Targeting connexins to promote functional neural repair and regeneration

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ABSTRACT

The connexins are a family of 21 proteins that represent the structural units of intercellular gap junctions and single membrane hemichannels. These channels provide a means for cells to exchange small metabolites and signaling molecules with adjacent cells and the extracellular space, respectively. Compelling evidence implicates connexins, and the more recently discovered pannexins, in the control of neural progenitor cell proliferation, survival and migration. Moreover, connexin and pannexin dysregulation following central nervous system injuries such as cerebral ischemia, spinal cord injury, and epilepsy contributes to the secondary expansion of lesions days and weeks after the initial insult. While these data suggest that connexins and pannexins represent novel therapeutic targets to both reduce the extent of neural injury and promote neural repair and regeneration, we currently lack the necessary repertoire of therapeutically useful connexin- and pannexin-specific compounds to test these hypotheses. In this thesis, I conducted targeted screening of a large, ethnobotanically-derived library to address my overarching objective of identifying compounds that selectively alter connexin and/or pannexin channel function. To accomplish this, I characterized the repertoire of connexins and pannexins expressed by neural progenitor cell-like NT2/D1 cells, quantified the intercellular flux of calcein through connexin gap junctions, and measured the uptake of lucifer yellow and propidium iodide through pannexin hemichannels. Collectively, these screens identified several promising lead compounds and ethanolic plant extracts that selectively alter connexin and pannexin channel activity.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
CBX	carbenoxolone
CNS	central nervous system
Cx	connexin
Da	dalton
DiI	1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Ham)
DMSO	dimethyl sulfoxide
EC50	median effective concentration
ER	endoplasmic reticulum
FITCD	fluorescein isothiocyanate dextran
GJIC	gap junctional intercellular communication
IP_3	inositol 1,4,5-triphosphate
kDa	kilodalton
LC50	median lethal concentration
LogP	octanol/water partition coefficient
LY	lucifer yellow
MCAO	middle cerebral artery occlusion
NMDA	*N*-methyl-D-aspartate
Panx	pannexin
PI	propidium iodide
RD	rhodamine B isothiocyanate dextran
RT-PCR	reverse transcription polymerase chain reaction
SGZ	subgranular zone
shRNA	short hairpin RNA
siRNA	small interfering RNA
SVZ	subventricular zone

LIST OF FIGURES

LIST OF TABLES

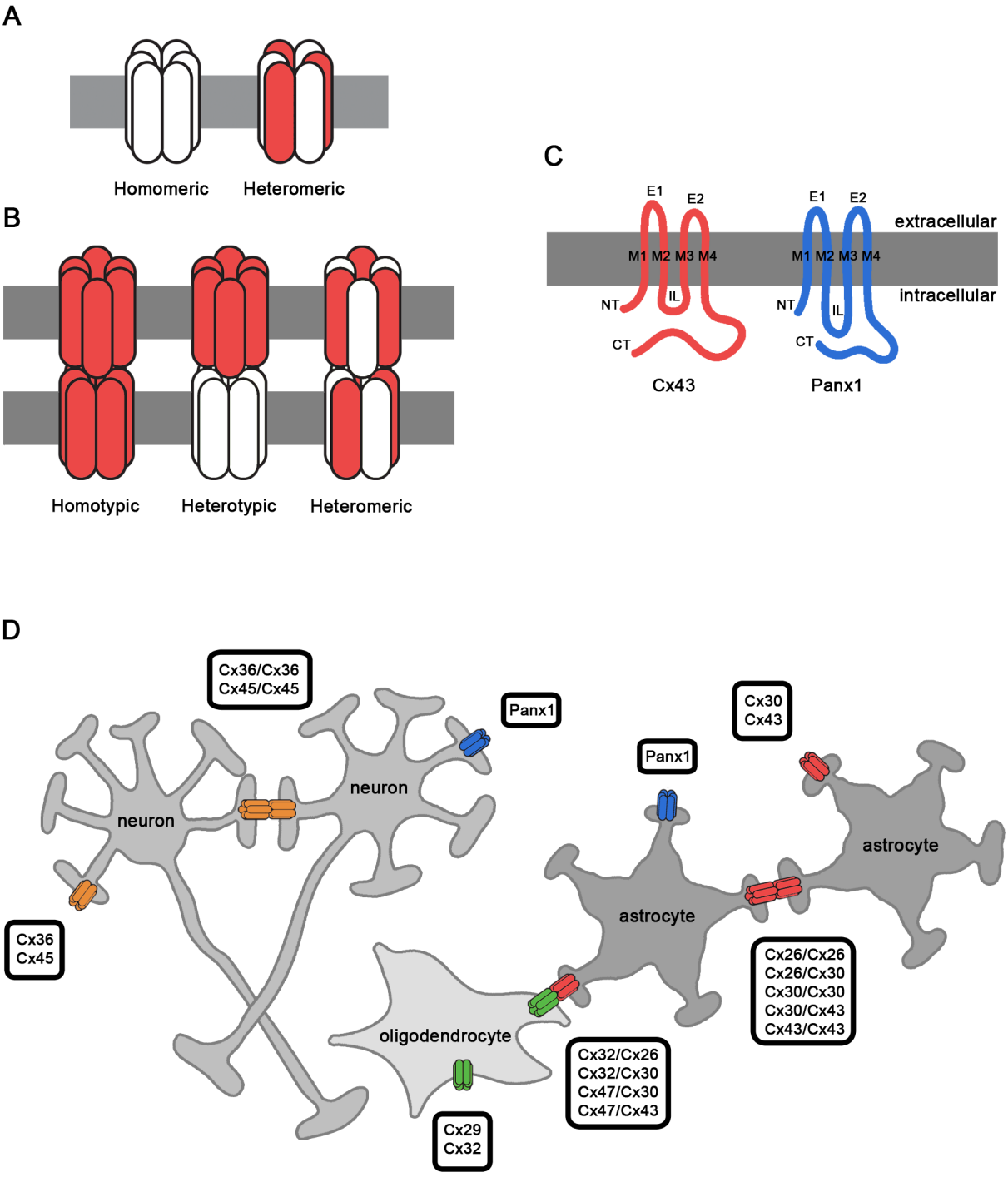
1 INTRODUCTION

1.1 The connexin family of protein channels

The connexins are a family of membrane proteins that range in size from 26 to 62 kilodaltons (kDa), each named using the prefix ‘Cx’ followed by the predicted molecular weight (196). In humans, 21 different connexins are expressed. Six connexins hexamerize into a single-membrane channel termed a connexon or hemichannel (Figure 1A). The docking of two connexons inserted into apposing membranes forms an aqueous, intercellular gap junction channel that enables adjacent cells to directly exchange ions and small metabolites up to approximately 1.5 kDa in mass (Figure 1B) (206). Typically, tens to thousands of these channels are located in close proximity, morphologically defining a gap junction plaque (85). Gap junctions and hemichannels composed of different connexins have different physiological properties (157). Tissue- and cell type-specific connexin expression determine which cell types are capable of functional intercellular communication (245). Importantly, cell to cell communication mediated by connexin channels (i.e., gap junctional intercellular communication, GJIC) underlies tissue homeostasis and enables coordinated cellular activities (84).

The large number of connexin family members suggests that a staggering number of connexons and gap junction channel combinations are possible. In reality, only a subset of these combinations is permitted. Connexons formed by connexins of a single type are referred to as homomeric (Figure 1A), while gap junctions formed by

Figure 1. Connexin and pannexin channel structure and expression in the central nervous system. (A) Six connexins oligomerize to form a single membrane channel termed a connexon, or hemichannel. Hemichannels composed of one or multiple connexin types are referred to as homomeric or heteromeric, respectively. When inserted into non-junctional membranes, connexin hemichannels allow for passage of small metabolites between cells and the extracellular space. (B) Hemichannels inserted into the plasma membrane of neighbouring cells may dock to form an intercellular channel termed a gap junction. Gap junctions are characterized as homotypic, heterotypic, or heteromeric depending on their composition. Homotypic gap junctions are formed by two identical homomeric hemichannels. Heterotypic gap junctions are formed by two different homomeric hemichannels. Heteromeric gap junctions are formed when a heteromeric hemichannel docks with either a second heteromeric hemichannel or with a homomeric hemichannel. (C) A typical connexin in monomeric form (i.e. Cx43, red) has intracellular amino and carboxyl termini domains (NT, CT), four transmembrane domains (M1-M4), one intracellular loop domain (IL), and two extracellular loop domains (E1, E2). Despite the lack of an evolutionary relationship between the connexin and pannexin families, the membrane topology of a typical pannexin in monomeric form (i.e. Panx1, blue) is strikingly similar to that of a connexin. Topology schematic adapted from (14). (D) Cell-specific connexin and pannexin expression determines which cell types are capable of functional intercellular and single membrane communication. The glial-neuronal nexus is illustrated here.


A
Homomeric
Heteromeric
B
Homotypic
Heterotypic
Heteromeric
C
E1
E2
M1 M2 M3 M4
NT
IL
CT
extracellular
intracellular
Cx43
Panx1
D
Cx36/Cx36
Cx45/Cx45
Panx1
Cx30
Cx43
neuron
neuron
Panx1
astrocyte
astrocyte
Cx36
Cx45
Cx26/Cx26
Cx26/Cx30
Cx30/Cx30
Cx30/Cx43
Cx43/Cx43
oligodendrocyte
Cx32/Cx26
Cx32/Cx30
Cx47/Cx30
Cx47/Cx43
Cx29
Cx32

identical homomeric connexons are referred to as homotypic (Figure 1B) (85). When two homomeric connexons composed of different connexins dock, they form a heterotypic gap junction (Figure 1B). Certain compatible combinations of connexins may also assemble into heteromeric connexons in which each single membrane channel is composed of multiple connexins (Figure 1B). Docking of a homotypic connexin with a heteromeric connexin or docking of two heteromeric connexons forms a heteromeric channel (Figure 1B).

1.1.1 Connexin, hemichannel and gap junction channel structure

Each connexin retains the same basic membrane topology with intracellular amino and carboxyl termini, four transmembrane domains, one cytoplasmic loop and two extracellular loops (Figure 1C) (85). The N-terminus, transmembrane domains and extracellular loops are well-conserved between family members (84). However, there is considerable variation in the cytoplasmic loop and C-terminus, accounting for the molecular weight differences between family members. The four transmembrane domains possess α-helical secondary structure (90). Further, six highly conserved cysteine residues form disulfide linkages between the extracellular loops within a connexin and stabilize these domains in a stacked, antiparallel β-sheet configuration (84, 106).

Connexin hemichannels (connexons) have a diameter of approximately 70 Å and protrude 15-20 Å into the extracellular space and at least 15-20 Å into the intracellular space depending on the length of the C-terminus (90). When a connexin

hemichannel docks with a hemichannel in an apposing membrane, each hexamer is rotationally staggered 30° from its partner (225). Each hemichannel in a gap junction spans a transmembrane region of approximately 40 Å and creates a characteristic gap between its own and the apposing membrane of 20-30 Å (90). The pore size of Cx26, of which the highest resolution crystal structure is available, is 14 Å in diameter (132). Within a gap junction, the first extracellular loops plays a role in channel selectivity while the second extracellular loop is important in determining heterotypic gap junction compatibility (90, 221). Within heterotypic and heteromeric channels, the limiting pore size and junctional conductance is generally determined by the most restrictive of the component connexons (35, 139, 157, 180).

1.1.2 Life cycle of connexins

The biosynthesis of a connexin commences with its translation to protein by ribosomes located on the rough endoplasmic reticulum (ER) membrane (116). The ER membrane is also the site at which connexins establish their final tetraspan topology with cytosolic amino and carboxyl termini. Depending on the particular connexin(s) being assembled, oligomerization into homomeric and heteromeric hemichannels occurs somewhere along the continuum between the ER and the trans-Golgi network (2, 52, 58, 68, 150). For example, Cx26 oligomerizes into homotypic connexons in the ER, while homotypic channels composed of Cx32 and Cx43 are assembled later in the ER-Golgi intermediate compartment and trans-Golgi network, respectively (58, 150). Following oligomerization, most hemichannels are trafficked to the plasma membrane from the Golgi apparatus in vesicles along

microtubules (120). An exception to this rule is Cx26, which has been shown to be capable of transferring directly from the ER to the plasma membrane independent of both the Golgi apparatus and microtubules (137).

Newly assembled hemichannels are distributed evenly throughout the plasma membrane and are can remain as non-junctional entities (i.e., single membrane channels) (192). Alternatively, they can diffuse laterally to the perimeters of gap junctional plaques located at cell-to-cell junctions. Here, they can dock with a hemichannel on a neighbouring cell and create an intercellular gap junction channel. The docking of hemichannels and the maintenance of gap junctional plaque stability are accomplished with the assistance of a network of associated proteins, including ZO-1, cadherins and occludin, that function in cell adhesion and structural support (79, 109, 145).

In comparison to most integral membrane proteins, connexins have relatively short half-lives of under six hours, suggestive of a capacity for rapid regulation in response to physiological changes (17, 51, 69, 117, 219). Several mechanisms by which connexin disassembly takes place have been described to date. The most well-characterized process begins with the internalization of large sections of whole gap junctions from the centre of plaques in double membrane vesicles, termed ‘annular junctions’ or ‘connexosomes’ (116). Additionally, intact gap junctions and non-junctional hemichannels can also be internalized via clathrin-mediated endocytosis (208). Regardless of which pathway is taken, connexins removed from the plasma membrane are primarily destined for disassembly in lysosomes,

although proteasomes also contribute in some cases (115, 122, 177). It is thought that the proteasomal pathway is responsible for the disassembly of misfolded connexins that have been discarded by the ER whereas biologically functional connexins are degraded in the lysosome (116).

Connexins undergo various post-translational modifications depending on the connexin isoform and the tissue in which they are expressed. For example, all connexins except Cx26 are phosphoproteins, and phosphorylation of connexins has diverse effects on channel gating, protein trafficking and channel stability depending on the residue that is modified (118, 119). There is also evidence of connexin ubiquitination, with monoubiquitination serving as a signal for channel internalization and polyubiquitination signaling proteasomal degradation (116). Although several connexins contain consensus sequences for N-glycosylation, it does not appear that connexins are glycosylated *in vivo*, although inhibition of protein glycosylation has, indirectly, been shown to alter Cx43 trafficking and channel function (108, 138).

1.1.3 Endogenous regulators of connexin gating

Connexin channels are subject to gating by several endogenous factors. Early experiments demonstrated that the electrical resistance of gap junctions could be regulated by transjunctional voltage. In these experiments, pairs of coupled amphibian blastomeres remained electrically coupled when small current pulses were applied to either cell, but rapidly uncoupled when pulses reached a sufficiently high level (198, 200). Voltage gating of connexins is now understood to be mediated

by fast and slow gate components (31). Fast gating is regulated by a sensor in the N-terminus that plugs the channel pore and brings about an intermediate state characterized by reduced channel permeability (132, 231). The structural underpinnings of the slow gate have been proposed to be located towards the extracellular domain of a hemichannel (174). Interestingly, this mechanism may function to preserve electrical coupling between cells but restrict passage of certain metabolites (31).

Gap junctions and hemichannels are reversibly closed by reductions in intracellular pH (101, 199, 220, 224). The proposed mechanism by which this occurs is the direct protonation of residues on the C-terminus and possibly cytoplasmic loop, leading to a conformational change in the connexon and channel closure (90). Given that these two domains represent the most variable sequences among the connexin family (84), this may explain the differences in pH sensitivity between different isoforms. Since gap junctions in healthy cells are unaffected by normal physiological pH changes, the sensitivity of connexins to low pH may represent a mechanism to abolish communication between healthy and dying cells (31).

Non-junctional connexin hemichannels are gated by millimolar concentrations of extracellular calcium (111, 125, 173). Using atomic force microscopy, calcium was shown to induce reversible conformational changes in the Cx26 hemichannel, reducing the diameter of the extracellular pore from 15 to 6 Å (149). Interestingly, this effect was specific to calcium, as magnesium had no effect, suggesting specific binding of calcium to an extracellular domain on the connexin molecule. The gating

of hemichannels by physiological calcium concentrations is thought to be crucial in order to prevent the efflux of biologically important molecules and the depletion of ionic gradients (202).

1.1.4 Channel functions

The most well-studied function of the connexins is their capacity to form a conduit for molecular signals to pass between cells. Intercellular exchange of ions also underlies connexin control of the “electrical synapse.” Although gap junctions have been classically regarded as large non-selective pores, more detailed studies have revealed that connexin channels demonstrate complex patterns of selectivity involving mass, charge and spatial orientation (157). This selectivity enables close regulation of the passage of soluble second messengers, amino acids, nucleotides and ions through gap junctions composed of different connexins (184).

Connexins have important junctional roles in the central and peripheral nervous system. Cx36 and Cx45 are expressed by neurons, in which they form electrical synapses that facilitate synchronous electrical activity within neuronal networks (Figure 1D) (57, 141). Astrocytic Cx26, Cx30 and Cx43 couple with each other as well as oligodendrocytic Cx32 and Cx47 to form the glial syncytium, a vast network that helps to maintains neural homeostasis in part through nutrient transport, the spatial buffering of extracellular potassium ions and glutamate uptake (Figure 1D) (215). In myelinating Schwann cells of the peripheral nervous system, Cx32 also forms reflexive gap junctions between layers of wrapped myelin, which expedite the

intracellular redistribution of potassium ions released during the propagation of action potentials (215).

Another important role of gap junctions lies in mediating the transmission of calcium waves between astrocytes. Astrocytes coordinate their function and respond to neuronal activity through complex signaling mechanisms initiated by elevations in intracellular calcium levels (50). One pathway by which these signals can be transmitted to neighbouring cells is via the passage of calcium and inositol 1,4,5-triphosphate (IP_3) through gap junctions (190). These second messengers then bring about calcium-dependent cellular events by modulating enzyme activity, gene transcription and gliotransmitter release (191).

The junctional functions of connexins are also recognized to play important roles in nearly all non-nervous tissues in the body. In the cardiovascular system, Cx40, Cx43 and Cx45 are differentially expressed in the intercalated discs of cardiomyocytes and facilitate their electrical coupling and the propagation of the cardiac action potential (55). In the liver, Cx26 and Cx32 gap junctions extensively couple hepatocytes (227). These channels provide a low resistance pathway for calcium wave propagation and enable rapid mobilization of glucose in response to sympathetic stimulation (185). Cx26 is also expressed, along with Cx30, in the inner ear and helps to maintain potassium concentrations in the endolymph (179). Mutations that cause a loss of Cx26 or Cx30 channel function are among the most common causes of nonsyndromic deafness (196). In the female reproductive system, Cx37 gap junctions are the primary means by which the somatic cells of the

follicle direct the growth of the oocyte, and the loss of Cx37 results in female infertility (194). Lens fiber cells express Cx46 and Cx50 (166, 242). In the post-natal lens, Cx46 and Cx50 gap junctions play a crucial role in maintaining homeostasis and lens transparency, since mice deficient in either of these connexins develop cataracts shortly after birth (185).

Connexins also have non-junctional functions as single membrane hemichannels. Generally speaking, hemichannels have low open channel probability, are closed under normal physiological conditions and may be viewed as precursors to intercellular channels (61). However, hemichannels open to the extracellular space in response to certain signals, such as membrane depolarization, metabolic inhibition and extracellular calcium depletion (61, 105). Under these conditions, connexin hemichannels can pass gap junction-permeable metabolites, including adenosine triphosphate (ATP) and glutamate (217). It has been proposed that astrocytic Cx43 hemichannels mediate an extracellular, ATP-dependent pathway for calcium wave propagation under normal physiological conditions, however this finding has come into question recently with evidence that this event also occurs in the absence of Cx43 (209).

