these responses occur *in vivo*, nonetheless it is evident that inflammation alters the balance of the astrocyte syncytium, which may have dramatic consequences on the homeostasis of CNS tissue and quite possibly repair processes following injury.

Involvement of gap junctions in the immune system

Since many inflammatory and infectious diseases of the CNS are associated with a peripheral immune infiltrate, a discussion of gap junctions in immune cells is warranted. There is a substantial body of evidence implicating gap junctions as a means of both homocellular and heterocellular communication between various cell types in the immune system (Oviedo-Orta and Howard Evans 2004; Wong et al. 2004; Chanson et al. 2005). Cx43 has been identified as the principle Cx isoform expressed in various immune cells with minor contributions by other family members (Oviedo-Orta and Howard Evans 2004; Wong et al. 2004; Chanson et al. 2005). An important role for Cx43, and presumably GJC, in regulating hematopoiesis was demonstrated by the fact that T and B cell development was impaired in Cx43 homozygous-deficient mice (Cx43 −/−) (Montecino-Rodríguez et al. 2000). However, Cx43 −/− mice do not survive long after birth due to heart failure (Reaume et al. 1995), necessitating the use of Cx43 +/− heterozygous mice or a targeted deletion of Cx43 in a specific cell type by Cre-lox technology to study the importance of Cx43 in adult animals (Theis et al. 2003). Even the loss of one Cx43 allele in Cx43 +/− mice led to a delay in lymphocyte maturation; however, this was rectified by 4 weeks of age when normal T and B cell maturation was detected (Montecino-Rodríguez et al. 2000). Despite these observations, it remains unclear as to how Cx43 influences lymphocyte development and interactions with stromal cells within primary lymphoid organs (i.e. bone marrow and thymus).

With regard to immune cells, gap junctions have been described either morphologically or functionally by means of dye or electrical coupling to facilitate homocellular communication between neutrophils, monocytes/macrophages, dendritic cells (DCs), and T and B lymphocytes (Oviedo-Orta and Howard Evans 2004; Wong et al. 2004; Chanson et al. 2005). A recent study has implicated gap junctions in regulating fundamental processes of lymphocyte function including the production of immunoglobulins and cytokines such as IL-10 (Oviedo-Orta et al. 2001). In general, studies with DCs and monocytes/macrophages are in agreement that under normal, resting conditions, these phagocytic cells are not coupled via gap junctions. However, in response to the appropriate combination of stimuli, both cell types exhibit homocellular coupling (Eugenín et al. 2003; Matsue et al. 2006; Corvalan et al. 2007). For example, treatment of either DCs or monocytes/macrophages with LPS or individual cytokines is usually not

sufficient to induce coupling or augment Cx43 expression. However, when a combination stimulus is applied (i.e. TNF-α + IL-1β or LPS + IFN-γ), Cx43 expression is enhanced which coincides with the induction of functional GJC (Eugenín et al. 2003; Matsue et al. 2006; Corvalan et al. 2007; Mendoza-Naranjo et al. 2007). It is interesting to note that this transformation to gap junction competency is concomitant with the transition to a mature cell phenotype as assessed by the expression of characteristic surface markers (Corvalan et al. 2007; Mendoza-Naranjo et al. 2007). In addition, many cell adhesion molecules are also induced in immune cells during the activation/maturation process, which is a prerequisite for cellular aggregation to facilitate the establishment of GJC (Musil et al. 1990; Lin et al. 2002; Giepmans 2004). When considering the circumstances leading to gap junction formation in these antigen presenting cells, the fact that activation steps are required to reach this endpoint are not unexpected. For example, DCs are highly migratory by nature, assigned the task of sampling antigens in a local tissue environment and transporting them to regional lymph nodes where they can stimulate antigen-specific T and B cells. Because of this migratory potential, it would appear counterintuitive to establish gap junctions between resident DCs and neighboring cells under physiological conditions. However, upon reaching draining lymph nodes in response to an inflammatory stimulus, DCs could be programmed to associate with other DCs or lymphocytes via gap junctions. A similar scenario could be envisioned for macrophages when interacting with infiltrating lymphocytes in areas of active inflammation. In both scenarios, the local microenvironment likely plays a pivotal role in dictating the activation status of DCs and macrophages and whether GJC will be established. Indeed, homocellular GJC has been described to occur between DCs, macrophages, and lymphocytes, as well as heterocellular communication between DCs and lymphocytes, or macrophages and lymphocytes (Oviedo-Orta and Evans 2002).

