PASSING THE TORCH: THE EFFECT OF GAP JUNCTION PLAQUE STABILITY ON NEURAL MEMBRANE PROTEINS

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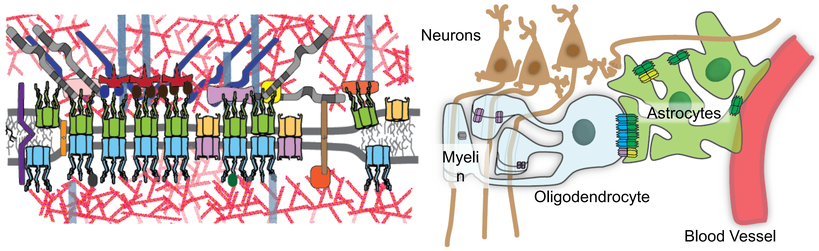
Abstract

Gap junction (GJ) channels permit small molecules, i.e. ions, metabolites/nutrients and second messengers, to transfer between cells. Their function is critical for a spectrum of cellular interactions including, but not limited to long-range propagation of calcium, spread and containment of apoptosis, and cancer invasiveness. Astrocytes and oligodendrocytes express mutually exclusive GJ proteins, or Connexins (Cx). Astrocytes express Cx26, Cx30, and Cx43. Oligodendrocytes express Cx29, Cx32, and Cx47. Cxs together with molecules that interact with GJ channels make up a supramolecular structure known as the GJ Nexus. In recent studies, we have examined the molecular stability and fluidity of both channel-forming and accessory GJ Nexus components using Fluorescence Recovery After Photobleaching (FRAP). We found that Cx32, Cx43, Cx47 form stable GJ plaques while Cx26 and Cx30 GJ plaques are fluid. We recently discovered that the formation of stable plaques (supramolecular arrays) is dependent on C-terminus (CT) cysteine residues. To reveal this fundamental property of GJs, we mutated the cysteine residues the CT of Cx32 and Cx43 to alanine, which resulted in significantly more fluid GJ structures. Based on these results, we hypothesized that GJ stability controls the location and mobility of other Nexus components of neural cells. (e.g. ZO-1, DLG1, EAAT2b and others). While GJ plaques excluded some proteins, others could infiltrate. Fluidity of GJ plaques had diverse effects on diffusion of proteins within the membrane.



The Gap Junction Nexus

Major Research Question:

- **What parts of gap junctions control Nexus structure?**

Hypothesis: Increased mobility of the core components of the gap junction Nexus (connexins) will determine localization and mobility of cellular components within 1μm of the Nexus.

Methods

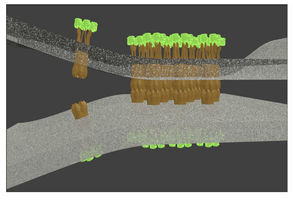
(See Stout and Spray, MBoC, 2017 and Stout and Spray, Ch.3, CNC Press, Methods, 2016)

Cells: Since we are focused on connexins and interacting molecules and not how cell-type affects gap junctions we use model gap junction deficient cell types: **Neuro2A** (N2A, mouse neuroblastoma cells), **HeLa cells**, and **immortalized Cx43KO mouse astrocytes**.

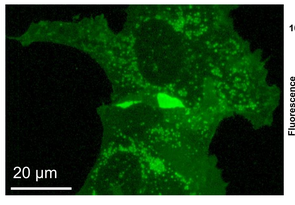
Transgenes and Fluorescent Proteins: We use plasmids to overexpress human and rat connexin proteins with monomerized fluorescent protein tags. **Constraints:** Tags on the Cx amino-terminus (NT) block channel function but preserve most interactions with other proteins. Tags on the Cx carboxyl-terminus (CT) preserve channel function but alter channel gating and block many protein interactions. CT tags that are not completely monomeric (A206K mutation in GFP) completely alter experimental results.

Fluorescence Recovery After Photobleaching (FRAP): Data was collected with a Zeiss 5Live laser-scanning confocal microscope using 405, 488, and 561 nm lasers and a 63x 1.5NA PlanApoFluor objective. Cells were maintained at 37 degrees in buffered media as described in Stout and Spray, 2017, MBoC.