1.2 Pannexins are evolutionarily distinct but functionally analogous to connexins

Within the last decade, a family of connexin-like proteins termed the pannexins (Panxs) was identified as the mammalian homologues to the invertebrate gap

junction family, the innexins (13). Three members of this family are found in the human genome: Panx1, Panx2 and Panx3 (13). Panx1 (molecular weight 46.7 kDa) is expressed in multiple tissues throughout the body, while Panx2 (molecular weight 69.5 kDa) expression is predominantly (although not exclusively) found in the central nervous system (CNS) (13). Panx3 (molecular weight 44.7 kDa) expression is restricted to skin and osteoblasts (169). Pannexins appear to be relatively long-lived in comparison to connexins, although only qualitative measurements have been published to date (169).

Although pannexins and connexins evolved independently to each other, their basic topology has striking similarities. Like connexins, pannexins are tetraspan proteins with intracellular amino and carboxyl termini (Figure 1C) (197). In the extracellular loops, pannexins have four conserved cysteine residues that are postulated to form intramolecular disulfide bonds analogous to those between the extracellular loops of a connexin (13). Panx1 oligomerizes into a hexameric channel, while Panx2 forms an octamer (5). The oligomeric structure for Panx3 is not definitively known but is predicted to be hexameric based on its sequence similarity to Panx1 (22).

Unlike connexins, pannexins are glycoproteins: Panx1 is N-glycosylated at asparagine 254 of the second extracellular loop, while Panx2 and Panx3 are N-glycosylated on the first extracellular loop at asparagine 86 and asparagine 71, respectively (171). Experiments with site-directed mutagenesis of Panx1 and Panx3 indicate that their N-glycosylation functions primarily in channel trafficking to the plasma membrane (169, 170). Whether other post-translational modifications play

an important role in the life cycle of pannexins is an area of research that requires further development.

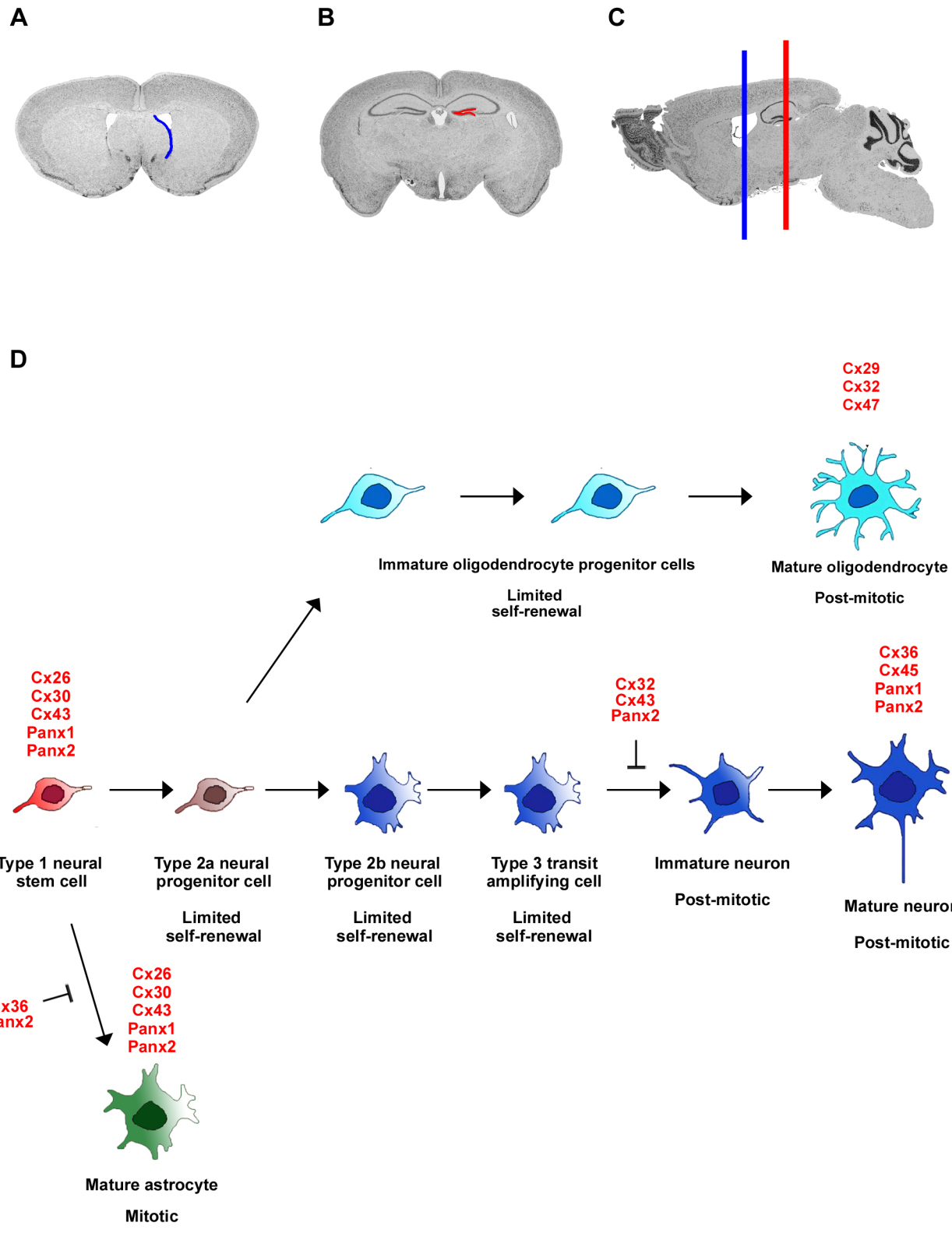
Pannexins are capable of forming functional channels when heterologously expressed in *Xenopus* oocytes (30). In unpaired oocytes, Panx1 forms homomeric hemichannels and heteromeric hemichannels in tandem with Panx2, however these heteromeric channels are very unstable. In paired oocytes, homotypic Panx1 and heterotypic Panx1/Panx2 gap junctions were also observed. Despite the demonstration that pannexins can form intercellular channels *in vitro*, there is no evidence that they form gap junctions under normal conditions *in vivo* in mammals (171). To date, physiological evidence for functional single membrane channel formation has been provided for only Panx1 and Panx2 (5, 30). Like connexin hemichannels, pannexin hemichannels are large, single membrane pores that are permeable to ions, second messengers and fluorescent dyes up to approximately 1.5 kDa in size (49, 236). To date, Panx1 has received the most attention in the pannexin field, and research has shed light on a number of its paracrine functions (171). One of these functions is as a mediator for calcium wave propagation. Panx1 hemichannels are permeable to ATP and are activated by mechanical stimulation and elevations in intracellular calcium (12, 131). Extracellular ATP binds to purinergic P2X and P2Y receptors and induces calcium release from the ER via the liberation of IP_3. This increase in intracellular calcium then induces activation of Panx1, ATP release and the propagation of the calcium wave (14).

1.3 Connexins and pannexins regulate post-natal neurogenesis and gliogenesis

It was previously thought that the regenerative capacity of the CNS was limited and that little could be done to restore the function lost as a result of neuronal death during stroke, spinal cord injury and neurodegenerative disease. However, the discovery of populations of neural progenitor cells in distinct regions of the adult brain and spinal cord has shown that functional neural regeneration is indeed possible following injury with potential for therapeutic enhancement (65). Three such niches have been identified in the adult CNS: the subventricular zone (SVZ) of the lateral ventricles (Figure 2A), the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Figure 2B) and the ependymal zone of the central canal in the spinal cord (74, 95). Under normal conditions, newborn neurons generated in the SVZ migrate to the olfactory bulb and play a role in olfactory discrimination (4). In the hippocampus, newborn neurons are integrated into the granule cell layer of the dentate gyrus and may function in learning and memory (250). The nascent progenitors of the spinal cord predominantly produce glia (95), though there is evidence that they are capable of producing neurons if removed from their niche or provided with the appropriate biochemical cues (193).

In addition to having well-documented roles in CNS development (64), there is substantial evidence that connexins have important functions in postnatal neural progenitor cells as well (Figure 2D). Our lab has shown that Cx26, Cx29, Cx30, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45 and Cx47 are differentially expressed in the

Figure 2. Connexins and pannexins regulate post-natal neurogenesis and gliogenesis. In the adult brain, there are two niches where neurogenesis occurs: (A) the subventricular zone of the lateral ventricles (blue) and (B) the subgranular zone of the hippocampal dentate gyrus (red). (C) Sagittal section indicating the location of the coronal sections shown in (A) and (B). (D) Newborn neurons and glia in the adult hippocampus originate from a population of radial glia-like cells termed Type 1 cells. Type 1 cells express Cx26, Cx30, Cx43, Panx1, and Panx2 and are capable of self-renewal or differentiation along astrocytic, neuronal and oligodendrocytic lineages. Cx36 inhibits the differentiation of Type 1 cells towards an astrocytic lineage. Cx30, Cx43, and Panx2 positively regulate Type 1 cell proliferation. The Type 2a progeny of Type 1 cells may further specify into neuronally-committed Type 2b cells or may differentiate along an oligodendrocytic lineage. Type 2b cells further specify into Type 3 transit amplifying cells before permanently exiting the cell cycle and permanently differentiate into post-mitotic neurons. Cx32, Cx43, and Panx2 inhibit neuronal commitment of Type 3 transit amplifying cells.


A
B
C
D
Cx29
Cx32
Cx47
Immature oligodendrocyte progenitor cells
Limited
self-renewal
Mature oligodendrocyte
Post-mitotic
Cx26
Cx30
Cx43
Panx1
Panx2
Cx32
Cx43
Panx2
Cx36
Cx45
Panx1
Panx2
Type 1 neural
stem cell
Type 2a neural
progenitor cell
Limited
self-renewal
Type 2b neural
progenitor cell
Limited
self-renewal
Type 3 transit
amplifying cell
Limited
self-renewal
Immature neuron
Post-mitotic
Mature neuron
Post-mitotic
Cx36
Panx2
Cx26
Cx30
Cx43
Panx1
Panx2
Mature astrocyte
Mitotic

SGZ over the course of specification (100). Here, Cx30 and Cx43 biochemically couple radial glia-like progenitors and act as positive regulators of proliferation (114). Similar observations regarding Cx43 have been made in the SVZ (130) and central canal (183), suggesting that there are some fundamental similarities between the progenitors in these niches. Recently, Cx45 hemichannel activity has been shown to be important for transit amplifying cell proliferation in the SVZ (112). Changes in connexin expression are also associated with neural progenitor cell fate decisions. Using a single knockout strategy, Cx43 was shown to negatively regulate the rate of neurogenesis (188). Conversely, Cx36 expression is correlated with increased rates of neurogenesis and oligodendrogenesis, whereas loss of Cx36 function is sufficient to decrease neurogenesis and increase astrogenesis (92).

The functions of pannexins in neural progenitor cells are increasingly being appreciated. The expression, post-translational modification and cellular localization of Panx2 are dynamically regulated over the course of postnatal hippocampal neurogenesis (212). Further, knockdown of Panx2 expression *in vitro* significantly increases the rate of neurogenesis. Panx1 expression is found in SVZ progenitors, where it positively regulates proliferation (243). Therefore, Panx1 and Panx2 may play antagonistic roles in regulating postnatal progenitor proliferation and neuronal commitment.

In rats, the rate of neurogenesis is acutely accelerated in both the SGZ and SVZ following middle cerebral artery occlusion (MCAO) (104). In the spinal cord, the proliferation of progenitor cells lining the central canal also increases markedly

following injury (148). These may represent endogenous compensatory changes to repair damaged tissue, however the vast majority of these cells do not survive and are unable to functionally integrate into the existing neural circuitry (175). The role of connexins and pannexins in regulating neural progenitor cell proliferation and differentiation suggests that they represent potentially valuable cellular targets to promote the sustained proliferation, survival and integration of newborn neurons to improve functional outcome following injury.

1.4 Connexins and pannexins are implicated in the propagation of neural injury

Given the importance of connexins and pannexins in maintaining tissue homeostasis, it is unsurprising that they also play determinative roles following tissue dysfunction. It is well-documented that connexin expression is spatially and temporally regulated following stroke, spinal cord injury and epilepsy (152). However the functional significance of these changes is subject to considerable debate, largely owing to the contradictory conclusions drawn from studies relying on non-specific reagents to inhibit GJIC and the relatively recent discovery of pannexins in the last decade (172). This has led to an appreciation of the complexity involved and the recognition that whether communication mediated by connexin and pannexin channels is beneficial or detrimental is highly dependent on the context and the channel-forming protein in question.

For example, mice with Cx43 conditionally deleted in astrocytes suffer larger infarcts and increased neuronal death following MCAO than wild type mice, suggesting that Cx43 is neuroprotective in this context (153). In contrast, the loss of Cx43 reduces inflammation and lesion size and improves motor recovery following traumatic spinal cord injury (97). The precise mechanisms underlying each of these effects remain controversial, however several have been described. First, astrocytic gap junctions remain open during injury and can mediate the spread of neurotoxic metabolites through a tissue, resulting in an increase in bystander cell death (43, 128). Second, Cx43 hemichannels can open during metabolic inhibition and contribute to cell death by destroying ionic and metabolic gradients (42). Third, and in contrast to the first two mechanisms discussed, Cx43 can exert protective effects through the uptake and spatial buffering of potassium ions and glutamate (7) or through channel-independent mechanisms (129). In actuality, the balance of these events in a particular injury likely dictates whether Cx43 is helpful or harmful.

Additional connexins have also been implicated in neuronal survival decisions following injury. Mice lacking Cx32 are more susceptible to neuronal death in the CA1 region of the hippocampus following global ischemia (160). Since oligodendrocytes mediate potassium clearance from the periaxonal space and Cx32 is capable of forming heterotypic gap junctions with astrocytic Cx26 and Cx30 (48, 241), it is possible that loss of Cx32 renders mice impaired in the spatial buffering of the excess potassium ions released during neuronal injury through the glial syncytium (161), leading to increased neuronal death. Conversely, a reduction in neuronal coupling through Cx36 gap junctions is associated with increased neuronal

survival (16, 19, 238, 239). Mechanistically, this is thought to be beneficial by preventing the spread through Cx36 gap junctions of apoptosis-promoting second messengers, such as calcium ions, potassium ions or IP_3 (18).

Recently, pannexins have been recognized as important therapeutic targets in excitotoxic injury. The first study implicating pannexins demonstrated that oxygen/glucose deprivation *in vitro* induced the opening of neuronal channels with electrophysiological characteristics resembling Panx1 (218). Later, it was shown that Panx1 hemichannels open in response to *N*-methyl-D-aspartate (NMDA) receptor activation and compound neuronal dysfunction (216). Since these publications, converging evidence has come from the use of Panx1 and Panx2 single and double knockouts (15). Here, Panx1 and Panx2 hemichannel activity was detected in neurons but not astrocytes *in vitro* in response to metabolic inhibition. Further, double knockouts developed smaller infarcts and sustained less motor impairment than single knockouts and wild type mice. These results suggest that activation of both Panx1 and Panx2 in neurons contributes to ischemic brain damage.

A common limitation to these studies using null-mutant models is an inability to control for compensatory changes in gene expression resulting from the knockout of the gene(s) in question. This is a valid concern, given that examples of functional compensation have been demonstrated within both the connexin and pannexin families (15, 97). Additionally, it is currently not possible to pharmacologically discriminate between connexin gap junctions and connexin hemichannels in a connexin-specific manner. New reagents capable of selectively modulating

connexins are necessary to validate the observations published to date in null-mutant models and to move this strategy towards pre-clinical intervention.

1.5 The reagents that are currently available are insufficient to manipulate the function of connexins or pannexins in a therapeutic context

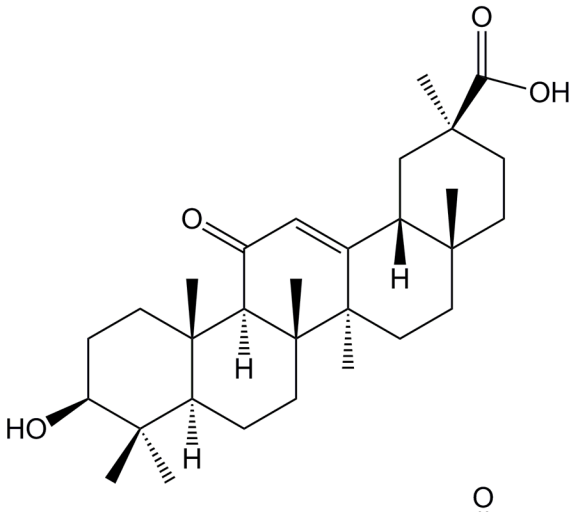
In order to target connexins to promote neural repair and regeneration following CNS injury, we require reagents that are able to discriminate between the various types of connexins and pannexins, are non-toxic at effective concentrations *in vivo* and are able to pass the blood-brain barrier. Although many of the compounds that are commonly used in research to elucidate connexin and pannexin function meet some of these criteria, most reagents are notoriously non-selective and are often too toxic for therapeutic use due to the high doses that are required to exert appreciable effects on connexins or pannexins *in vivo* (93, 201). For this reason, the identification of novel drugs that improve upon existing compounds is an area of great interest in the connexin and pannexin fields. The pharmaceutical repertoire of reagents used to manipulate connexin and pannexin channel function and expression and their prospects for therapeutic use is reviewed below.

1.5.1 Glycyrrhetinic acid

Glycyrrhetinic acid is a pentacyclic triterpenoid saponin isolated from the root of licorice (*Glycyrrhiza*) (Figure 3). This compound and its derivatives possess anti-inflammatory, antiviral and antitumour effects and have been used for the treatment

Figure 3. Pentacyclic triterpenoid inhibitors of connexin and pannexin channels. (A) Glycyrrhetinic acid. (B) Carbenoxolone. (C) Glycyrrhizic acid.