Besides establishing communication between various immune cell populations, gap junctions have been identified morphologically at leukocyte-endothelial cell contacts and studies have demonstrated that gap junctions are involved in regulating leukocyte transendothelial migration. The latter has been suggested by the ability of Cx mimetic peptides to interfere with transendothelial migration in *in vitro* culture models (Jara et al. 1995; Oviedo-Orta et al. 2002; Eugenín et al. 2003; Zahler et al. 2003; Oviedo-Orta and Evans 2004; Haddad et al. 2007). With regard to the impact of Cx43 on leukocyte recruitment into diseased tissues, others have demonstrated significantly fewer inflammatory cells in atherosclerotic LDLR-deficient mice crossed to Cx43 +/− animals (Kwak et al. 2003) as well as reduced numbers of macrophages/microglia in the CNS of Cx43 +/− mice following cerebral ischemia (Nakase et al. 2004). In addition, administration of Cx43 anti-sense oligonucleotides to wounded skin in mice led to a reduction in leukocyte influx into the damaged site (Qiu et al. 2003). However, the functional implications of gap junctions in modulating changes in adhering leukocytes and endothelial cells as well as the impact of inflammation on this process have not yet been defined.

Despite the reported expression of Cxs in immune cells and their ability to facilitate dye transfer via gap junctions in a homocellular or heterocellular manner, relatively little information is available regarding the implications of such associations. However, recent studies have provided a functional correlate for the role of gap junctions in the transport of small peptides between cells, a process referred to as “cross-presentation” (Heath and Carbone 2005; Li and Herlyn 2005; Neijssen et al. 2005; Handel et al. 2007). The term cross-presentation refers to the ability to transfer extracellular proteins into the MHC class I pathway, which is normally reserved for the presentation of intracellular peptide antigens to $CD8^+$ T cells. The ability of antigen presenting cells to acquire exogenous antigens released from virus infected or tumor cells and present them on MHC class I to stimulate $CD8^+$ cytolytic T cells provided a mechanism by which infected or tumor cells could be targeted by the immune system. With regard to the role of gap junctions in this process, an elegant study by Neijssen et al. demonstrated that Cx43-containing gap junctions facilitated the intercellular transfer of small

peptides between 4–10 amino acids in length (Neijssen et al. 2005). The efficiency of peptide transfer decreased with increasing peptide size and the confirmation of the peptide also dictated whether passage was facilitated via gap junctions. Specifically, linear peptides were propagated to neighboring gap junction coupled cells, whereas molecules with an identical size but containing secondary structure were not transferred (Neijssen et al. 2005). With regard to the functional implications of this phenomenon, the authors went on to show that the transfer of peptides to neighboring cells via gap junctions resulted in their recognition and killing by peptide-specific $CD8^+$ cytotoxic T cells via an innocent bystander pathway (Neijssen et al. 2005). This mode of antigen propagation inherently raises some fundamental concerns in terms of how this process is controlled to prevent the excessive destruction of neighboring gap junction coupled cells that are not necessarily affected but yet have obtained the antigenic peptide by means of gap junction transport. One answer is that cells are equipped with a high level of cytosolic amino peptidase activity, such that intracellular peptides are rapidly degraded (Neijssen et al. 2005). Therefore, this cytosolic peptidase activity would limit, but not completely prevent, the transfer of peptides via gap junctions to neighboring cells, effectively minimizing the extent of innocent bystander cell involvement. This fact is supported by the finding that the number of cells receiving peptides via gap junction transport was rather limited (Neijssen et al. 2005). Conversely, the collateral damage afforded by gap junction-mediated cross-presentation of peptides could also prove beneficial during viral infections or tumor growth. In both circumstances, the effective elimination of bystander cells that received antigenic peptides would prevent a nearby nidus for additional viral replication or disrupt a favorable microenvironment for virus survival or tumor growth and metastasis (Li and Herlyn 2005). Recent studies by other groups have also demonstrated a role for gap junctions in the transfer of tumor antigens between DCs (Mendoza-Naranjo et al. 2007). Additional reports have demonstrated siRNA transfer to neighboring cells through gap junctions (Valiunas et al. 2005; Wolvetang et al. 2007); however, it remains unclear as to whether this can occur *in vivo*.