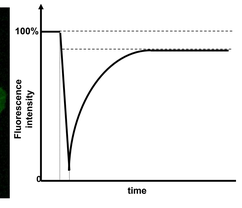
CGI rendering of a GJ made up of Cx43-GFP



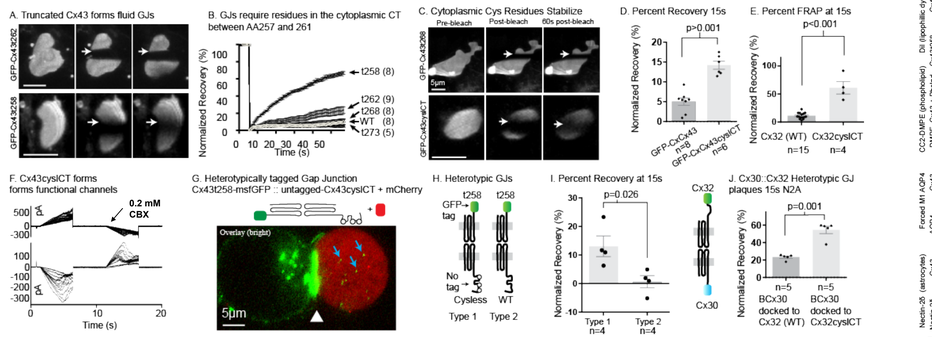
Immortalized Cx43KO astrocytes expressing msfGFP-Cx43 by transient transfection



FRAP quantitation of percent recovery

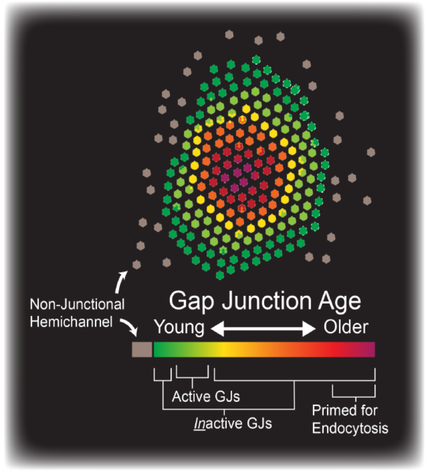


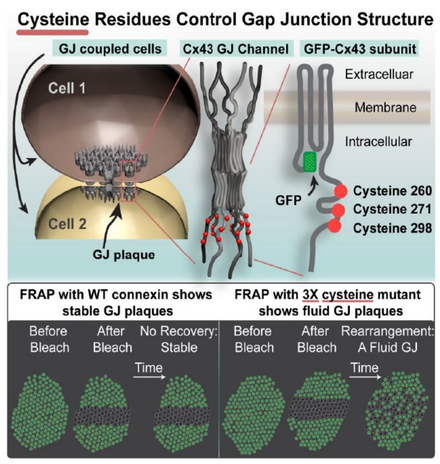
Results



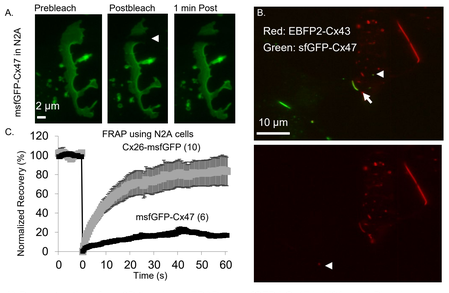
Determinants of GJ Plaque Stability

A, B) 3D time-lapse FRAP compared mobility of progressivley truncated constructs. **C, D, E)** Full-length Cx43 and Cx32 with cysteine-to-alanine mutations in the CT significantly destabilizes GJ plaque arrangement. **F)** Dual whole-cell patch clamp on Neuro2A cells expressing Cx43cysICT indicates that untagged Cx43cysICT (used in panels to the right) form functional gap junction channels. **G)** Approach to measure mobility of untagged connexins incorporated into functional gap junction channels. **H, I)** Wild-type Cx43 immobilizes a normally highly mobile connexin mutant (which forms functional channels) Cx43K258stop-msfGFP within gap junctions to a significantly greater degree than Cx43cysICT. **J)** Cysteine residues within the cytoplasmic CT of Cx32 immobilize Cx30 at the form of heterotypic GJs found at oligodendrocyte-astrocyte Nexuses. Adapted from Stout and Spray, *MBoC*, 2017.