A



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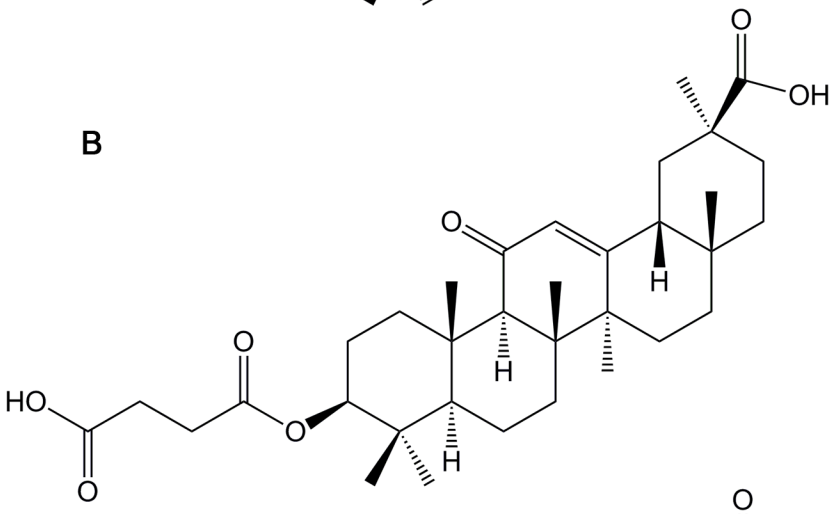

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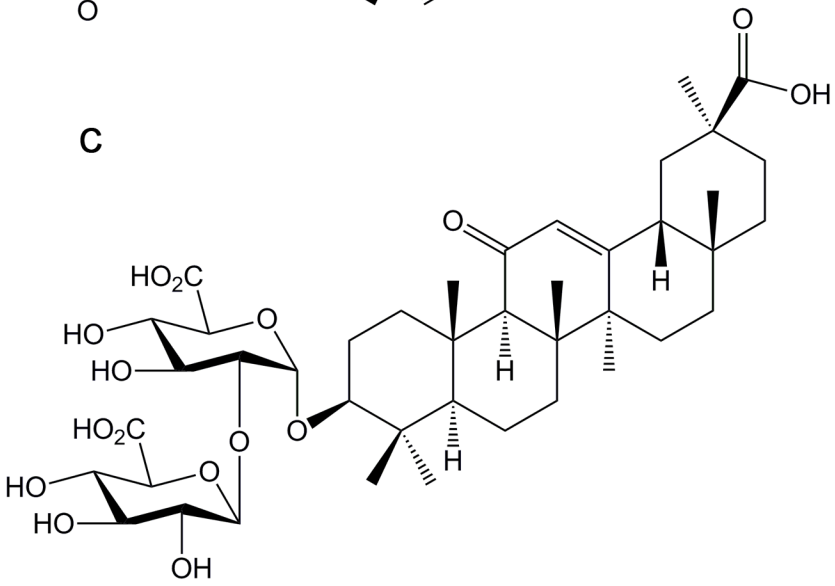


of peptic ulcer disease and dermatological problems (10). The primary mechanism by which glycyrrhetinic acid acts is though the inhibition of 11β-hydroxysteroid hydroxygenase, leading to an accumulation of anti-inflammatory glucocorticoids. If licorice is consumed in large enough quantities, glycyrrhetinic acid can cause an overactivation of renal mineralocorticoid receptors that results in hypertension, visual problems and headache (10).

The first example of an effect by glycyrrhetinic acid on GJIC was in human fibroblasts, where 18-α-glycyrrhetinic acid, 18-β-glycyrrhetinic acid and their derivative carbenoxolone (CBX) strongly and reversibly inhibited metabolic coupling with minimal toxicity at effective concentrations (54). Since this early study, these compounds have been regularly used to non-selectively block both connexin gap junctions and hemichannels in a wide range of cell types *in vitro* (93). Interestingly, CBX also inhibits pannexin hemichannels and does so at concentrations an order of magnitude below those required to significantly alter connexin function, thus enabling pharmacological distinction between connexin and pannexin hemichannels (29).

The mechanism by which glycyrrhetinic acid and its derivatives inhibit connexin and pannexin channel activity has not been definitively proven. The most-cited hypothesis is that these compounds insert themselves into the plasma membrane and induce a conformational change that closes the channel (53). They may also exert a biphasic effect, as prolonged treatment (1-4 hours) induce a change in Cx43 phosphorylation and eventual disassembly of gap junctional plaques (88).

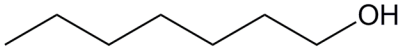
Unfortunately, these compounds do not act selectively on individual connexins at their effective concentrations and a number of other off-targets exist within the CNS, limiting their therapeutic potential (39, 232). In some cases, this has complicated the interpretation of experimental findings when CBX or glycyrrhetinic acid have been employed to elucidate connexin function, necessitating the use of the inactive analog glycyrrhizic acid to control for nonspecific effects (201). Further, CBX likely does not cross the blood-brain barrier following systemic administration, therefore more invasive methods of delivery would be necessary to exert CNS effects (123).

1.5.2 Alkanols and volatile anesthetics

The effect of anesthetics (Figure 4) on electrical synapses was first identified in 1980 (107). This study found that extracellular application of heptanol and octanol blocked the electrical propagation of action potentials between coupled crayfish giant axons at millimolar concentrations. Intracellular application of octanol at ten-fold higher concentrations had no effect, suggesting its binding site is only accessible from the extracellular space. In 1989, Burt and Spray showed that similar concentrations of halothane and ethrane also uncouple neonatal cardiomyocytes (34). In addition to these junctional effects, later studies have confirmed that alkanols and volatile anesthetics inhibit connexin hemichannels as well, although heptanol has been shown to have no effect on Panx1 conductance (66, 168, 214, 247). Some of these reports provide examples of selectivity between connexin subtypes, however detailed studies are lacking that look at a substantial repertoire of connexins.

Figure 4. Examples of alkanols and volatile anesthetics known to modulate connexin gap junction and hemichannel function. (A) Heptanol. (B) Octanol. (C) Halothane. (D) Ethrane.

A



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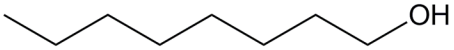

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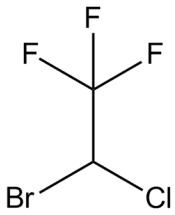
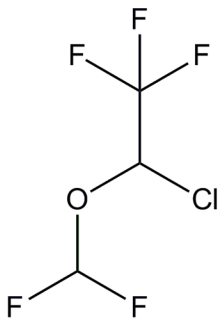


D



The mechanism by which anesthetics uncouple gap junctions is very rapid and reversible and occurs by reducing the time in which the channel is open rather than reducing its unitary conductance (33, 34, 214). They are generally understood to elicit these changes by disrupting the fluidity of the local membrane surrounding the channel. This type of non-specific effect seems more likely than a direct interaction given that anesthetics alter the function of numerous other membrane channel as well (154). This, along with the fact that their effects on connexins occur at concentrations used for inhalation anesthesia (201), makes them unsuitable for the specific targeting of connexins *in vivo*.

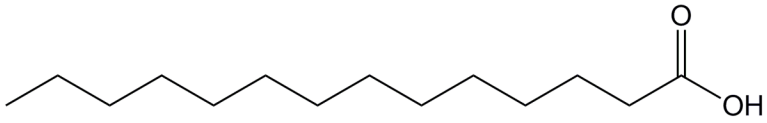
1.5.3 Fatty acids

Various unsaturated and saturated fatty acids disrupt gap junctional coupling at micromolar concentrations (Figure 5). As a general trend, the optimal chain length for inhibition increases with the degree of unsaturation. Among unsaturated fatty acids, lauric acid (12:0) and myristic acid (14:0) are the most potent, while palmitoleic acid (16:1, n-9) and oleic acid (18:1, n-9) represent the strongest monounsaturated uncouplers (32). A number of polyunsaturated fatty acids with chain lengths between 18 and 22 carbons have also been tested and shown to be effective, including γ-linolenic acid (18:3, n-6), arachidonic acid (20:4, n-6), eicosapentaenoic acid (20:5, n-3) and docosahexaenoic acid (22:6, n-3) (94).

The fact that free fatty acids and a number of their metabolites, including anandamide (230), oleamide (87) and thromboxane A_2 (9), are able to modulate gap

Figure 5. Fatty acid and fatty acid metabolite uncouplers of gap junctional intercellular communication. (A) Myristic acid. (B) Palmitoleic acid. (C) Arachidonic acid. (D) Anandamide. (E) Oleamide.

A



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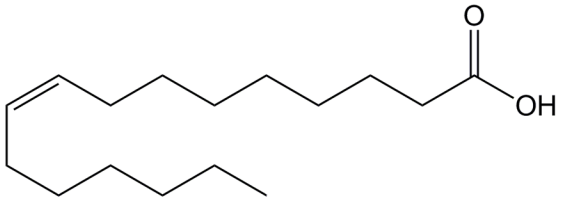

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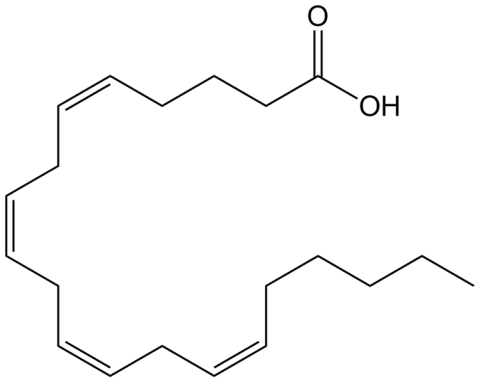

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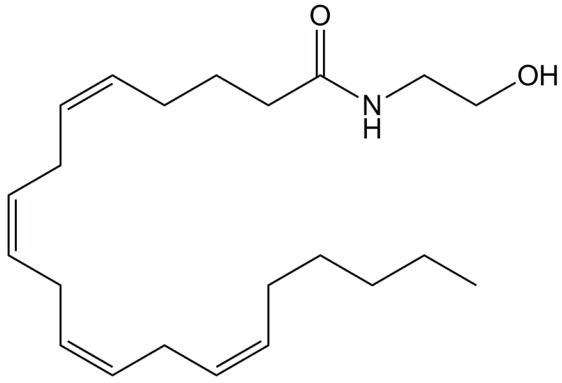

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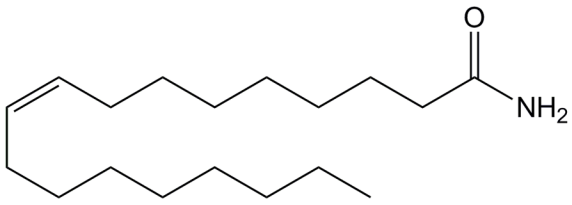


junction function has important biological ramifications, as these metabolites may represent elegant endogenous mechanisms to rapidly regulate intercellular coupling directly at the membrane (140). While the potential relationship between lipid metabolism and connexin function is enticing, evidence that targeting this interaction is a viable therapeutic strategy has not been demonstrated.

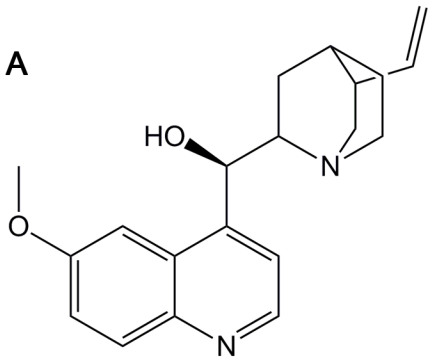
1.5.4 Quinine

Quinine (Figure 6) is an alkaloid that was first isolated from the bark of the cinchona tree, native to South America. Globally, it has been the most important anti-malarial drug for nearly 400 years and to this day is still the drug of choice in many parts of the world where most costly alternatives are not widely available (1). A relatively recent finding in the history of quinine is that this compound reversibly inhibits connexin channels (203).

N2a neuroblastoma cells transfected with plasmids encoding the transcripts of various connexins were used to show that quinine is remarkably selective for gap junctions formed by Cx36 (EC50 = 32 μM) in comparison to Cx50 (EC50 = 73 μM), while having little to no effect on Cx26, Cx32, Cx40, Cx43 or Cx45 gap junctions at concentrations as high as 300 μM (203). Similar effects were also seen when non-junctional hemichannels were studied. A later study demonstrated that the synthetic derivative mefloquine is even more potent than quinine, blocking Cx36 (EC50 = 300 nM) and Cx50 (EC50 = 1.1 μM) at concentrations 10- to 100-fold lower than were required for other connexins (47). Despite this unique selectivity for Cx36 over other

Figure 6. Anti-malarial quinoline alkaloids used as selective blockers of connexin and pannexin channels. (A) Quinine. (B) Mefloquine.

A



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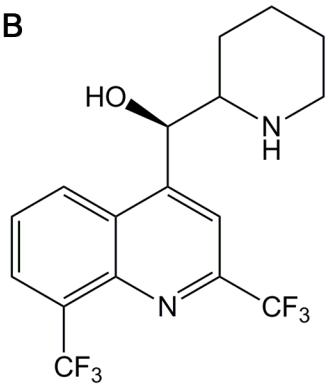


connexins, quinine and mefloquine are unlikely to be useful to discriminate between Cx36 and Panx1 function, since mefloquine is even more selective for Panx1 hemichannels than it is for Cx36, having an EC50 of only 53 nM (99).

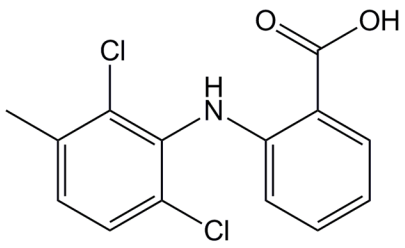
The mechanism by which quinine and its derivatives act on connexins is by direct binding to an intracellular location on the channel, possibly located in the pore, which decreases the open probability of the channel (203). The mechanism by which mefloquine acts on Panx1 has not yet been investigated. Although the ability of quinine and mefloquine to selectively modulate certain connexin and pannexin subtypes is promising, their weakness is the fact that they are not specific for these channels. However, these studies convincingly illustrate that small modifications in the structure of quinine can alter its selectivity. Therefore, this class holds promise as the basis for more specific connexin and pannexin inhibitors.

1.5.5 Fenamates

Fenamates (Figure 7) are a class of synthetic non-steroidal anti-inflammatory drugs derived from fenamic acid. They produce their therapeutic actions by inhibiting cyclooxygenase, the key enzyme in prostaglandin synthesis (228). At higher concentrations, their nonspecific effects on membrane ion channels are well documented; fenamates also alter the activity of various potassium (70, 86, 121, 163), calcium (127), chloride (240) and nonselective cation channels (82).

Figure 7. Derivatives of fenamic acid known to interact with connexin channels. (A) Meclofenamic acid. (B) Niflumic acid. (C) Flufenamic acid.

A



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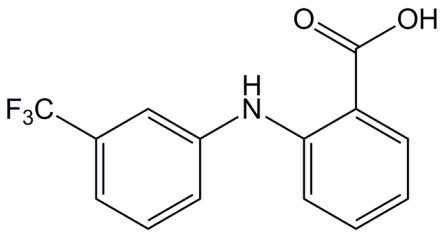

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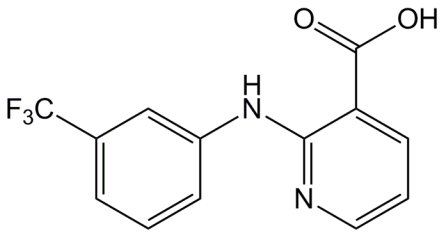


The first example of fenamates altering connexin function showed that meclofenamic acid, niflumic acid and flufenamic acid rapidly, concentration-dependently and reversibly decreased electrical and dye coupling of rat kidney fibroblasts endogenously expressing Cx43 (89). These effects were later demonstrated to be relatively indiscriminant for gap junctions and hemichannels formed by different connexins, as EC50 values for flufenamic acid on Cx26, Cx32, Cx40, Cx43, Cx46 and Cx50 ranged from 20 to 60 μM (204). Of note, fenamates may be used to distinguish between pannexin and connexin hemichannel activity. Flufenamic acid had only a modest inhibitory effect on Panx1 hemichannel currents in microinjected *Xenopus* oocytes at concentrations up to 300 μM, while currents through Cx46 hemichannels were dose dependently and significantly inhibited following flufenamic acid treatment at concentrations as low as 30 μM (29).

The activity of fenamates in inhibiting gap junctions correlates with their octanol/water partition (LogP) coefficients (204). Their mechanism of action is proposed to be through allosteric modification of the channel by binding to a site within the membrane that is inaccessible from the cytoplasm (66, 204). Unfortunately, the micromolar concentrations that are required to exert these effects on connexins also modify additional ion channels targets as well (93).

1.5.6 Mimetic peptides

Connexin-mimetic peptides are perhaps the best tools currently available to specifically inhibit connexin channels. These short peptides correspond to

sequences on the extracellular loops of connexins and, in many cases, are able to target gap junctions or hemichannels formed by a particular connexin (67). A key advantage of these peptides over the compounds previously mentioned is that they are non-toxic to cells. The first mimetic peptides to be widely used, called Gap26 and Gap27, correspond to sequences on the extracellular loops of Cx43, though they are not entirely specific for Cx43 as they also inhibit channels formed by Cx26, Cx32, Cx37 and Cx40 (38, 67). Derivatives of Gap26 and Gap27 and additional mimetic peptides targeted to connexin- and pannexin-specific sequences are actively being tested and some have improved in target specificity.

The exact mechanism by which connexin mimetic peptides inhibit gap junctions and hemichannels is not definitively known but it has been observed that hemichannel inhibition occurs early and gap junctional inhibition follows (124). To explain this, it is thought that mimetic peptides bind to a domain on the extracellular loop of a connexin and induce channel closure (67). The delayed effects on gap junctions versus hemichannels are therefore explained by the fact that this domain would be hidden on a junctional channel and a reduction in gap junctional intercellular communication would require protein turnover.

The main limitations of mimetic peptides are that they do not completely inhibit GJIC, require high concentrations to exert their effects and are relatively unstable and short-lived (201). Taken together, this suggests that mimetic peptides are unlikely to be therapeutically useful *in vivo* in their current form.

1.5.7 Nucleic acids

Various nucleic acid-based approaches to knockdown connexin and pannexin expression have been used. The first approach employed antisense oligonucleotides. These are short sequences of DNA or RNA that bind to complementary messenger RNA sequences and prevent their translation to protein (234). As such, they have the capability to knockdown the expression of protein encoded by a specific transcript. The major advantages of these techniques over a traditional knockout are greater temporal and spatial specificity and the opportunity to transiently knockdown the expression of a particular gene when complete knockout is lethal. These advantages have led to the use of antisense approaches to study connexin function in several experimental paradigms, including neuronal differentiation (92), spinal cord injury (46), stroke (72) and wound healing (178), among others.

More recent knockdown approaches have taken advantage of modern small interfering RNA (siRNA) and short hairpin RNA (shRNA) techniques (188, 212). Despite much enthusiasm regarding their potential, the widespread adoption of therapeutics based on these technologies is hampered by some general issues that include poor pharmacokinetics and lack of penetrance to the CNS following systemic or oral administration, necessitating local or vector delivery systems (110). In terms of the targeting of connexins, some further issues include the incompleteness of knockdown and the inability to selectively target gap junctions or hemichannels exclusively. Further, as in connexin knockout mice, the possibility of functional

compensation between connexins in tissues expressing multiple subtypes is high and must not be overlooked (146).

1.6 Ethnobotany is a useful approach to drug discovery

The field of ethnobotany studies the traditional knowledge of a people concerning plants and their medical, religious and cultural uses. The related field of ethnopharmacology is concerned with the study of the active principles, modes of action and biological effects of plant-derived drugs. Plants have been the most important source of pharmaceuticals throughout history due to the fact that have evolved to produce an extremely diverse portfolio of bioactive secondary metabolites as chemical signaling and defense mechanisms (44).

An ethnobotanical approach to drug discovery has several inherent advantages over a conventional combinatorial library screen. By testing plants that have a tradition of medicinal use for the treatment of particular diseases or ailments, one can be confident in the safety and efficacy of the active principles (44). Further, the active principles in plants used for their neuroactive effects are likely to pass the blood-brain barrier, a major hurtle in the rationalized design of pharmaceuticals destined for CNS use (45).

Historically, many well-known neuroactive compounds have been derived from plants, having analgesic, stimulant, depressant and hallucinogenic effects (142). More recently, physostigmine from *Physostigma venenosum* and galantamine from

Galanthus woronowii have received attention as acetylcholinesterase inhibitors in use for the treatment of Alzheimer's disease (222). Interestingly, two of the most effective inhibitors of connexins and pannexins that are currently available were originally isolated from plants having medicinal value: glycyrrhetinic acid from the rhizome and root of *Glycyrrhiza glabra* (licorice) and quinine from bark of *Cinchona*. Collectively, these lines of evidence suggest that a targeted ethnopharmacological approach may be an effective means to identify novel connexin- and pannexin-specific compounds.