The implications for such gap junction-mediated cross presentation of antigens could have important consequences in other diseases where $CD8^+$ T cells play a role in pathology. For example, with regard to the CNS, $CD8^+$ T cells have been implicated in the pathogenesis of MS (Babbe et al. 2000; Friese and Fugger 2005; Weiss et al. 2007) and the transfer of self-antigens via gap junctions in the CNS could contribute to disease severity. In addition, several virus infections that afflict the CNS such as HIV-1 and LCMV have also been associated with $CD8^+$ cells (Nansen et al. 2000; McCrossan et al. 2006; Petito et al. 2006; Roberts et al. 2006; Storm et al. 2006) and the propagation of viral peptides via gap junctions could conceivably influence the nature of viral dissemination or the resultant host immune response. However, some viruses and viral proteins are capable of inhibiting GJC (Ennaji et al. 1995; Oelze et al. 1995; Knabb et al. 2007) and although it is not known whether other CNS tropic viruses have similar effects or whether CNS gap junctions would be regulated in a similar manner as other cell types in peripheral tissues, this remains one way in which the virus may escape a cross-presentation mechanism to ensure survival. Likewise, the spread of tumor-associated peptides via gap junctions to neighboring antigen presenting cells in malignant brain tumors such as GBM could facilitate immune recognition of tumor targets. However, it is well established that gliomas downregulate Cx43 expression and GJC, which correlates with cell cycle dysregulation and invasion (Naus et al. 1992; Zhu et al. 1992; Soroceanu et al. 2001; Lin et al. 2002). Therefore, strategies to augment Cx expression in these tumors may be required to reap any potential benefit from peptide cross-presentation and cell destruction. Although intriguing, these possibilities remain highly speculative at the present time but warrant further investigation to determine their impact on disease pathogenesis.

Gap junctions in models of non-infectious CNS inflammation

Several recent studies have reported that Cx43 expression is reduced in demyelinating plaques within the lumbar spinal cord white matter in rodent models of EAE (Brand-Schieber et al. 2005; Roscoe et al. 2007a; Roscoe et al. 2007b). The regions of Cx43 loss corresponded to areas of active microglia/macrophage aggregation and astrocyte activation and hypertrophy (Brand-Schieber et al. 2005; Roscoe et al. 2007b). Interestingly, Cx43 expression within plaques recovered during the remyelination phase and exceeded levels found in control tissues (Roscoe et al. 2007b). Despite these intriguing observations, it is not yet clear whether the increase in Cx43 expression observed during remyelination has functional implications or whether it represents a surrogate marker for the remyelination process (Roscoe et al. 2007b). An argument against a pivotal role for Cx43 in neuroprotection during EAE was revealed by a recent study demonstrating that disease severity was identical in Cx43 +/− and Cx43 +/+ mice (Roscoe et al. 2007a). However, this does not necessarily discount a role for Cx43 since it remains possible that a loss of both Cx43 alleles is required to detect significant changes in pathology and/or alternative Cx isoforms functionally compensate for the reduction in Cx43 expression in Cx43 +/− animals during EAE. However, Brand-Schieber reported that Cx30, another Cx isoform expressed by astrocytes, was not detected in demyelinated plaques when Cx43 was lost (Brand-Schieber et al. 2005). This argues against a compensatory role for alternative Cx isoforms during EAE development; however additional studies are needed to definitely reach this conclusion. Studying EAE development in mice that selectively lack Cx43 in astrocytes (Theis et al. 2003) would provide additional insights into the potential role of astrocytic Cx43 in plaque formation and inflammation during CNS autoimmunity.

Another CNS disorder typified by neuroinflammation is Alzheimer's disease (AD) (Eikelenboom et al. 2002; Griffin and Mrak 2002; McGeer and McGeer 2002). One report exists in the literature where Cx43 immunoreactivity was compared in brain tissues from AD patients versus healthy controls (Nagy et al. 1996). Interestingly, unlike what has been reported in rodent models of EAE, Cx43 expression was augmented in AD plaques, which was found to co-localize with activated astrocytes and β-amyloid deposition (Nagy et al. 1996). The apparent dichotomy between Cx43 expression in EAE models versus AD could be explained by several factors. First, these changes could be species specific and to date, no one has reported whether Cx43 levels change in plaques from MS patients. Second, the nature of the inflammatory infiltrate may dictate the subsequent change in Cx43 expression. For example, the degree of inflammation observed in EAE/MS is more overt and involves significant peripheral immune infiltrates, whereas the extent of inflammation in AD is less severe and primarily involves the activation of resident glia (i.e. microglia and astrocytes) although reports of T cell and macrophage infiltrates have been described (Eikelenboom et al. 2002; Griffin and Mrak 2002; McGeer and McGeer 2002). Finally, the nature of the inflammatory milieu may dictate whether Cx43 expression is augmented or reduced. It is worth noting that with regard to EAE, several proinflammatory mediators, including IL-1β, TNF-α, and NO, have been reported to have both positive and negative effects on disease progression (Arnett et al. 2001; Mason et al. 2001; Smith and Lassmann 2002; Dalton and Wittmer 2005). Specifically, these disparate effects are likely attributable to the timing and concentration of mediator release. Therefore, it may be dangerous to fall into the mindset that cytokine expression always leads to a down-regulation in Cx43 expression (as observed in cytokine-treated astrocytes). In fact, it remains possible that at lower levels (that might be associated with reparative stages typical of re-myelination) cytokines could conceivably facilitate an increase in Cx43 expression.