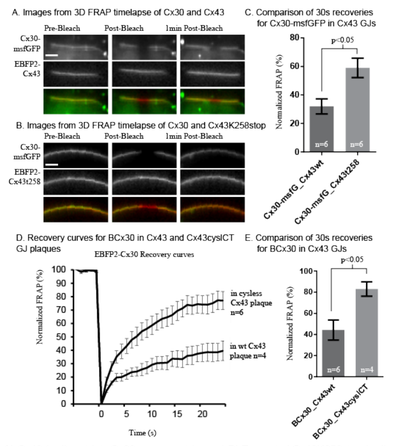


Cx47 forms stable GJs (Like the other oligodendrocyte Cx, Cx32)



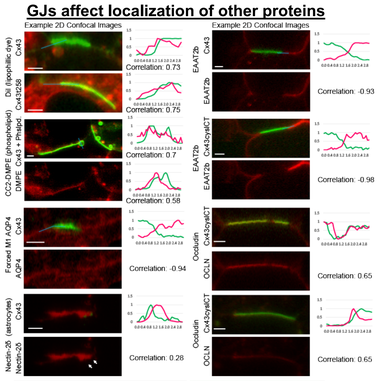
A) Reconstructions from 3D time-lapse FRAP on msfGFP-hCx47 in N2A cells. Bleach location is indicated by white arrowhead. There is no rearrangement of Cx47 1 min after Photobleach. **B)** msfGFP-Cx47 forms heterotypic GJs with EBFP2-Cx47. Homotypic and heterotypic (arrow) plaques are present. **C)** FRAP experiments indicate Cx47 has much lower mobility than Cx26. Error bars are SEM.

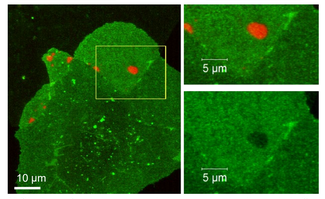
Connexin 30 (The other major astrocyte connexin)



A) Cx43 (red) restricts Cx30 movement (green) **B)** Truncated Cx43K258stop (red) permits Cx30 to undergo rapid recovery. **C)** Recovery for Cx30-msfGFP is significantly lower in stably arranged Cx43 plaques as assessed by % recovery at 30s post-bleach. **D)** Recovery curves for EBFP2-Cx30 within stably arranged (sfGFP-Cx43) and fluid (sfGFP-Cx43cysICT) GJ plaques. **E)** Percent recovery from FRAP curves in (D).

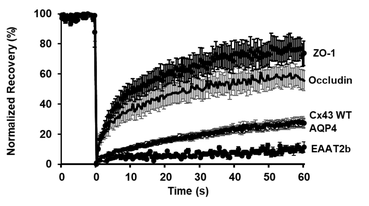
GJs affect localization of other proteins





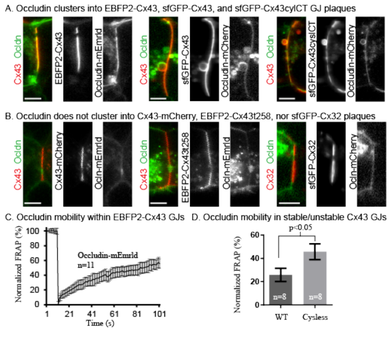
A) Single confocal plane example images showing the varied effects of Cx43 gap junctions on other proteins that are expressed, or can become expressed in some conditions, in astrocytes. Cx43 and Cx43 mutants are shown in green. The other Nexus component is shown in red. Images from N2A cells except where noted. No major differences were noted between cell types. **B)** Cultured mouse immortalized Cx43KO astrocytes expressing EBFP2-Cx43 (red dots are plaques) and EAAT2b-EGFP (green). The glutamate transporter is excluded from locations where red Cx43 GJ plaques formed.

Non-plaque Protein Mobility



FRAP experiments indicate ZO-1 and Occludin are mobile outside of the GJ plaque, wild type Cx43 and AQP4 are modestly mobility (more mobile that mobile that plaque or orthogonal arrays of particles, respectively), and EAAT2b is immobile. n = 3 for EAAT2b, n = 9 for other proteins.

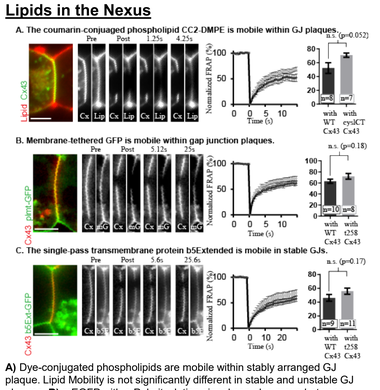
Occludin (expressed in astrocytes upon injury)



A) Occludin is localized to GJ plaques made of Cx43 with free distal C-termini. **B)** When a tag is attached to the CT of Cx43, Cx43 is truncated, or plaques are made up of Cx32 the localization pater of Occludin is reversed with it being excluded from the plaque-membrane area. **C)** Surprisingly Occludin is mobile within stably arranged GJs but is significantly more mobile in fluid GJs (D). **E)** Cartoon illustration of the authors interpretation of these data.