1.7 Members of the *Piperaceae* family are preferentially used by traditional healers for the treatment of neurological symptoms

The Q'eqchi' Maya have a rich medical tradition that is practiced today by groups of healers living throughout roughly 30 Q'eqchi' villages in Southern Belize. These healers recognize the symptoms of a wide range of systemic, neurological and culture-bound syndromes and treat them with plants selectively chosen from the extremely diverse tropical flora in which they live (6). Among the plants used to treat disorders of the CNS (i.e. *susto*, epilepsy, headache, madness, insomnia, stress, numbness and depression), species belonging to the *Piperaceae* family were most preferentially used (26), strongly hinting at the presence of neuroactive metabolites within these plants. Supporting this, ethanolic extracts of *Piperaceae* species used by the Q'eqchi' to treat epilepsy have been shown to possess potent anti-epileptic effects *in vitro* (11).

Epileptic seizures are characterized by electrical disturbances in the brain that result from pathological increases in neuronal excitability and synchronization (36). These changes in neuronal activity can be precipitated by imbalances in neurotransmitter metabolism at chemical synapses or by mechanisms independent to neurotransmitter activity, usually involving voltage-gated cation channels (181). A growing body of evidence indicating that connexin and pannexin channels play a primary role in the generation and propagation of seizures suggests that these proteins warrant attention as a potential therapeutic target.

For example, it has been repeatedly noted in the literature by multiple groups that non-specific connexin and pannexin blockers like CBX, halothane and octanol decrease the incidence and severity of epileptic events *in vitro* (59, 80, 81, 103, 113, 136, 164, 182, 186, 187) and *in vivo* (24, 75-77, 81, 96, 143, 176, 213, 246). Further, there are multiple reports of changes in connexin expression in epilepsy in post-mortem human tissue (8, 40, 71, 156) and in a wide range of rodent models *in vitro* (126, 186) and *in vivo* (41, 75, 213), with the general trend being an increase in the expression of glial connexins post-injury. Conflicting reports have been published regarding whether neuronal Cx36 expression is increased, decreased or unchanged (41, 75, 103, 186, 195), likely reflecting differences in the models used. Recent studies *in* vivo have demonstrated that Panx1 and Panx2 expression is also increased in the hippocampus of mice following seizure (151, 189). These results suggest that any number of these channel-forming proteins might play important roles during epilepsy.

Additional insight has come from *in vivo* studies using connexin knockouts and moderately selective pharmacological inhibitors. Cx36 knockout mice are more sensitive to induced seizures, suggesting that the presence of Cx36 is anticonvulsant (102). This is in disagreement with several published studies using quinine, a "selective" inhibitor of Cx36 function, which have consistently shown that quinine has antiepileptic properties *in vivo* (24, 76, 144, 155). A potential explanation for this discrepancy is that quinine is also a potent inhibitor of Panx1 and it may be through inhibition of Panx1 hemichannel activity that quinine exerts its antiepileptic effects. This is plausible, given that Panx1 hemichannels mediate pathological ATP release following kainic acid administration (189). The contribution of astrocytic Cx30 and Cx43 to seizure generation is controversial, with contradictory reports claiming protection from and exacerbation of epileptic activity *in vitro* (187, 235). Taken together, these ethnobotanical and experimental data suggest that members of the *Piperaceae* family, traditionally used to manage seizures by Q'eqchi' Maya healers, may potentially interact with connexin and pannexin channels.

1.8 Objectives and hypothesis

We currently lack the compounds that are necessary to target connexins and pannexins for neural repair and regeneration following excitotoxic and ischemic injury *in vivo*. I hypothesized that a combination of ethnobotanical, structural and synthetic approaches would allow us to identify novel pharmacological tools that alter connexin and pannexin channel function with potential for therapeutic use. To test this hypothesis, I prioritized and screened an extensive library of

ethnobotanically-derived compounds and complex extracts curated by Dr. John Thor Arnason for capacity to alter connexin and pannexin channel function *in vitro.* Library extracts were prioritized for testing based on their ethnobotanical use to control epileptic activity. Pure compounds were then selected for testing based on their predicted enrichment within these extracts and/or based on structural similarities to known connexin and pannexin inhibitors. The cell model chosen for bioassay was the NT2/D1 cell line. These cells are derived from a human teratocarcinoma, have a multipotent neural progenitor cell phenotype, are metabolically coupled by connexin gap junctions and exhibit functional hemichannel activity (25). To establish the complete repertoire of connexins and pannexins expressed by NT2/D1 cells, these cells were profiled at the mRNA level by reverse transcriptase polymerase chain reaction (RT-PCR). To assess intercellular connexin gap junction activity, I performed the parachute assay, which measures the intercellular flux of fluorescent calcein between coupled cells through gap junction channels. To assess single-membrane hemichannel activity, I measured the uptake of both anionic lucifer yellow (LY) and cationic propidium iodide (PI) in calcium-free medium.

2 MATERIALS AND METHODS

2.1 Cell culture

NT2/D1 cells (passage number 68-74) were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Ham) (1:1) (DMEM/F12) (Invitrogen 12400, lot 1124811) supplemented with 10% Fetal Bovine Serum (Invitrogen 12483, lot 1129906), 1% Penicillin Streptomycin (Invitrogen 15140, lot 980006) and 1% L-Glutamine (Invitrogen 25030, lot 767886). Cells were maintained at 37°C and 5% CO_2 in 100 mm tissue culture dishes (BD Falcon 353003, lot 2062663).

2.2 RNA isolation

Total RNA from NT2/D1 cells, post-mortem human hippocampus and HeLa cells was isolated using TRIzol reagent (Invitrogen 15596018, lot 28120) following the manufacturer's recommended protocol. Briefly, cultures were washed with ice cold PBS and lysed with 1 ml of TRIzol reagent per 100 mm dish. Following a 5 minute incubation at room temperature, 200 µl of chloroform per 1 ml of TRIzol reagent was added to the homogenized cell lysates. Tubes were capped and shaken vigorously for 15 seconds and incubated at room temperature for an additional 3 minutes before being centrifuged at 12,000 g for 15 minutes at 4°C. Following centrifugation, the upper aqueous phase was transferred to a new 1.5 ml tube and 500 µl isopropanol per 1 ml of TRIzol reagent was added. Samples were then incubated for 10 minutes at room temperature to precipitate RNA and centrifuged at 12,000 g for

10 minutes at 4°C to pellet the RNA. Following centrifugation, the supernatant was removed and the pellet was washed with 1 ml 75% DEPC-ethanol per 1 ml TRIzol reagent and centrifuged at 7,400 g for 5 minutes at 4°C. The supernatant was then removed and RNA pellets were allowed to dry before being resuspended in nuclease-free water (Invitrogen 10977-015, lot 1237837).

2.3 DNAse treatment

To eliminate genomic DNA contamination, RNA samples were treated with DNAse-1 (Sigma-Aldrich D5307, lot SLBD2329V). 10 µg RNA was added to 10 µl of DNAse-1, 4 µl 10X buffer (Sigma-Aldrich R6273, lot SLBC7096V) and nuclease-free water to a final reaction volume of 40 µl and incubated for 30 minutes at 37°C. After incubation, 160 µl nuclease-free water and 200 µl phenol/chloroform were added to each sample to precipitate RNA. Samples were then vortexed and centrifuged at 16,100 g for 1 minute at 4°C. The upper aqueous phase was transferred to a new tube, to which 22 µl 3 M sodium acetate and 900 µl ethanol were added. Samples were gently mixed and incubated overnight at -80°C. The following day, samples were centrifuged at 16,100 g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed twice with 500 µl 75% DEPC-ethanol and spun at 16,100 g for 1 minute at 4°C. The supernatant was then removed and RNA pellets were allowed to dry before being resuspended in nuclease-free water.

2.4 Reverse transcription

Following DNAse-1 treatment, RNA samples and QPCR Human Reference Total RNA (Stratagene 750500, lot 0006153539) were reverse transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen 18064-014, lot 924845C). For RT+ reactions, 2 µg DNAse-1-treated RNA was added to 1 µl pdN6 random primers (Promega C1181, lot 0000017994) and nuclease-free water to a final volume of 12 µl. For reactions processed in the absence of RT reactions, 2 µg DNAse-1-treated RNA was added to 1 µl pdN6 random primers and nuclease-free water to a final volume of 13 µl. For no template controls, 1 µl pdN6 random primers was added to 12 µl nuclease-free water. Samples were incubated at 70°C for 10 minutes and then 4°C for 2 minutes, at which point 4 µl 1st strand buffer, 2 µl DTT and 1 µl dNTPs (Fermentas 0181) were added to all reactions. Samples were then incubated at 37°C for 2 minutes and brought to 25°C, at which point 1 µl of reverse transcriptase was added to the RT reactions. Samples were then incubated at 25°C for 10 minutes, 42°C for 60 minutes, 50°C for 30 minutes, and brought to 4°C.

2.5 PCR

PCR was performed using the primers listed in Table 1.

Gene	Strand	Primer sequence (5' to 3')	Amplicon size (bp)
GAPDH	Sense	TGGTGCTGAGTATGTCGTGGAGT	270
	Antisense	AGTCCTCTGAGTGGCAGTGATGG	
Cx26	Sense	CTGCAGCTGATCTTCGTGTC	308
	Antisense	AAGCAGTCCACAGTGTTG	
Cx30	Sense	GTGACGAGCAAGAGGACTTC	512
	Antisense	CAGCAGCAGGTAGCACAACT	
Cx32	Sense	CTGCTCTACCCGGGCTATGC	386
	Antisense	ACGGCTGAGCATCGGTCGCTCTT	
Cx36	Sense	AACGCCGCTACTCTACAGTC	596
	Antisense	CCTTGGCAGGTCCTTGTTAC	
Cx37	Sense	ATCTGGCTGACGGTGCTCTT	619
	Antisense	GCACCAACTCCAGCAGGTTA	
Cx43	Sense	CTCAGCAACCTGGTTGTGAA	709
	Antisense	TCGCCAGTAACCAGCTTGTA	
Cx45	Sense	AGTTCTGGACAACAGGGCAT	408
	Antisense	GGTGTTGTTTCCAGCGCATT	
Cx47	Sense	GACGAGCAGGCCAAGTTCAC	572
	Antisense	ACCTCGAAGCCGTACAGCAG	
Panx1	Sense	CTCCTGTACCTGCCCCCGCT	854
	Antisense	TTCATACCTTGGAGCTCTGCCGT	
Panx2	Sense	ACCAAGAACTTCGCAGAGGA	925
	Antisense	CCACGTTGTCGTACATGAGG	

Table 1. PCR primer pair sequences and amplicon sizes.

All primers were synthesized by IDT Integrated DNA Technologies. Cx26, Cx32, Cx37 and Cx43 were amplified in reactions containing 1 µl cDNA, 2.5 µl 10X Taq buffer, 800 µM dNTPs, 12.5 pmol primers, 0.5 µl Titanium Taq DNA Polymerase (Clontech 639209, lot 1205040A) and nuclease-free water to a final volume of 25 µl using the following cycling conditions: 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 60°C for 60 seconds and 72°C for 120 seconds, 72°C for 7 minutes and brought to a final temperature of 4°C. For Cx36, the annealing temperature was adjusted to 55°C. For Cx30 and Cx47, the annealing temperature was adjusted to 55°C and 58°C, respectively and 7.5% dimethylsulfoxide (DMSO) (Sigma-Aldrich

D2650, lot RNBB4487) was added. For Cx45, primer concentrations were reduced to 10 pmol per reaction. For GAPDH, primer concentrations were reduced to 5 pmol per reaction and annealing temperature was reduced to 59°C and annealing time reduced to 50 seconds. The denaturing time for Panx1 and Panx2 was adjusted to 45 seconds and the annealing temperature was adjusted to 62°C. Panx1 reactions contained 5 pmol primers, while Panx2 reactions contained 25 pmol primers.

2.6 Preparation of compounds and extracts

Piperaceae leaves were collected in Peru by Gabriel Picard (laboratory of Dr. John Thor Arnason, University of Ottawa) and identified to species level by Dr. Ricardo Callejas (University of Antioquia, Colombia). Upon collection, plants were immediately stored in ethanol for the duration of the fieldwork. Upon returning to the University of Ottawa, leaves were ground, extracted, filtered and roto-evaporated by Gabriel Picard to prepare the solid extract. AF101 and analogs were synthesized by Ana Francis Carballo Arce (laboratory of Dr. Tony Durst, University of Ottawa). All extracts were resuspended in ethanol to a final concentration of 20 mg/ml, passed through a 0.2 µm polytetrafluorethylene (PTFE) filter (Chromspec TI35530500) and stored at -80°C. All flavonoids were prepared in ethanol to a stock concentration of 50 mg/ml, passed through a 0.2 µm PTFE filter and stored at -80°C. All remaining compounds were prepared in DMSO to a stock concentration of 200 mg/ml and stored at -80°C. They were freeze-thawed a maximum of two times.

2.7 WST assay

Compound and extract toxicity in NT2/D1 cultures was tested using the WST-1 formazan dye assay (Roche 11644807001, lot 12426400). On day 0, 10,000 cells per well were plated in 96-well plates (BD Falcon 353072). On day 1, cells were treated in replicates of 5 for 24 hours with 5 concentrations of each compound or extract in regular growth medium. Vehicle concentration was held constant at 0.1% total medium. On day 2, cells were treated for 45 minutes at 37°C with WST-1 at a final concentration of 10% in regular growth medium. Absorbances were read at a test wavelength of 450 nm and a reference wavelength of 655 nm using an iMark Microplate Reader (Bio Rad). To determine cell numbers, a standard curve of untreated cells was also treated with WST-1. Compounds and extracts were treated at concentrations 10-fold lower than the median lethal concentration (LC50) in each cell line. Compounds that did not exhibit any toxicity were treated at a maximal concentration of 100 μM.

2.8 Parachute assay

The parachute assay was used to assess gap junctional intercellular communication as has been previously described (83). On day 0, 20,000 recipient NT2/D1 cells were plated in uncoated optical 96 well plates (Greiner Bio-One 655090). At this time, 500,000 donor cells were also plated in a 60 mm tissue culture dish (Corning 430196). On day 1, donor cells were treated for 30 minutes with 4 μM calcein AM (Sigma-Aldrich C1359) and 10 μM 1,1'-Dioctadecyl-3,3,3',3'-

tetramethylindocarbocyanine perchlorate (DiI) (Sigma-Aldrich 468495, lot 13021HO) in normal growth medium. Donor cells were then washed with PBS, trypsinized for 4 minutes with 0.05% trypsin-EDTA (Invitrogen 25300, lot 688247) and counted. Donor cells were then seeded on top of recipient cells at a density of 200 cells per well (1:100 donor to recipient ratio) and incubated with treatments and 0.1 µg/ml Hoechst 33342 trihydrochloride trihydrate (Invitrogen H1399, lot 908746) for 75 minutes in normal growth medium at 37°C prior to imaging. Vehicle concentration was held constant and did not exceed 0.1% total medium. Experiments were imaged on an Opera High Content Screening System (Perkin Elmer) at 20X magnification using the 20xW_UAPO20xW3/340_NA=0.7 objective. Hoechst was excited at a wavelength of 405 nm and its emission was detected at a wavelength of 450 nm. Calcein was excited at a wavelength of 488 nm and its emission was detected at a wavelength of 540 nm. DiI was excited at a wavelength of 561 nm and its emission was detected at a wavelength of 690 nm. Image analysis was performed using Columbus Image Analysis System version 2.3.0 (Perkin Elmer). Replicate experiments were analyzed with each treatment in replicates of 12 wells and 20 images per well. All plates were controlled with untreated cells, vehicle-treated cells and cells treated with 10 and 100 µM carbenoxolone disodium salt (C4790, lot 020M1257V).

2.9 Zero-calcium dye uptake assay

Uptake of Lucifer Yellow CH dilithium salt (Sigma L0259, lot MKBJ5898V) and Propidium Iodide (Invitrogen P1304MP, lot 890805) in the absence of extracellular

calcium was used to assess hemichannel activity in NT2/D1 cells. On day 0, 20,000 NT2/D1 cells were plated in optical 96 well plates (Greiner Bio-One 655090, lot E1110CV) coated with 100 µg/ml poly-L-lysine hydrobromide (Sigma-Aldrich P1274, lot 030K5101). On day 1, cells were incubated for 5 minutes in either 250 µg/ml LY or 50 µg/ml PI in PBS containing 1 mM EGTA. Cells were then washed with PBS and fixed with 3.7% formaldehyde in PBS for 10 minutes. Before imaging, cells were washed and incubated in 0.1 µg/ml Hoechst 33342 in PBS for 30 minutes at room temperature. LY assays were controlled for dye uptake nonspecific to hemichannels using rhodamine B isothiocyanate dextran, 10,000 MW (RD) (Sigma R8881, lot 090M5307V). PI assays were controlled for dye uptake nonspecific to hemichannels using fluorescein isothiocyanate dextran, 10,000 MW (FITCD) (Sigma FD10S, lot 021M5302V). Experiments were imaged on an Opera High Content Screening System (Perkin Elmer) at 20X magnification using the 20xW_UAPO20xW3/340_NA=0.7 objective. Hoechst 33342 was excited at a wavelength of 405 nm and its emission was detected at a wavelength of 450 nm. LY was excited at a wavelength of 405 nm and its emission was detected at a wavelength of 600 nm. RD was excited at a wavelength of 561 nm and its emission was detected at a wavelength of 690 nm. PI was excited at a wavelength of 561 nm and its emission was detected at a wavelength of 690 nm. FITCD was excited at a wavelength of 488 nm and its emission was detected at a wavelength of 540 nm. Image analysis was performed using Columbus Image Analysis System version 2.3.0 (Perkin Elmer). Replicate experiments were analyzed with each treatment in replicates of 12 wells and 20 images per well. Controls included assays performed in normal growth medium and medium containing 100 µM CBX.