Although these studies suggest that the inflammatory milieu shapes changes in GJC in the CNS, the classical "chicken or the egg" question remains; namely, whether changes in Cx isoform expression contribute to disease severity or rather, are merely a secondary consequence

of tissue damage. Addressing this question will prove challenging and perhaps can be further clarified through the use of conditional Cx-deficient mice and the development of new highly specific gap junction blockers.

In vivo studies from several groups have produced conflicting results regarding whether GJC is beneficial or detrimental in the context of other CNS diseases (Pérez Velazquez et al. 2003; Farahani et al. 2005; Talhouk et al. 2008). For example, Cx43 +/− mice showed a significantly increased infarct size and enhanced apoptosis after brain ischemia, suggesting that GJC plays a neuroprotective role in this setting (Naus et al. 2001; Nakase et al. 2003; Nakase et al. 2004). An interesting study has demonstrated that pre-conditioning, a phenomenon where an initial exposure to a sub-threshold stimulus reduces damage from a subsequent more severe injury, leads to enhanced Cx43 expression in astrocytes by blocking its lysosomal degradation (Lin et al. 2008). The protective mechanism of pre-conditioning, afforded by several distinct stimuli, was identified as an increase in Cx43 hemichannel activity and subsequent release of ATP which was catabolized to adenosine, a well known neuroprotective agent (Lin et al. 2008). The authors went on to show that the reduction in infarct volume afforded by hypoxic pre-conditioning requires Cx43 expression in astrocytes (Lin et al. 2008). In contrast, other groups have reported that gap junctions exacerbate CNS tissue damage by potentially propagating the spread of toxic/stress molecules to neighboring cells throughout glial syncytia (Rawanduzy et al. 1997; Frantseva et al. 2002; de Pina-Benabou et al. 2005). Since the brain is a complex organ with intricate relationships among its cellular constituents, it is not unexpected that different experimental paradigms such as tissue injury location, type of injury, and the resultant chemical and structural changes that occur in gap junctions may be key determinants of whether such alterations are detrimental or beneficial for disease outcome following CNS injury.

Connexins: beyond traditional roles of second messenger or signaling molecule transport

In recent years, Cxs have been shown to play multiple roles in addition to being an integral component of gap junction channels (Giepmans 2004; Stout et al. 2004; Jiang and Gu 2005). For example, Cx expression facilitates ATP release independent of gap junction coupling, which is thought to occur via hemichannels (Cotrina et al. 1998; Stout et al. 2002; Stout et al. 2004). In addition, numerous proteins have been identified to associate with Cxs including those involved with cytoskeletal and transcriptional regulation (Giepmans 2004). With regard to infectious disease, a recent study has suggested that *Shigella* infection of epithelial cells triggers ATP release through hemichannels, resulting in the activation of purinergic receptors on neighboring cells and bacterial dissemination (Tran Van Nhieu et al. 2003). Other gap junction-independent activities of Cxs that have been reported include regulation of cell proliferation (Huang et al. 1998; Moorby and Patel 2001; Qin et al. 2002; Kardami et al. 2007) and resistance to injury via anti-apoptotic actions (Lin et al. 2003). In astrocytes there are at least two additional roles for Cxs independent of traditional gap junction channels, namely regulation of neuronal migration and differentiation (Elias et al. 2007; Wiencken-Barger et al. 2007) and regulation of ATP receptor expression (Scemes 2008).

Recent work from Spray and Iacobas has introduced the concept that deletion of the Cx43 gene can influence a wide range of genes and potentially cellular functions in the brain in addition to their well described roles in GJC (Iacobas et al. 2005; Iacobas et al. 2007; Spray and Iacobas 2007). Through the use of transcriptional profiling, the authors have identified that Cx43 represents a node for regulating the expression of a wide array of genes (Iacobas et al. 2005; Iacobas et al. 2007). A model has been proposed where the transcriptome is organized in parallel modules of genes that are coordinately expressed. These relationships are envisioned to stabilize the overall transcriptome and likely serve redundant functions to compensate for

the dysregulation of important regulatory genes during overt pathology (Iacobas et al. 2007; Spray and Iacobas 2007). Collectively, these results support gap junction-independent effects of Cx43 as well as the ability of Cx43 to influence the expression of alternative genes involved in a wide array of cellular functions. With this in mind, it is important to not dismiss the potential for neuroinflammation induced by cytokines, infectious agents, or during autoimmune responses to modulate important mechanisms of Cx43 function that are not associated with its gap junction properties. These issues have not yet been addressed but present an interesting avenue for future research efforts.