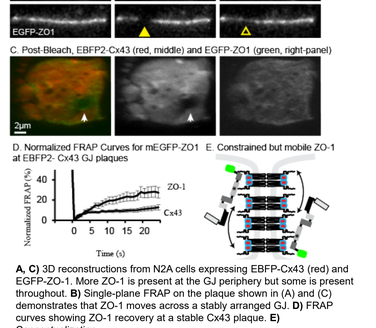
E. Green occludin moves between stably arranged Cx43

Lipids in the Nexus



A) Dye-conjugated phospholipids are mobile within stably arranged GJ plaque. Lipid Mobility is not significantly different in stable and unstable GJ plaques. **B)** mEGFP with a Palmitoylation signal can also move between stably arranged Cx43 in order to recover after photobleach. Recovery was not significantly faster in fluidly arranged GJs. **C)** The mutant plasma membrane localized b5Extended protein can also move rapidly between stably arranged Cx43 proteins. N2A cells, scale is 2 μm, T-test.

ZO-1 (TJP1, highly expressed in astrocytes)



A, C) 3D reconstructions from N2A cells expressing EBFP-Cx43 (red) and EGFP-ZO-1. More ZO-1 is present at the GJ periphery but some is present throughout. **B)** Single-plane FRAP on the plaque shown in (A) and (C) demonstrates that ZO-1 moves across a stably arranged GJ. **D)** FRAP curves showing ZO-1 recovery at a stable Cx43 plaque. **E)** Conceptualization.

References

Cone, Angela C., et al. "Protein kinase Cδ-mediated phosphorylation of Connexin43 gap junction channels causes movement within gap junctions followed by vesicle internalization and protein degradation." *Journal of Biological Chemistry* 289.13 (2014): 8781-8798.

Duffy, Heather S., Mario Delmar, and David C. Spray. "Formation of the gap junction nexus: binding partners for connexins." *Journal of Physiology-Paris* 96.3 (2002): 243-249.

Gilleron, Jérome, et al. "Molecular reorganization of Cx43, Zo-1 and Src complexes during the endocytosis of gap junction plaques in response to a non-genomic carcinogen." *J Cell Sci* 121.24 (2008): 4069-4078.

Horng, Sam, et al. "Astrocytic tight junctions control inflammatory CNS lesion pathogenesis." *The Journal of clinical investigation* 127.8 (2017): 3136-3151.

Lauf, Undine, et al. "Dynamic trafficking and delivery of connexons to the plasma membrane and accretion to gap junctions in living cells." *Proceedings of the National Academy of Sciences* 99.16 (2002): 10446-10451.

Lutz, Sarah E., et al. "Deletion of astrocyte connexins 43 and 30 leads to a dysmyelinating phenotype and hippocampal CA1 vacuolation." *Journal of Neuroscience* 29.24 (2009): 7743-7752

Miyata, M., et al. "Localization of nectin-2δ at perivascular astrocytic endfoot processes and degeneration of astrocytes and neurons in nectin-2 knockout mouse brain." *Brain research* 1649.Pt A (2016): 90.

Orthmann-Murphy, Jennifer L., et al. "Two distinct heterotypic channels mediate gap junction coupling between astrocyte and oligodendrocyte connexins." *Journal of Neuroscience* 27.51 (2007): 13949-13957.

Stout, Randy F., Erik Lee Snapp, and David C. Spray. "Connexin type and fluorescent protein fusion tag determine structural stability of gap junction plaques." *Journal of Biological Chemistry* 290.39 (2015): 23497-23514.

Stout, Randy F., and David C. Spray. "Cysteine residues in the cytoplasmic carboxy terminus of connexins dictate gap junction plaque stability." *Molecular Biology of the Cell* 28.21 (2017): 2757-2764.

Wang, Helen Yanran, et al. "Two-color fluorescent analysis of connexin 36 turnover: relationship to functional plasticity." *J Cell Sci* 128.21 (2015): 3888-3897.

Wasseff, Sameh K., and Steven S. Scherer. "Cx32 and Cx47 mediate oligodendrocyte: astrocyte and oligodendrocyte: oligodendrocyte gap junction coupling." *Neurobiology of disease* 42.3 (2011): 506-513.

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