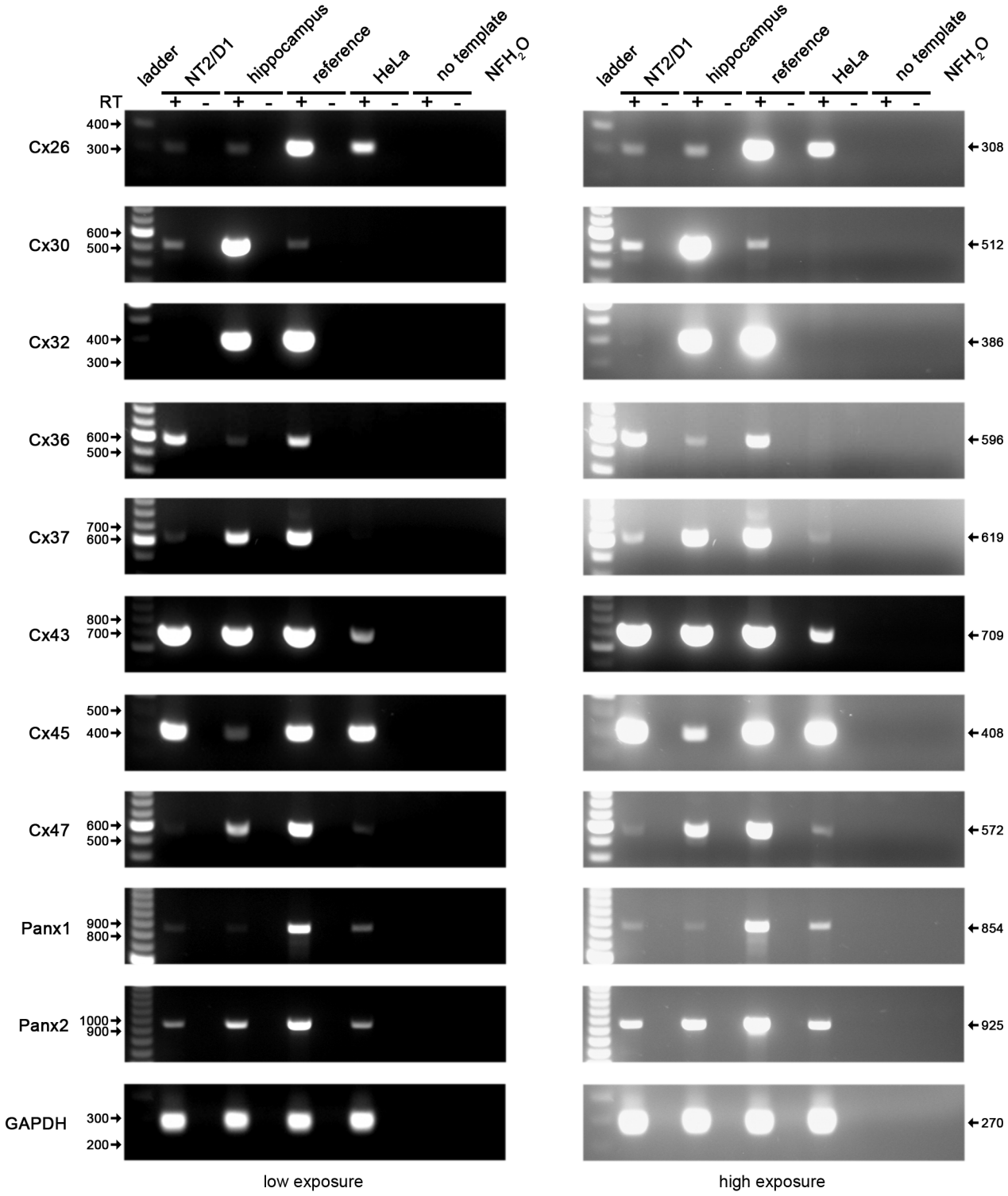
3 RESULTS

3.1 Characterization of the connexin and pannexin expression profile of naïve NT2/D1 neural progenitor-like cells using RT-PCR

It has been previously shown by our laboratory that naïve (undifferentiated) NT2/D1 cells are dye-coupled, exhibit functional hemichannel activity and express Cx30, Cx36, Cx37 and Cx43 (25). These studies were performed on cells cultured for 58-60 passages. In order to profile the complete repertoire of connexins and pannexins expressed by NT2/D1 cells at passages 68-74 as used in this study, RT-PCR was performed on total RNA isolated from NT2/D1 cells (Figure 8). To assess whether NT2/D1 cells represent a model of connexin/pannexin expression relevant to human brain, RT-PCR was performed on total RNA isolated from post-mortem human hippocampus. As a positive control, a commercial reference RNA composed of total RNA from ten human cell lines was profiled. Further, RT-PCR was also performed on HeLa cells, which are commonly used as a human cell model deficient in functional gap junctional intercellular communication (35, 63, 135).

Transcripts for Cx26, Cx30, Cx36, Cx37, Cx43, Cx45, Cx47, Panx1 and Panx2 were found in NT2/D1 cells (Figure 8). All transcripts detected in NT2/D1 cells, plus Cx32, were detected in hippocampus (Figure 8). Surprisingly, transcripts for Cx26, Cx37, Cx43, Cx45 and Cx47 were detected in HeLa cells. Standard practice in our laboratory is to use null-mutant controls to verify primer specificity. In light of the unavailability of appropriate human-specific null controls, select RT-PCR products

Figure 8. NT2/D1 cells express Cx26, Cx30, Cx36, Cx37, Cx43, Cx45, Cx47, Panx1, and Panx2 at the mRNA level. Total RNA was isolated from naïve NT2/D1 cells and treated with DNase-1 to eliminate residual genomic DNA. RNA was then reverse transcribed to cDNA using random primers (RT+). Following reverse transcription, PCR was performed to detect mRNA expression of the indicated connexin and pannexin transcripts. GAPDH was used as an internal control for template integrity. Additional controls included RT reactions performed without the addition of reverse transcriptase enzyme (RT-) or in absence of RNA template, and PCR reactions performed using NFH_2O in place of template. Following PCR, DNA was resolved by gel electrophoresis on a 1% agarose gel. Total RNA isolated from post-natal human hippocampus (hippocampus) and HeLa cells were processed at the same time. A commercial human reference RNA (reference) was used as a positive control for all RT-PCR reactions. Transcripts for Cx26, Cx30, Cx36, Cx37, Cx43, Cx45, Cx47, Panx1, and Panx2 were detected in NT2/D1 cells.


ladder
NT2/D1
hippocampus
reference
HeLa
no template
NFH2O
RT
+ - + - + - + - + -
Cx26
400
300
308
Cx30
600
500
512
Cx32
400
300
386
Cx36
600
500
596
Cx37
700
600
619
Cx43
800
700
709
Cx45
500
400
408
Cx47
600
500
572
Panx1
900
800
854
Panx2
1000
900
925
GAPDH
300
200
270
low exposure
high exposure

from Cx26, Cx36, Cx45, Panx1 and Panx2 were sequenced to verify transcript specificity (see Appendix, section 7.1). These results confirmed amplicon identify as the connexin or pannexin targeted.

3.2 Establishment of *in vitro* treatment concentrations

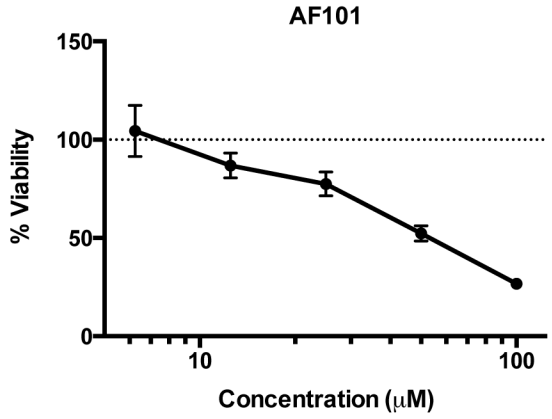
Prior to testing for capacity to alter connexin and pannexin channel activity, test compounds and extracts were screened to establish LC50s. NT2/D1 cells were treated with five dilutions of each test compound or extract for 24 hours before addition of WST-1, whose cleavage in viable cells yields a formazan dye that may be quantified using a spectrophotometer. LC50s were calculated by converting absorbance values to cell number in comparison to a standard curve of known cell numbers performed in parallel. Percent viability was calculated using these cell numbers relative to untreated cells. LC50s were established by linear regression. To ensure that functional GJIC and hemichannel assays were carried out at non-toxic concentrations, and in recognition of the limitations of the WST-1 assay stemming from the assumption that the effects of treatment on mitochondrial dehydrogenase enzymatic activity are negligible, treatments were diluted ten-fold lower than the calculated LC50 values. Treatments that did not exhibit any toxicity were assayed at the maximal concentration. Finally, compounds and extracts that reduced viability by greater than 20% but less than 50% at the maximal test concentration were assayed at a five-fold dilution. The results of this study are summarized in Figure 9A. AF101 demonstrated a typical concentration-dependent reduction in cellular viability over a two-fold dilution range up to a maximal concentration of 100 μM (Figure 9B).

Figure 9. LC50 values and treatment concentrations. (A) The WST-1 cell proliferation assay was used to establish appropriate *in vitro* treatment concentrations for test compounds and extracts in NT2/D1 cells. Compounds and extracts that exhibited greater than 50% toxicity at the maximal concentration tested were treated at concentrations ten-fold lower than their respective LC50 values. Compounds and extracts that did not demonstrate toxicity were assayed at a maximal concentration of 100 μM. Compounds and extracts that demonstrated less than 50% but greater than 20% at the maximal concentration tested were assayed at a five-fold dilution. (B) NT2/D1 exhibited a dose-dependent decrease in cell viability as a result of exposure to increasing concentrations of AF101 (LC50 = 64 μM).

A

	NT2/D1	
	LC_{50}	[treatment]
4,5-dihydropiperlongmine	> 100 µM	100 µM
AF101	64 µM	6 µM
Asiatic acid	37 µM	4 µM
Asiaticoside	> 100 µM	100 µM
Betulinic acid	24 µM	2 µM
Cinchonidine	> 100 µM	100 µM
Cinchoninic acid	> 100 µM	100 µM
Cubebin	100 µM	10 µM
Quercetin	50 µM	5 µM
Myricitrin	> 25 µM	25 µM
Piperine	> 100 µM	100 µM
Reserpine	18 µM	2 µM
Rutin	> 25 µM	25 µM
Piper subflavispicum C.DC.	> 20 µg/ml	10 µg/ml
Piper aff. euriphyllum Trel.	> 20 µg/ml	10 µg/ml
Piper denisii Trel.	> 20 µg/ml	10 µg/ml
Piper quimirianum Trel.	> 20 µg/ml	10 µg/ml
Piper aduncum L.	> 20 µg/ml	10 µg/ml
Piper aff cupreatum Trel.	> 20 µg/ml	10 µg/ml
Piper longifilamentosum Trel.	> 20 µg/ml	10 µg/ml
Piper margaritanum Trel.	> 20 µg/ml	10 µg/ml
Piper allardii Trel.	> 20 µg/ml	10 µg/ml
Piper propinquum C.DC.	> 20 µg/ml	10 µg/ml
Piper celer Trel.	> 20 µg/ml	10 µg/ml
Piper cf. dilatatum L.C. Rich.	> 20 µg/ml	10 µg/ml
Piper longifilamentosum Trel.	> 20 µg/ml	10 µg/ml
Piper loretoanum Trel.	> 20 µg/ml	10 µg/ml
Piper soledadense Trel.	> 20 µg/ml	10 µg/ml

B



3.3 Development of bioassays to assess connexin and pannexin channel function

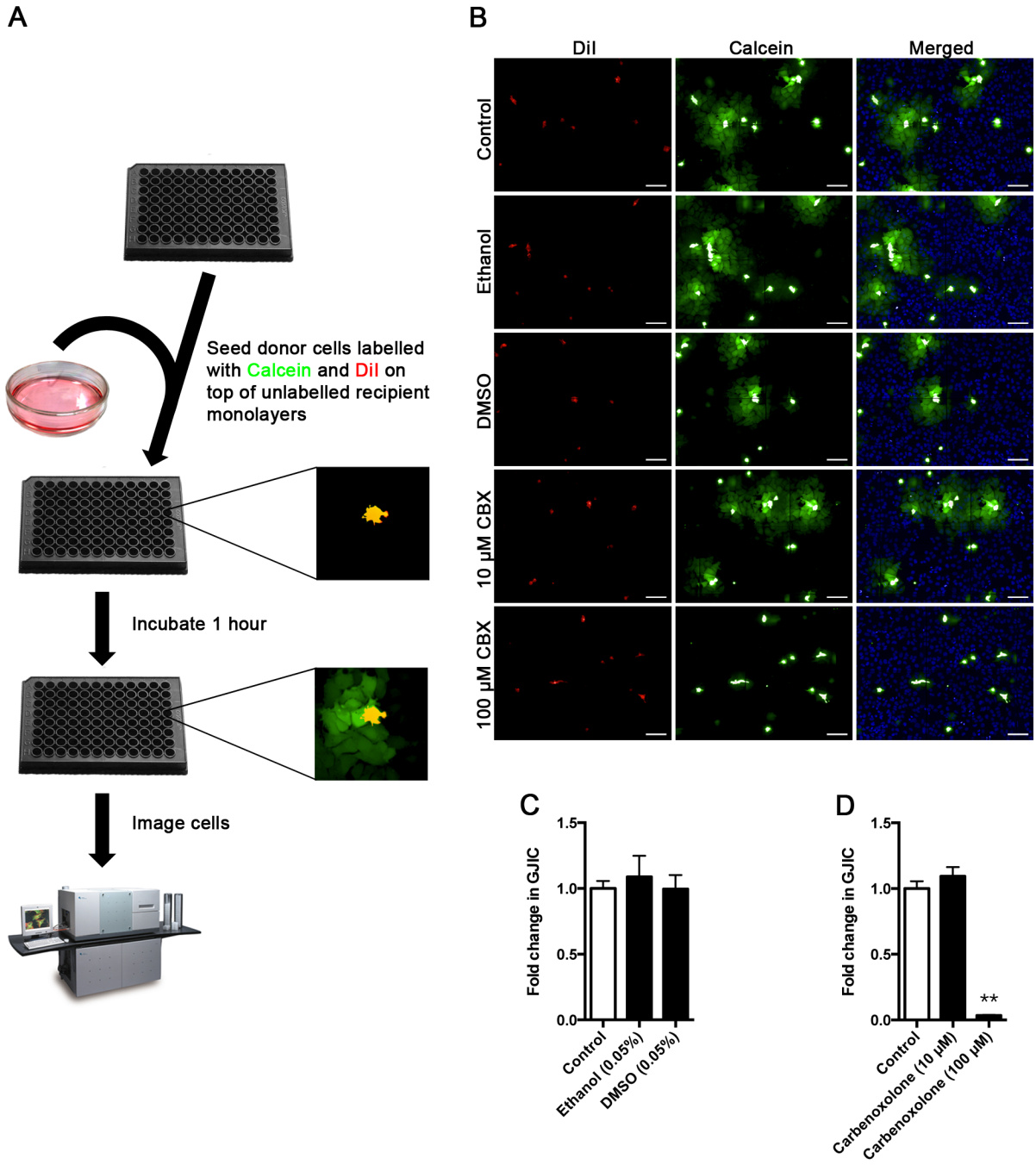
3.3.1 Parachute assay to assess anionic GJIC

The passage of small (under 1.5 kDa) fluorescent compounds between cells is an established method to assess GJIC (83). In this study, I performed the parachute assay, in which the intercellular flux of fluorescent calcein (charge 4-) between adjacent cells coupled through gap junctions is quantified. Donor cells were labeled with calcein and DiI and seeded at low density on top of monolayers of unlabeled recipient cells in 96-well plates (Figure 10A). Following a one hour incubation, cells were imaged and calcein diffusion was quantified.

Donor cells are distinguished from recipient cells by the presence of DiI, a lipophilic cyanine dye that integrates into membranes and stably marks cells without passage through connexin (or other) channels (Figure 10B). Control donor cells incubated in normal growth medium could clearly be seen to pass calcein to neighbouring DiI-negative recipient cells. The addition of 0.05% ethanol or 0.05% DMSO did not significantly alter GJIC (Figure 10C).

CBX, a non-selective inhibitor of GJIC, had no effect on calcein diffusion through gap junctions at a concentration of 10 μM, however near complete inhibition was observed when a concentration of 100 μM was used, validating assay methodology (Figure 10D).

Figure 10. Parachute assay to assess GJIC in NT2/D1 cells. (A) On day 0, recipient NT2/D1 cells were plated to reach confluency in 24 hours in 96-well optical microplates. On day 1, donor cells plated in 60 mm dishes were treated with calcein AM and DiI and were seeded at low density on top of unlabeled recipient cells. Following a one hour incubation at 37°C, cells were imaged. (B) Calcein (green) is an anionic dye (623 Da) that diffuses through gap junction channels to recipient cells. DiI (red) was used as a stable marker of donor cells. Hoechst (blue) was used to visualize donor and recipient cell nuclei. (C) Ethanol (0.05%) and DMSO (0.05%) did not alter GJIC. Data are expressed as the fold change in the number of calcein+/DiI- recipient cells per calcein+/DiI+ donor cell. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. (D) CBX (100 μM) significantly decreased GJIC relative to untreated control cells. ** denotes one-way ANOVA, *post-hoc* Dunnett's multiple comparisons test, $P < 0.01$. Scale bar = 100 μm.

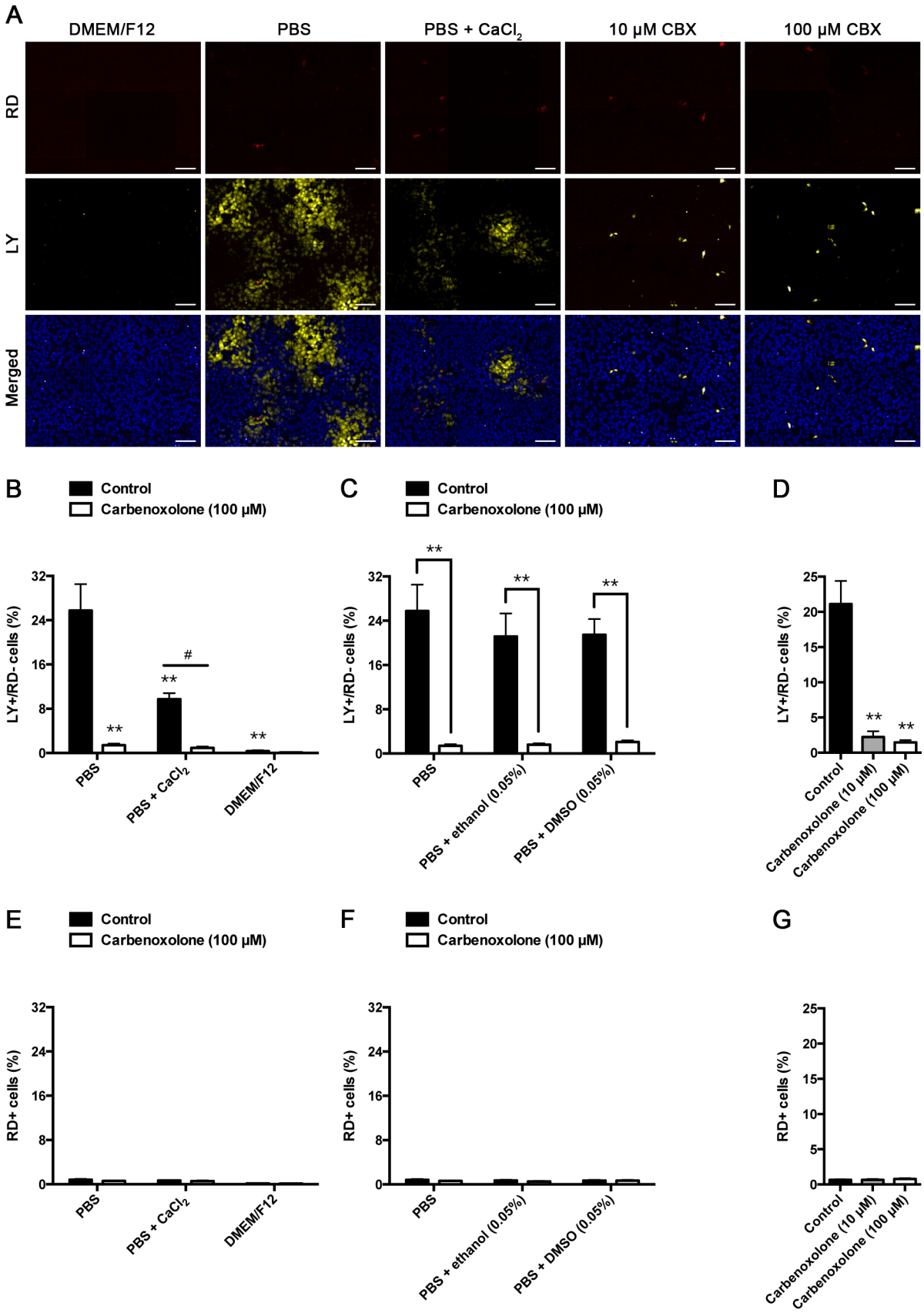

A
Seed donor cells labelled with Calcein and DiI on top of unlabelled recipient monolayers
Incubate 1 hour
Image cells
B
DiI
Calcein
Merged
Control
Ethanol
DMSO
10 µM CBX
100 µM CBX
C
Fold change in GJIC
1.5
1.0
0.5
0.0
Control
Ethanol (0.05%)
DMSO (0.05%)
D
Fold change in GJIC
1.5
1.0
0.5
0.0
**
Control
Carbenoxolone (10 µM)
Carbenoxolone (100 µM)

3.3.2 Zero-calcium dye uptake assay to assess anionic hemichannel activity

By the same principle that the intercellular diffusion of small fluorescent compounds is used to assess GJIC, the uptake of small fluorescent molecules from the extracellular medium in the absence of extracellular calcium can also be used to assess hemichannel activity (25). Here, cells were incubated for five minutes in dye uptake solution containing LY a small, anionic compound (charge 2-) that freely passes though connexin (63) and pannexin (78) channels (Figure 11A). Dye uptake solutions also contained RD, whose mass (10 kDa) is too large to pass through connexin or pannexin hemichannels. It was used to control for nonspecific uptake of LY resulting from the loss of membrane integrity.