Future directions- challenges facing the study of gap junctions in complex inflammatory conditions

Despite recent progress in characterizing the effects of inflammation on gap junction coupling in the CNS, several challenges remain. First and foremost is the issue of whether the descriptive changes in Cx isoform expression detected during CNS pathology equate with functional alterations in connectivity within glia syncytia. Progress in this area could be facilitated with the use of brain slice cultures in addition to cutting-edge *in vivo* microscopy techniques or perhaps the development of novel gap junction tracers that could be used in the setting of PET or SPECT scanning. Another obstacle relates to the complexity of Cx composition of gap junction channels and the potential for alternative Cxs to compensate for the loss of another isoform (i.e. in the case of Cx-deficient mice). There is also a need for pharmacological tools to block and discriminate between the two communicating functions of Cxs; namely gap junctions and hemichannels. These distinctions are not easily made using currently available technology. It will be interesting to identify the molecules that are capable of moving through astrocytic and microglial Cx gap junction channels and hemichannels under both normal and inflammatory conditions. In addition, the impact of changes in Cx isoform expression on neuronal fate (i.e. survival and differentiation) and how inflammation may affect these parameters *in vitro* and *in vivo* are warranted. Since much research emphasis has been placed on examining the role of Cx43 in glial GJC, the role of Cx30, an astrocytic Cx that is abundantly expressed *in vivo* along the glia-vascular interface, remains to be fully elucidated. Adding another level of intricacy to the situation is emerging evidence for glial cell heterogeneity throughout the CNS, where cells may inherently display different coupling properties that may be modulated by an inflammatory milieu. Although recent emphasis has been placed on the consequences of inflammatory stimuli on glial gap junction coupling, it will be equally important to consider the potential interplay between gap junctions in CNS cells and various immune cell populations under conditions where peripheral immune infiltrates are prominent. In summary, our knowledge characterizing the effects of inflammatory stimuli on glial GJC is ever expanding; however, future studies will need to define the biological consequences of such changes in the context of CNS pathology. This will be no small feat but could lead to the development of novel therapeutic strategies to combat various CNS diseases with an inflammatory component.

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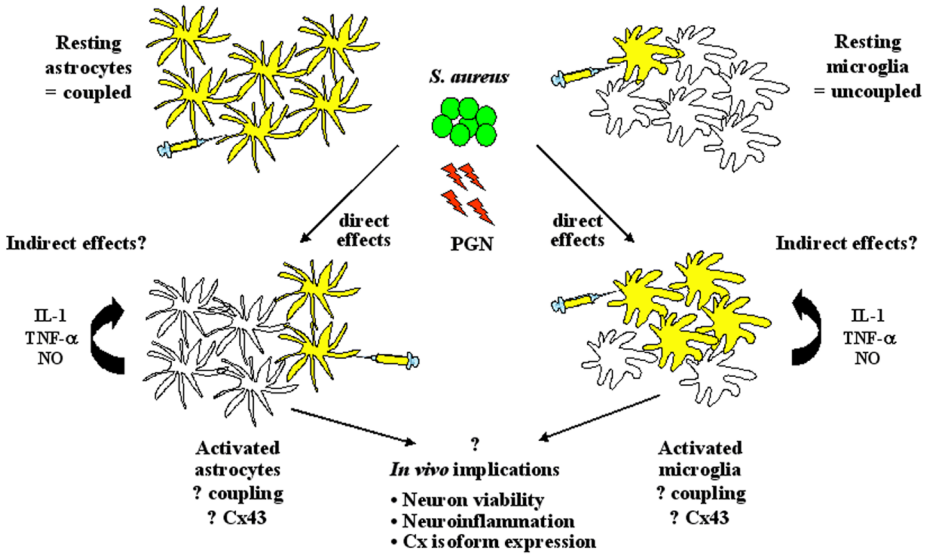

Figure 1. Conceptual overview of the "syncytial switch" in glia elicited by inflammatory stimuli
The yellow color in glia depicts the extent of Lucifer yellow dye transfer, representing the extent of functional gap junction channels.