Cells incubated with LY and RD in normal growth medium (DMEM/F12) had minimal uptake of LY (Figure 11A). Incubating cells in PBS containing 1 mM EGTA elicited a significant increase in LY+/RD- cells (Figure 11B). This increase was significantly reduced by the addition of either 100 µM CBX (which blocks both hemichannels and gap junctions) or 1.05 mM $CaCl_2$ (which blocks only hemichannels) to the dye uptake solution (Figure 11B). Interestingly, when CBX was added at the same time as $CaCl_2$, LY uptake was further reduced (Figure 11B). There was no significant change in LY uptake relative to PBS control due to the addition of 0.05% ethanol or 0.05% DMSO (Figure 11C). CBX was equally effective at blocking LY uptake at concentrations of 10 µM and 100 µM (Figure 10D). Lastly, $CaCl_2$ (Figure 11E), ethanol and DMSO (Figure 11F) and CBX (Figure 11G) did not increase RD uptake

Figure 11. Zero-calcium LY uptake assay to assess hemichannel activity in NT2/D1 cells. (A) Confluent NT2/D1 cells were treated for five minutes in zero-calcium medium (PBS with 1 mM EGTA) containing LY and RD. Cells were then washed, fixed with 3.7% formaldehyde in PBS and imaged. LY (yellow) is an anionic dye (444 Da) that diffuses through open connexin and pannexin hemichannels. RD (red) is a fluorescent dextran conjugate that is too large (10 kDa) to pass through connexin and pannexin channels. Here, it was used as a control for non-specific LY uptake resulting from membrane damage. Hoechst (blue) was used to visualize nuclei. Scale bar = 100 µm. (B) Incubating cells in zero-calcium medium (PBS) resulted in a significant increase in anionic hemichannel activity over complete medium control. Data are expressed as the mean percentage of LY+/RD- per Hoechst+ nuclei per well. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. Assay integrity was verified by treatment with the known hemichannel blocker, CBX (100 µM) or by added $CaCl_2$ (1.05 mM) to PBS. (C) Ethanol (0.05%) and DMSO (0.05%) do not alter CBX-sensitive anionic hemichannel activity. (D) A significant decrease in LY+/RD- cells relative to PBS control was observed in cells treated with either 10 µM or 100 µM CBX. (E-G) Treatment with $CaCl_2$, ethanol, DMSO, or CBX did not increase non-specific dye uptake due to membrane damage, as measured by quantifying the percent of RD+ cells. Statistics were one-way ANOVA, *post-hoc* Sidak's multiple comparisons test. ** denotes $P < 0.01$ from PBS control and $^{\#}$ denotes $P < 0.05$ from PBS + $CaCl_2$ control.

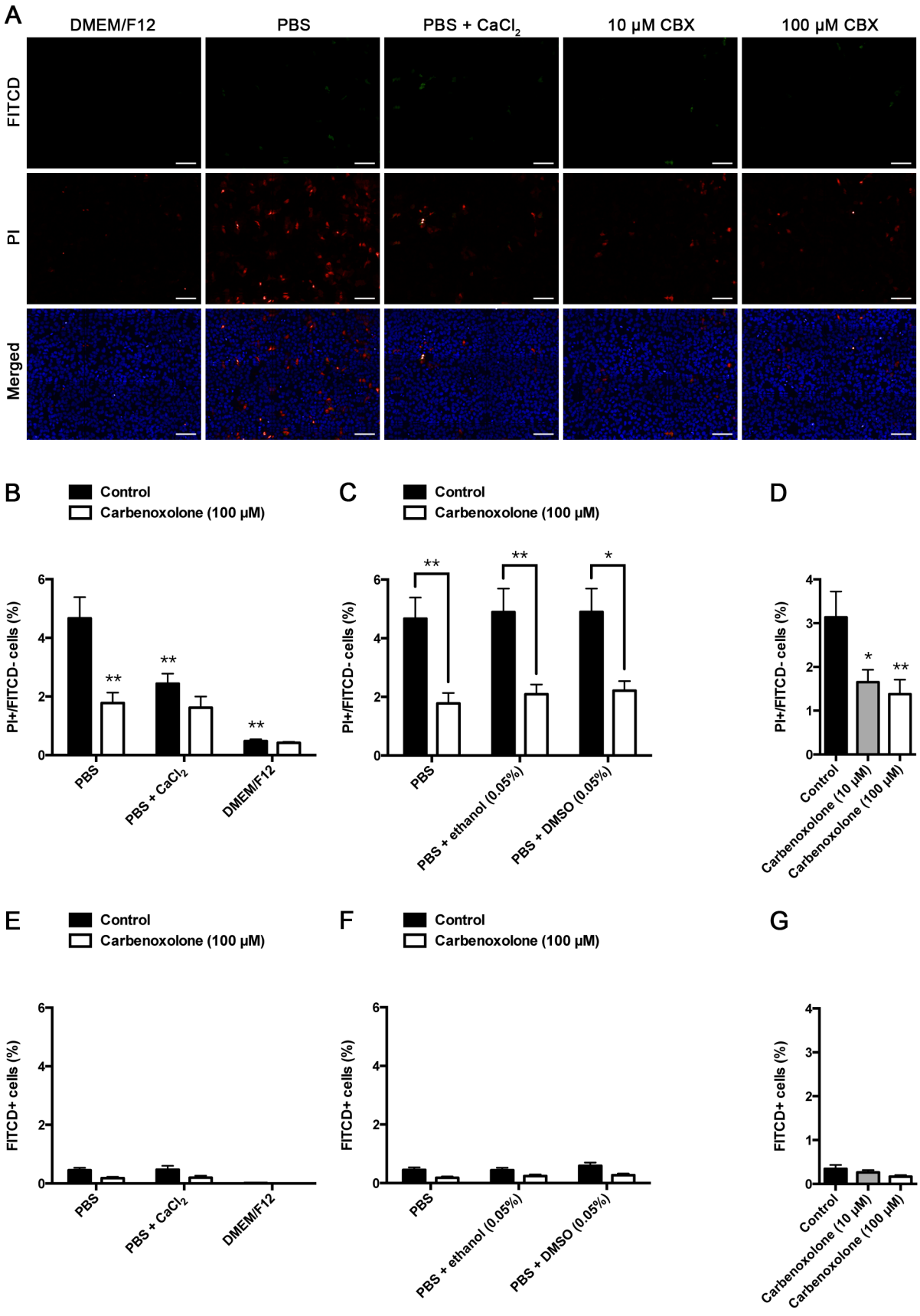

A
DMEM/F12
PBS
PBS + CaCl2
10 µM CBX
100 µM CBX
RD
LY
Merged
B
Control
Carbenoxolone (100 µM)
LY+/RD- cells (%)
PBS
PBS + CaCl2
DMEM/F12
C
Control
Carbenoxolone (100 µM)
LY+/RD- cells (%)
PBS
PBS + ethanol (0.05%)
PBS + DMSO (0.05%)
D
LY+/RD- cells (%)
Control
Carbenoxolone (10 µM)
Carbenoxolone (100 µM)
E
Control
Carbenoxolone (100 µM)
RD+ cells (%)
PBS
PBS + CaCl2
DMEM/F12
F
Control
Carbenoxolone (100 µM)
RD+ cells (%)
PBS
PBS + ethanol (0.05%)
PBS + DMSO (0.05%)
G
RD+ cells (%)
Control
Carbenoxolone (10 µM)
Carbenoxolone (100 µM)

relative to PBS control, indicating that they did not increase nonspecific LY uptake or compromise membrane integrity.

3.3.3 Zero-calcium dye uptake assay to assess cationic hemichannel activity

To assess cationic connexin hemichannel and pannexin channel activity, the uptake of PI (charge 2+) from the extracellular medium in the absence of extracellular calcium was measured. In this assay, dye uptake solutions also contained FITCD (10 kDa) as a control for nonspecific uptake of PI. Similar to what was observed when LY was used, there was a significant increase in PI uptake when cells were incubated in PBS containing 1 mM EGTA as compared to normal growth medium (DMEM/F12) (Figure 12A). When cells were incubated with either 1.05 mM $CaCl_2$ or 100 µM CBX, PI uptake was reduced relative to PBS control (Figure 12B). There was no significant change in the number of PI+/FITCD- cells as a result of the addition of 0.05% ethanol or 0.05% DMSO to PBS, and activity was sensitive to CBX (Figure 12C). As was observed with LY, both 10 and 100 µM CBX were effective in reducing PI uptake (Figure 12D). Finally, $CaCl_2$ (Figure 12E), ethanol and DMSO (Figure 12F) and CBX (Figure 12G) did not increase FITCD uptake relative to PBS control, indicating that they did not increase nonspecific PI uptake or compromise membrane integrity.

Figure 12. Zero-calcium PI uptake assay to assess hemichannel activity in NT2/D1 cells. (A) Confluent NT2/D1 cells were treated for five minutes in zero-calcium medium containing PI and FITCD. Cells were then washed, fixed with 3.7% formaldehyde in PBS and imaged. PI (red) is a cationic dye (668 Da) that diffuses through open connexin and pannexin hemichannels. FITCD (green) is a fluorescent dextran conjugate that is too large (10 kDa) to pass through connexin and pannexin channels. Here, it was used as a control for non-specific PI uptake resulting from membrane damage. Hoechst (blue) was used to visualize nuclei. Scale bar = 100 μm. (B) Incubating cells in zero-calcium medium (PBS) resulted in a significant increase in cationic hemichannel activity over complete medium control. Data are expressed as the mean percentage of PI+/FITCD- per Hoechst+ nuclei per well. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. Assay integrity was verified by treatment with the known hemichannel blocker, CBX (100 μM) or by added $CaCl_2$ (1.05 mM) to PBS. (C) Ethanol (0.05%) and DMSO (0.05%) do not alter CBX-sensitive anionic hemichannel activity. (D) A significant decrease in PI+/FITCD- cells relative to PBS control was observed in cells treated with either 10 μM or 100 μM CBX. (E-G) Treatment with $CaCl_2$, ethanol, DMSO, or CBX did not increase non-specific dye uptake due to membrane damage, as measured by quantifying the percent of FITCD+ cells. Statistics were one-way ANOVA, *post-hoc* Sidak's multiple comparisons test. ** denotes $P < 0.01$ from PBS control and $^{\#}$ denotes $P < 0.05$ from PBS + $CaCl_2$ control.


A
DMEM/F12
PBS
PBS + CaCl2
10 μM CBX
100 μM CBX
FITCD
PI
Merged
B
Control
Carbenoxolone (100 μM)
PI+/FITCD- cells (%)
PBS
PBS + CaCl2
DMEM/F12
C
Control
Carbenoxolone (100 μM)
PI+/FITCD- cells (%)
PBS
PBS + ethanol (0.05%)
PBS + DMSO (0.05%)
D
PI+/FITCD- cells (%)
Control
Carbenoxolone (10 μM)
Carbenoxolone (100 μM)
E
Control
Carbenoxolone (100 μM)
FITCD+ cells (%)
PBS
PBS + CaCl2
DMEM/F12
F
Control
Carbenoxolone (100 μM)
FITCD+ cells (%)
PBS
PBS + ethanol (0.05%)
PBS + DMSO (0.05%)
G
FITCD+ cells (%)
Control
Carbenoxolone (10 μM)
Carbenoxolone (100 μM)

3.4 An ethnobotanical approach to the identification of novel connexin and pannexin channel modulators

Fifteen ethanolic leaf extracts of species belonging to the *Piperaceae* family were screened for capacity to alter GJIC and/or hemichannel activity. The species that were tested were selected based on their traditional use in the pharmacopeia of the Q'eqchi' Maya in Belize and the Yanesha in eastern Peru for the treatment of the symptoms of connexin- and pannexin-related neurological disorders, including epilepsy (6, 11, 26, 226).

Two *Piper* species significantly increased GJIC in NT2/D1 cells (Figure 13A). The most effective extract belonged to *Piper aduncum* L., which increased calcein diffusion by nearly three-fold (Figure 13A). There was no change in anionic hemichannel activity due to treatment with any of the extracts tested, as measured by uptake of LY from the extracellular medium in the absence of extracellular calcium (Figure 13B). In contrast, 13 of the 15 extracts significantly reduced PI uptake, a measure of cationic hemichannel activity (Figure 13C). Among these extracts, *Piper subflavispicum* C.DC. was the most potent.

In an effort to elucidate which compounds are contributing to the activity observed in the complex extracts, three pure compounds that are found in high abundance in *Piper* species were tested: piperine, cubebin and 4,5-dihydropiperlongumine (Figure 14A). GJIC in cultures treated with two compounds, 4,5-dihydropiperlongumine and piperine, was less than 50% that of vehicle-treated cultures (Figure 14B). While

Figure 13. Members of the *Piperaceae* family alter gap junction and hemichannel activity in NT2/D1 cells. (A) GJIC data are expressed as the fold change in the number of calcein+/DiI- recipient cells per calcein+/DiI+ donor cell. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. Two extracts significantly altered GJIC in NT2/D1 cells. The most potent extract, *Piper aduncum* L. (10 µg/ml), increased GJIC by approximately 2.5-fold relative to ethanol control at a concentration of 10 µg/ml. Scale bar = 100 µm. (B) Anionic hemichannel assay data are expressed as the mean percentage of LY+/RD- per Hoechst+ nuclei per well. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. No extract tested significantly altered anionic hemichannel activity in NT2/D1 cells. A representative treatment, *Piper aff. cupreatum* Trel. (10 µg/ml), is shown. Scale bar = 100 µm. (C) Cationic hemichannel assay data are expressed as the mean percentage of PI+/FITCD- per Hoechst+ nuclei per well. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. Of the 15 *Piperaceae* extracts tested, a significant reduction in the uptake of cationic PI was observed in 13 treatments. The most potent extract, *Piper subflavispicum* C.DC. (10 µg/ml), significantly decreased cationic hemichannel activity in NT2/D1 cells. Scale bar = 100 µm. Statistics were one-way ANOVA, *post-hoc* Fisher's LSD test. ** denotes $P < 0.01$ from ethanol control.

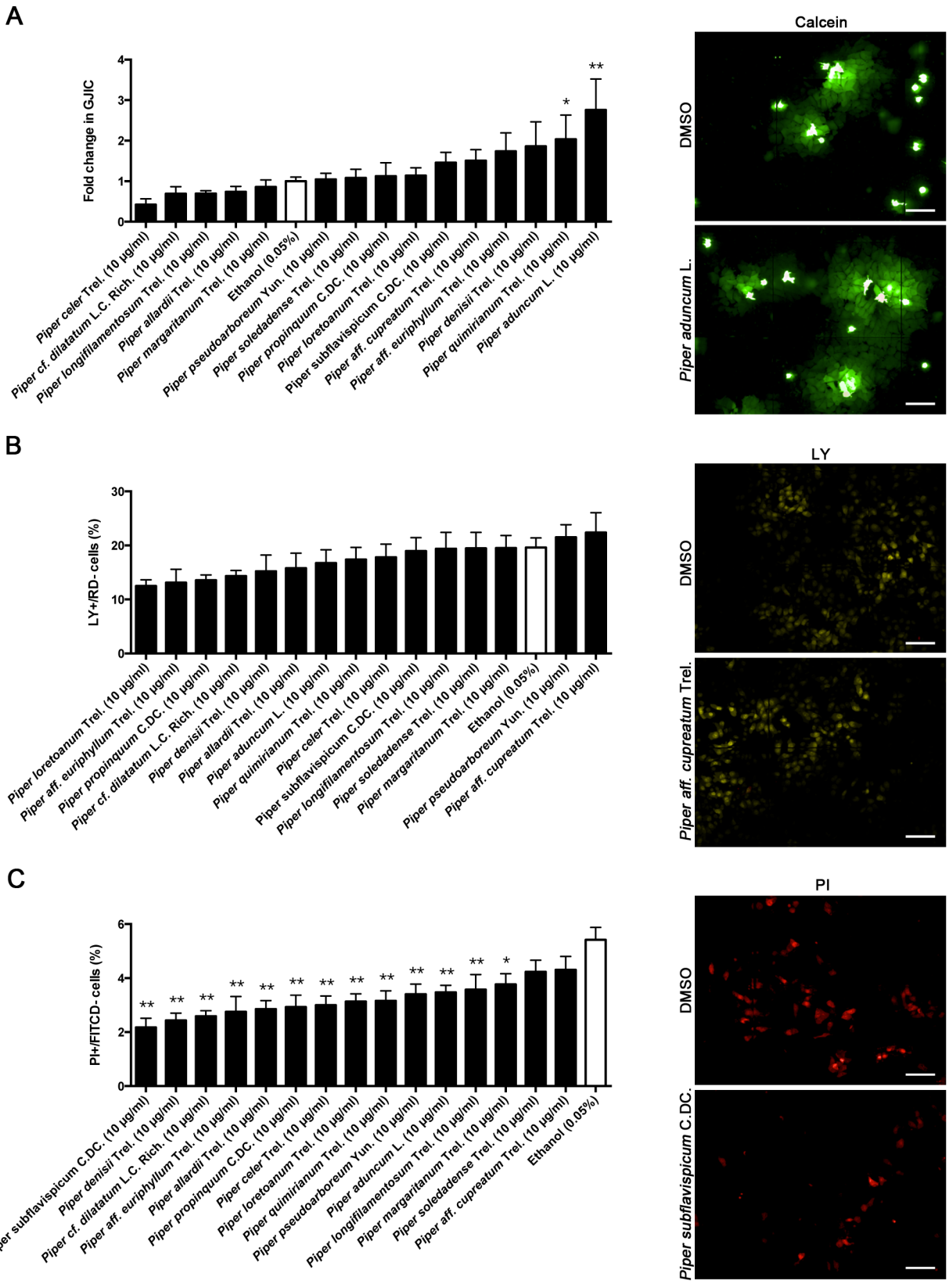
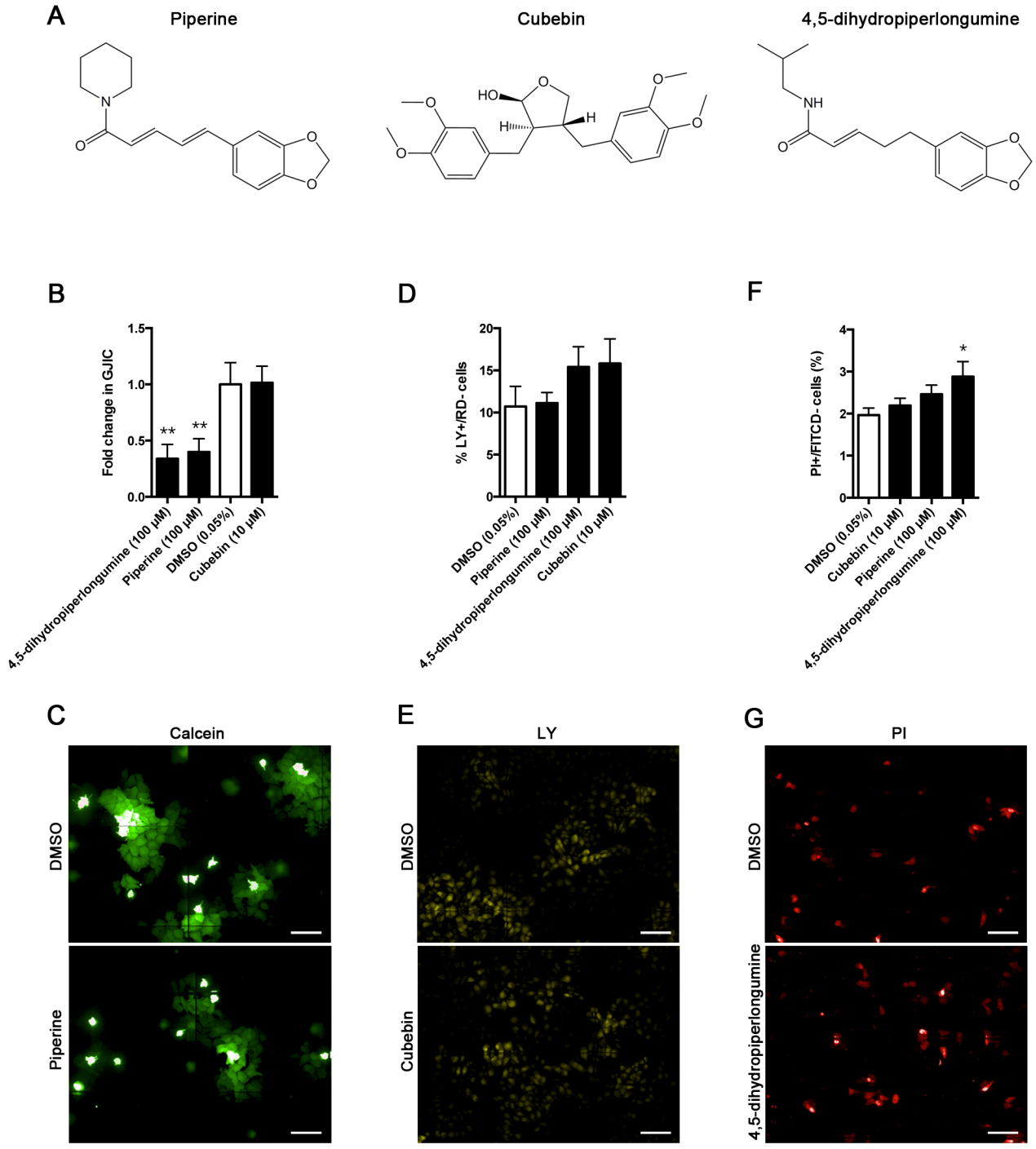

A
Fold change in GJIC
Piper celer Trel. (10 µg/ml)
Piper cf. dilatatum L.C. Rich. (10 µg/ml)
Piper longifilamentosum Trel. (10 µg/ml)
Piper allardii Trel. (10 µg/ml)
Piper margaritanum Trel. (10 µg/ml)
Ethanol (0.05%)
Piper pseudoarboreum Yun. (10 µg/ml)
Piper soledadense Trel. (10 µg/ml)
Piper propinquum C.DC. (10 µg/ml)
Piper loretoanum Trel. (10 µg/ml)
Piper subflavispicum C.DC. (10 µg/ml)
Piper aff. cupreatum Trel. (10 µg/ml)
Piper aff. euriphyllum Trel. (10 µg/ml)
Piper denisii Trel. (10 µg/ml)
Piper quimirianum Trel. (10 µg/ml)
Piper aduncum L. (10 µg/ml)
Calcein
DMSO
Piper aduncum L.
B
LY+/RD- cells (%)
Piper loretoanum Trel. (10 µg/ml)
Piper aff. euriphyllum Trel. (10 µg/ml)
Piper propinquum C.DC. (10 µg/ml)
Piper cf. dilatatum L.C. Rich. (10 µg/ml)
Piper denisii Trel. (10 µg/ml)
Piper allardii Trel. (10 µg/ml)
Piper aduncum L. (10 µg/ml)
Piper quimirianum Trel. (10 µg/ml)
Piper celer Trel. (10 µg/ml)
Piper subflavispicum C.DC. (10 µg/ml)
Piper longifilamentosum Trel. (10 µg/ml)
Piper soledadense Trel. (10 µg/ml)
Piper margaritanum Trel. (10 µg/ml)
Ethanol (0.05%)
Piper pseudoarboreum Yun. (10 µg/ml)
Piper aff. cupreatum Trel. (10 µg/ml)
LY
DMSO
Piper aff. cupreatum Trel.
C
PH+/FITCD- cells (%)
Piper subflavispicum C.DC. (10 µg/ml)
Piper denisii Trel. (10 µg/ml)
Piper cf. dilatatum L.C. Rich. (10 µg/ml)
Piper aff. euriphyllum Trel. (10 µg/ml)
Piper allardii Trel. (10 µg/ml)
Piper propinquum C.DC. (10 µg/ml)
Piper celer Trel. (10 µg/ml)
Piper loretoanum Trel. (10 µg/ml)
Piper quimirianum Trel. (10 µg/ml)
Piper pseudoarboreum Yun. (10 µg/ml)
Piper aduncum L. (10 µg/ml)
Piper longifilamentosum Trel. (10 µg/ml)
Piper margaritanum Trel. (10 µg/ml)
Piper soledadense Trel. (10 µg/ml)
Piper aff. cupreatum Trel. (10 µg/ml)
Ethanol (0.05%)
PI
DMSO
Piper subflavispicum C.DC.

Figure 14. Compounds present in *Piperaceae* species alter gap junction and hemichannel activity in NT2/D1 cells. (A) Piperine, cubebin, and 4,5-dihydropiperlongumine are compounds which are abundantly found in members of the *Piperaceae* family. Piperine and 4,5-dihydropiperlongumine are alkaloids. Cubebin is a lignan. (B) GJIC data are expressed as the fold change in the number of calcein+/DiI- recipient cells per calcein+/DiI+ donor cell. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. 4,5-dihydropiperlongumine (100 μM) and piperine (100 μM) significantly reduced GJIC related to ethanol control in NT2/D1 cells. Representative images depicting the effect of piperine on GJIC are shown. Scale bar = 100 μm. (C) Anionic hemichannel assay data are expressed as the mean percentage of LY+/RD- per Hoechst+ nuclei per well. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. None of the three compounds tested had a significant effect on anionic hemichannel activity in NT2/D1 cells. Scale bar = 100 μm. (D) Cationic hemichannel assay data are expressed as the mean percentage of PI+/FITCD- per Hoechst+ nuclei per well. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. 4,5-dihydropiperlongumine (100 μM) significantly increased cationic hemichannel activity relative to ethanol control in NT2/D1 cells. Scale bar = 100 μm. Statistics were one-way ANOVA, *post-hoc* Fisher's LSD test. * denotes $P < 0.05$ from ethanol control. ** denotes $P < 0.01$ from ethanol control.


A
Piperine
Cubebin
4,5-dihydropiperlongumine
B
Fold change in GJIC
1.5
1.0
0.5
0.0
**
**
4,5-dihydropiperlongumine (100 µM)
Piperine (100 µM)
DMSO (0.05%)
Cubebin (10 µM)
D
% LY+/RD- cells
20
15
10
5
0
DMSO (0.05%)
Piperine (100 µM)
4,5-dihydropiperlongumine (100 µM)
Cubebin (10 µM)
F
PI+/FITCD- cells (%)
4
3
2
1
0
*
DMSO (0.05%)
Cubebin (10 µM)
Piperine (100 µM)
4,5-dihydropiperlongumine (100 µM)
C
Calcein
DMSO
Piperine
E
LY
DMSO
Cubebin
G
PI
DMSO
4,5-dihydropiperlongumine

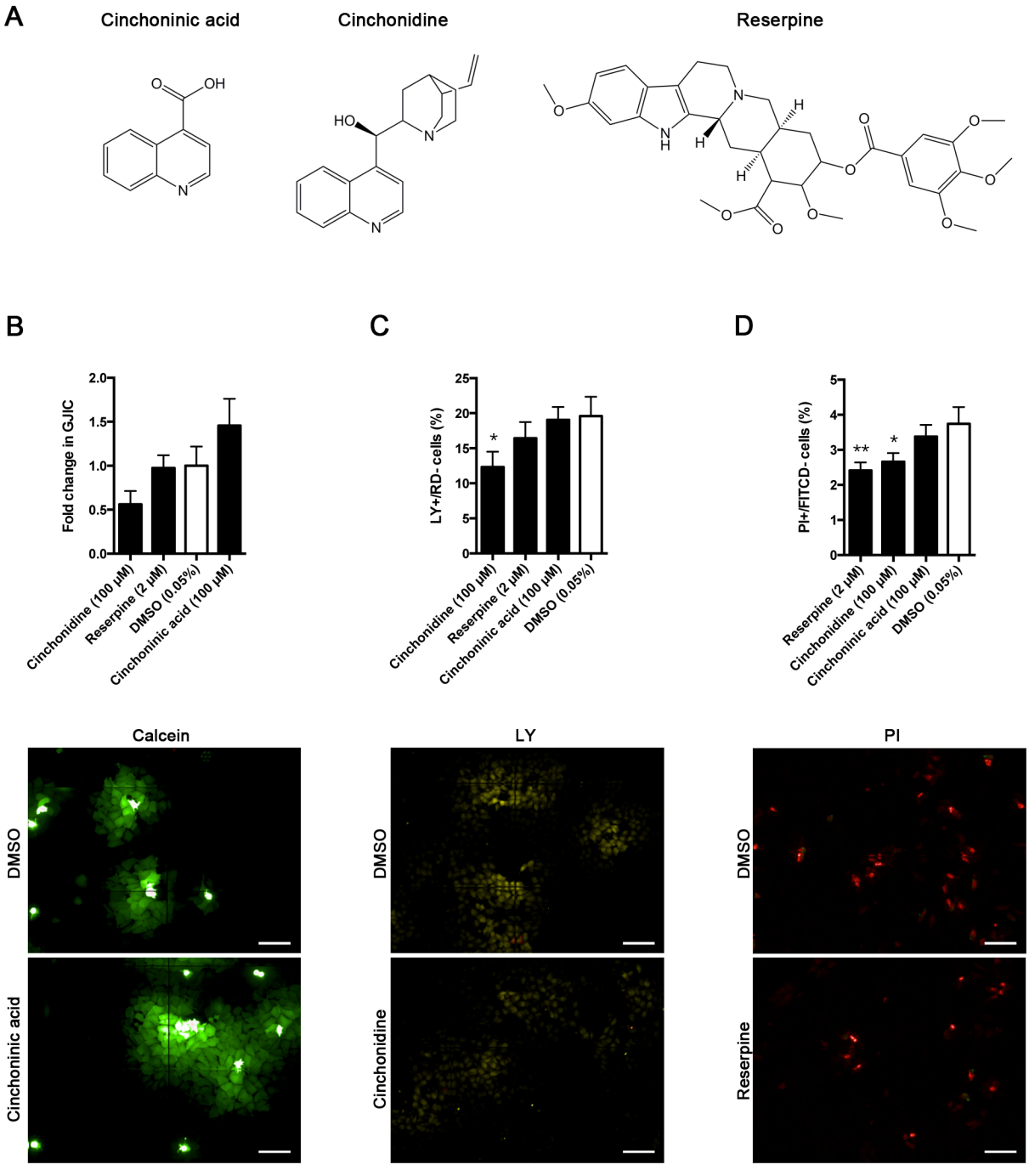
none of these compounds had an effect on anionic hemichannel activity (Figure 14C), 4,5-dihydropiperlongumine significantly increased cationic hemichannel activity (Figure 14D).

3.5 A structural approach to the identification of novel connexin and pannexin channel modulators

There are several classes of molecules that are effective in inhibiting GJIC and connexin- and pannexin-mediated hemichannel activity (reviewed in section 1.5). Existing evidence suggests that small changes in molecular structure are capable of conferring (or abolishing) activity as well as changing the degree of selectivity for individual connexin (and presumably pannexin) subtypes (47, 53, 203). Based on these observations, compounds from several classes were selected from the Arnason library for testing for capacity to alter connexin channel function.

Two alkaloids sharing the aromatic bicyclic quinoline ring with quinine and mefloquine were tested: cinchoninic acid and cinconidine (Figure 15A). Reserpine, an indole alkaloid, was also tested (Figure 15A). None of the three alkaloids tested had a significant effect on GJIC (Figure 15B). Cinchonidine treatment led to a modest decrease in both anionic (Figure 15C) and cationic (Figure 15D) hemichannel activity, while reserpine inhibited cationic hemichannel activity (Figure 15D).

Figure 15. The alkaloids cinchoninic acid, cinchonidine, and reserpine inhibit hemichannel activity in NT2/D1 cells. (A) Cinchoninic acid and cinchonidine are quinoline alkaloids related to the anti-malarial compound quinine. Reserpine is a terpenoid indole alkaloid. (B) GJIC data are expressed as the fold change in the number of calcein+/DiI- recipient cells per calcein+/DiI+ donor cell. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. No significant change in GJIC was observed following treatment with cinchoninic acid (100 μM), cinchonidine (100 μM), or reserpine (2 μM) in NT2/D1 cells. Scale bar = 100 μm. (C) Anionic hemichannel assay data are expressed as the mean percentage of LY+/RD- per Hoechst+ nuclei per well. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. A significant reduction in anionic hemichannel activity in NT2/D1 cells was found following treatment cinchonidine (100 μM). Scale bar = 100 μm. (D) Cationic hemichannel assay data are expressed as the mean percentage of PI+/FITCD- per Hoechst+ nuclei per well. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. Reserpine (2 μM) and cinchonidine (100 μM) significantly reduced cationic hemichannel activity in NT2/D1 cells. Scale bar = 100 μm. Statistics were one-way ANOVA, *post-hoc* Fisher's LSD test. * denotes $P < 0.05$ from DMSO control. ** denotes $P < 0.01$ from DMSO control.


A
Cinchoninic acid
Cinchonidine
Reserpine
O
OH
N
HO
N
O
N
H
H
H
O
O
O
O
O
O
O
B
Fold change in GJIC
2.0
1.5
1.0
0.5
0.0
Cinchonidine (100 μM)
Reserpine (2 μM)
DMSO (0.05%)
Cinchoninic acid (100 μM)
C
LY+/RD- cells (%)
25
20
15
10
5
0
*
Cinchonidine (100 μM)
Reserpine (2 μM)
Cinchoninic acid (100 μM)
DMSO (0.05%)
D
PI+/FITCD- cells (%)
5
4
3
2
1
0
**
*
Reserpine (2 μM)
Cinchonidine (100 μM)
Cinchoninic acid (100 μM)
DMSO (0.05%)
Calcein
LY
PI
DMSO
Cinchoninic acid
DMSO
Cinchonidine
DMSO
Reserpine

Three flavonoids were tested: quercetin, myricitrin and rutin (Figure 16A). Quercetin is a flavonol aglycone, while myricitrin and rutin are flavonol glycosides. Rutin is a glycoside of quercetin and the disaccharide rutinose. Myricitrin is a glycoside of the alglycone myricetin and the monosaccharide rhamnose. None of the three flavonoids tested had a significant effect on GJIC (Figure 16B) or anionic hemichannel activity (Figure 16C). Quercetin, myricitrin and rutin all significantly reduced cationic hemichannel activity (Figure 16D).

Three pentacyclic triterpenoids related to glycyrrhetinic acid were also tested: betulinic acid, asiatic acid and asiaticoside, a glycoside of asiatic acid conjugated to two glucose residues and one rhamnose residue (Figure 17A). None of the three compounds significantly altered GJIC (Figure 17B). There was also no significant effect on anionic hemichannel activity observed (Figure 17C), however asiaticoside and betulinic acid both significantly reduced cationic hemichannel activity (Figure 17D).

3.6 A synthetic approach to the identification of novel connexin and pannexin channel modulators

AF101 is a β-amino sulfoxide compound containing three phenyl groups (Figure 18A). One phenyl group is attached to carbon, one to nitrogen, and the third to the sulfoxide group. It was identified as a lead compound in preliminary screens as a result of its ability to inhibit GJIC (Figure 18B). To gain more information on the

Figure 16. The flavonoids quercetin, myricitrin and rutin inhibit cationic hemichannel activity in NT2/D1 cells. (A) Quercetin is a flavonol aglycone. Rutin is a glycoside of quercetin and the disaccharide rutinose. Myricitrin is a glycoside of the algycone myricetin and the monosaccharide rhamnose. (B) GJIC data are expressed as the fold change in the number of calcein+/DiI- recipient cells per calcein+/DiI+ donor cell. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. No significant change in GJIC was observed following treatment with quercetin (5 μM), myricitrin (25 μM), or rutin (25 μM) in NT2/D1 cells. Scale bar = 100 μm. (C) Anionic hemichannel assay data are expressed as the mean percentage of LY+/RD- per Hoechst+ nuclei per well. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. Quercetin (5 μM), myricitrin (25 μM) and rutin (25 μM) did not significantly alter anionic hemichannel activity in NT2/D1 cells. Scale bar = 100 μm. (D) Cationic hemichannel assay data are expressed as the mean percentage of PI+/FITCD- per Hoechst+ nuclei per well. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. All three flavonoids tested significantly reduced cationic hemichannel activity in NT2/D1 cells. Scale bar = 100 μm. Statistics were one-way ANOVA, *post-hoc* Fisher's LSD test. * denotes $P < 0.05$ from ethanol control. ** denotes $P < 0.01$ from ethanol control.

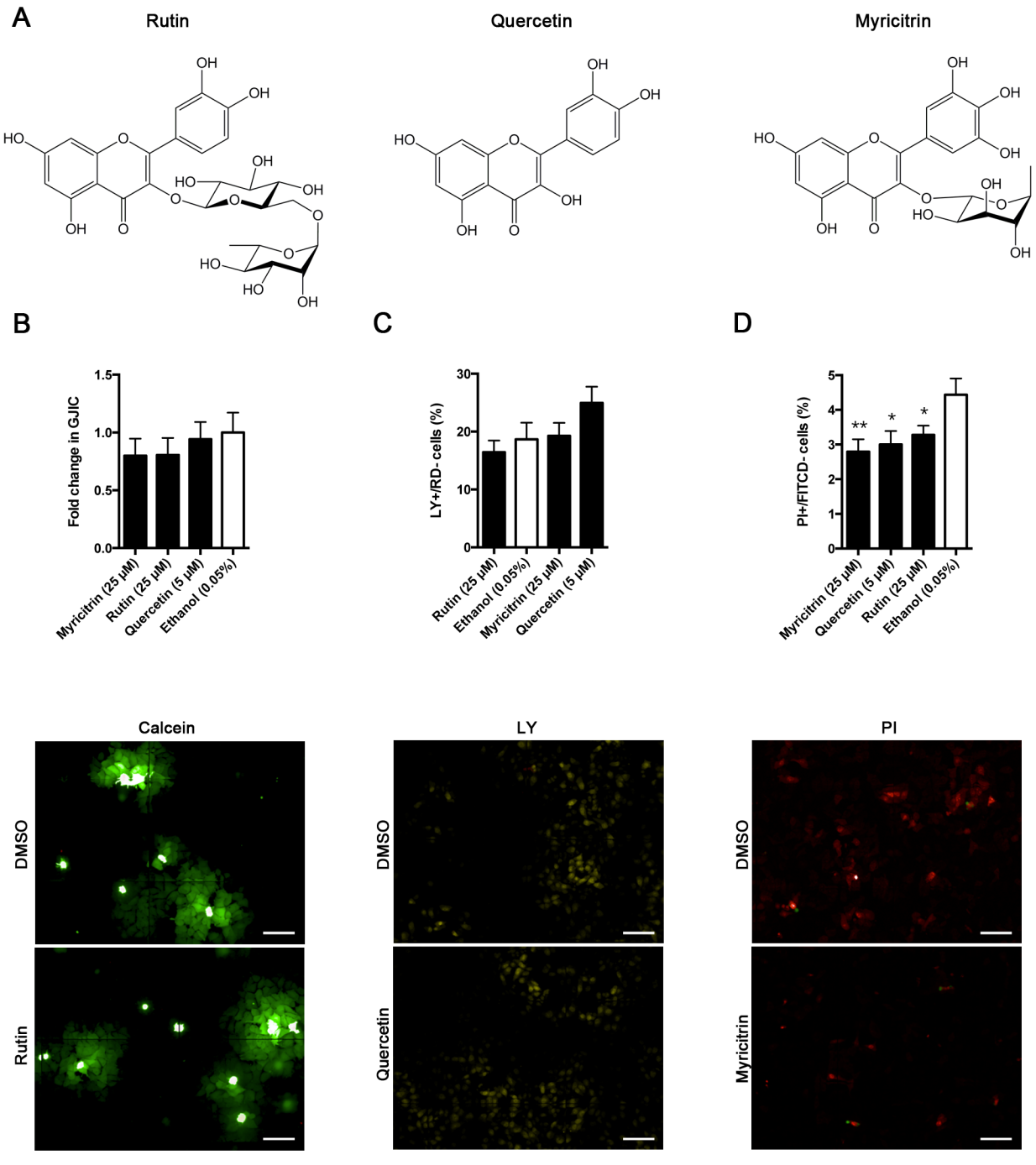

A
Rutin
Quercetin
Myricitrin
B
Fold change in GJIC
Myricitrin (25 μM)
Rutin (25 μM)
Quercetin (5 μM)
Ethanol (0.05%)
C
LY+/RD- cells (%)
Rutin (25 μM)
Ethanol (0.05%)
Myricitrin (25 μM)
Quercetin (5 μM)
D
PI+/FITCD- cells (%)
Myricitrin (25 μM)
Quercetin (5 μM)
Rutin (25 μM)
Ethanol (0.05%)
Calcein
LY
PI
DMSO
Rutin
DMSO
Quercetin
DMSO
Myricitrin

Figure 17. The triterpenoids betulinic acid and asiaticoside inhibit cationic hemichannel activity in NT2/D1 cells. (A) Betulinic acid and asiatic acid are pentacyclic triterpenoids. Asiaticoside is a glycoside of asiatic acid and the sugars glucose (Glu), glucose, and rhamnose (Rha). (B) GJIC data are expressed as the fold change in the number of calcein+/DiI- recipient cells per calcein+/DiI+ donor cell. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. No significant change in GJIC was observed following treatment with betulinic acid (2 μM), asiatic acid (4 μM), or asiaticoside (100 μM) in NT2/D1 cells. Scale bar = 100 μm. (C) Anionic hemichannel assay data are expressed as the mean percentage of LY+/RD- per Hoechst+ nuclei per well. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. All three triterpenoids tested had no significant effect on anionic hemichannel activity in NT2/D1 cells. Scale bar = 100 μm. (D) Cationic hemichannel assay data are expressed as the mean percentage of PI+/FITCD- per Hoechst+ nuclei per well. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. Asiaticoside (100 μM) and betulinic acid (2 μM) significantly reduced cationic hemichannel activity in NT2/D1 cells. Scale bar = 100 μm. Statistics were one-way ANOVA, *post-hoc* Fisher's LSD test. * denotes $P < 0.05$ from DMSO control. ** denotes $P < 0.01$ from DMSO control.

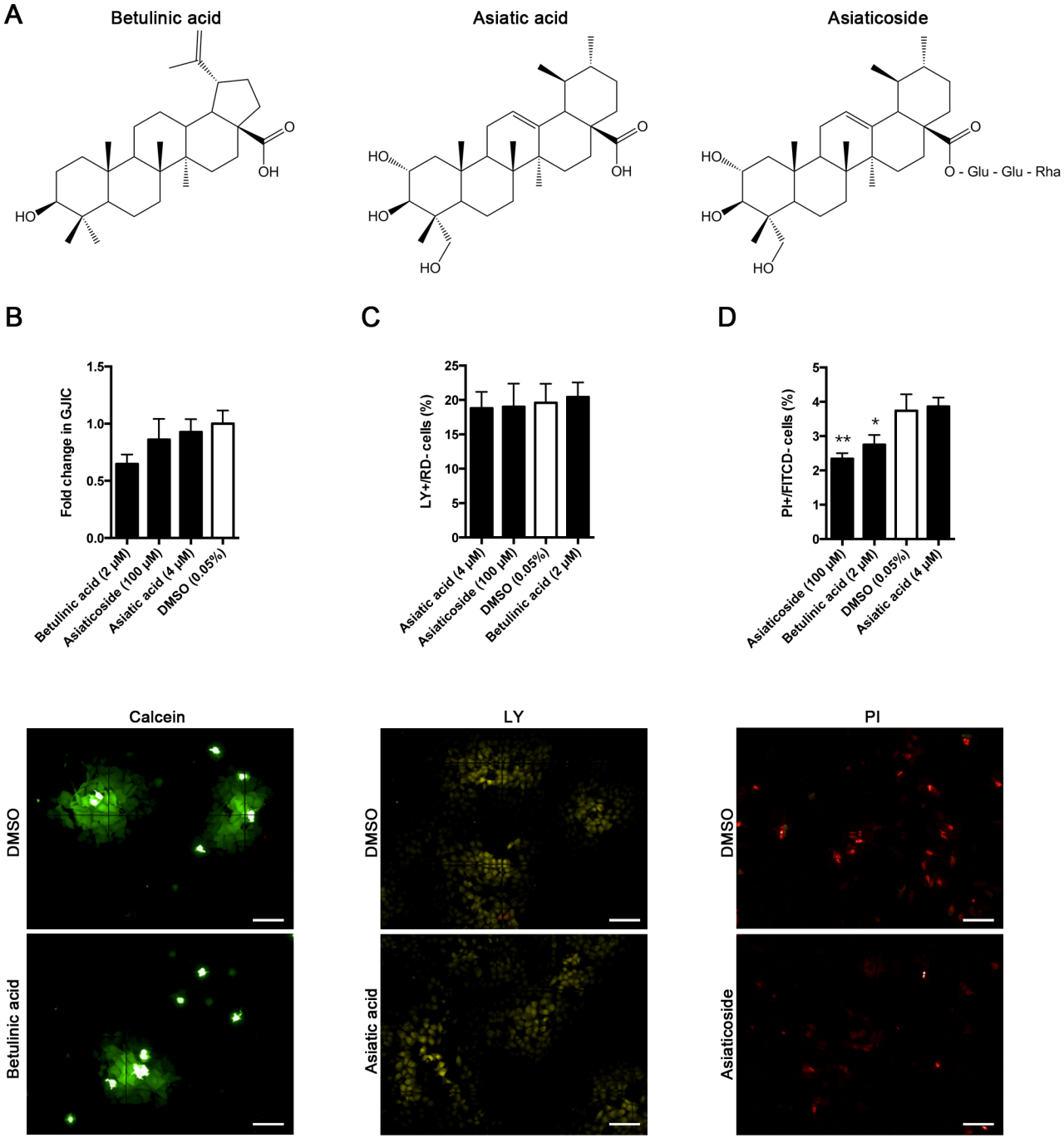
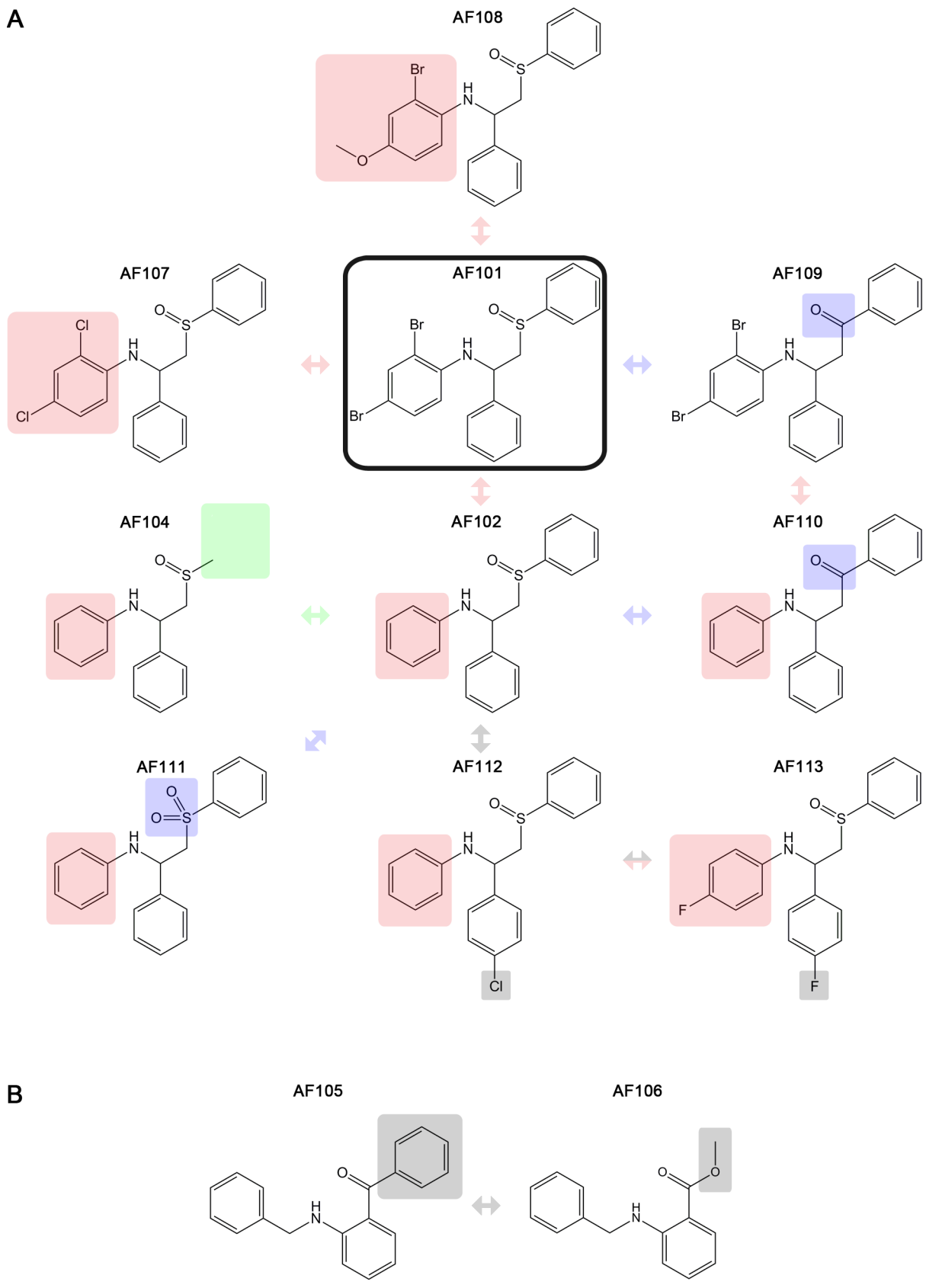

A
Betulinic acid
Asiatic acid
Asiaticoside
HO
OH
O
O - Glu - Glu - Rha
B
Fold change in GJIC
1.5
1.0
0.5
0.0
Betulinic acid (2 μM)
Asiaticoside (100 μM)
Asiatic acid (4 μM)
DMSO (0.05%)
C
LY+/RD- cells (%)
25
20
15
10
5
0
Asiatic acid (4 μM)
Asiaticoside (100 μM)
DMSO (0.05%)
Betulinic acid (2 μM)
D
PI+/FITCD- cells (%)
5
4
3
2
1
0
**
*
Asiaticoside (100 μM)
Betulinic acid (2 μM)
DMSO (0.05%)
Asiatic acid (4 μM)
Calcein
LY
PI
DMSO
Betulinic acid
Asiatic acid
Asiaticoside

Figure 18. Structures of AF101 and its analogs. (A) AF101 is a β-amino sulfoxide compound containing three phenyl groups. One phenyl group is attached to carbon, one to nitrogen, and the third to the sulfoxide group. Nine analogs of AF101 were synthesized in which changes were made to the sulfoxide group and to the substituents on the phenyl groups. The relationship of each compound to parent AF101 is depicted. (B) AF105 and AF106 are structural isomers of AF101.


A
AF108
AF107
AF101
AF109
AF104
AF102
AF110
AF111
AF112
AF113
B
AF105
AF106

functional groups responsible for this activity, a structural deconvolution was carried out and 11 related compounds were tested (Figure 18A).

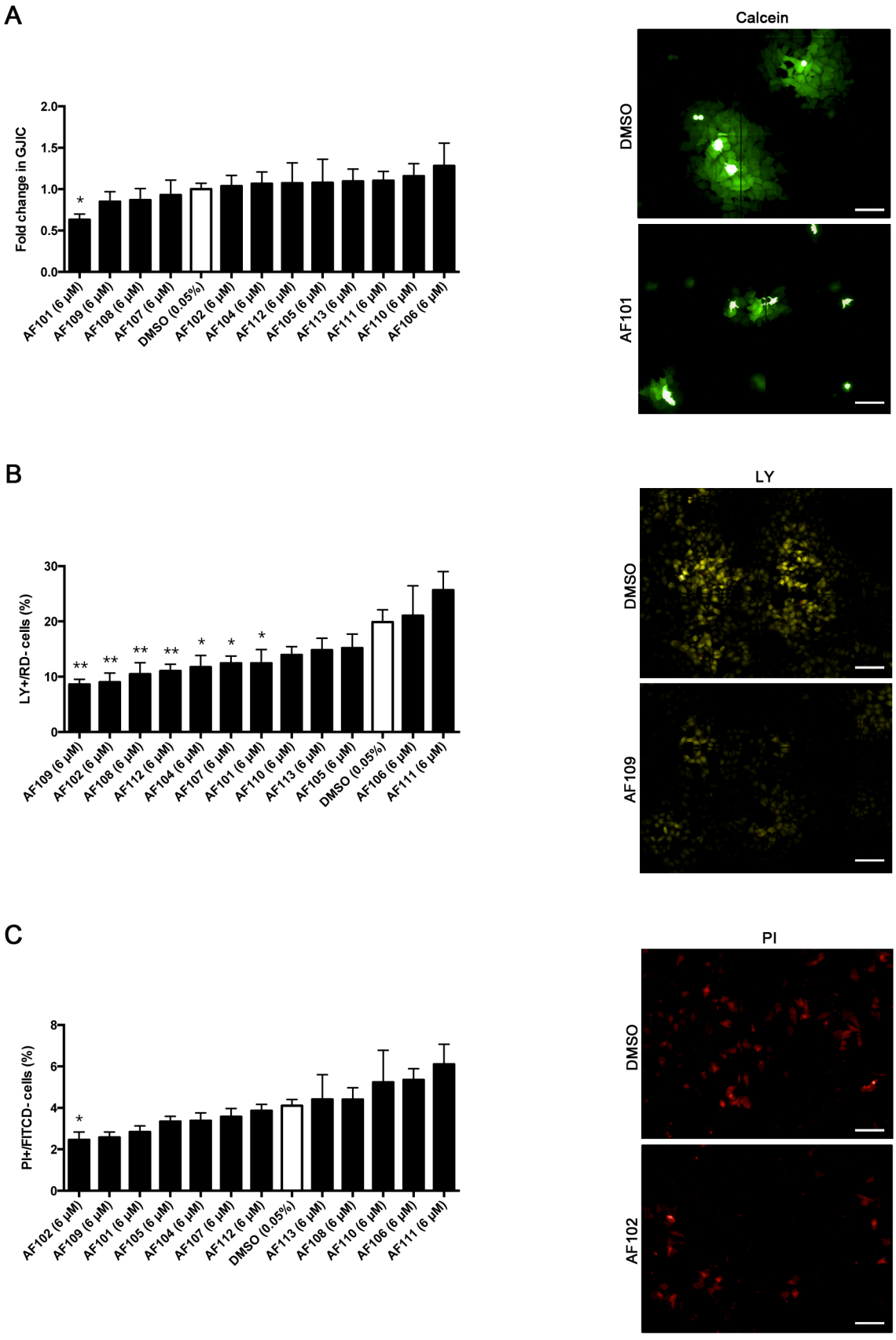
Four of these compounds are directly related to AF101. AF102 and AF107 contain a phenyl group and an m-dichlorobenzene group, respectively, in place of the amine-linked m-dibromobenzene group found in AF101. AF108 substitutes a methoxy group for the bromine para to the nitrogen in AF101. Finally, AF109 contains a carbonyl group in place of the sulfoxide in AF101.

A further five of the analogs are directly related to AF102. AF104 substitutes a methyl group for the S-linked phenyl group in AF102. AF110 and AF111 contain a carbonyl and a sulfone, respectively, in place of the sulfoxide group in AF102. AF112 and AF113 contain a chlorine and fluorine, respectively, on the C-linked phenyl group. AF113 additionally contains a fluorine on the amine-linked phenyl group, located para to the nitrogen.

The last two analogs have the phenyl groups arranged in a different orientation. AF105 is a structural isomer of AF110 in which the carbon chain linking the nitrogen and carbonyl carbon is fixed by a phenyl ring (Figure 18B). AF106 has a methoxy group in place of the carbonyl-linked phenyl group in AF105.

AF101 was the only compound that significantly inhibited anionic GJIC (Figure 19A). AF101, along with AF102, AF104, AF107, AF108, AF109 and AF112 significantly

Figure 19. AF101 and its derivatives are novel inhibitors of gap junction and hemichannel activity. (A) GJIC data are expressed as the fold change in the number of calcein+/DiI- recipient cells per calcein+/DiI+ donor cell. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. AF101 (6 μM) was found to significantly decrease GJIC in NT2/D1 cells. Scale bar = 100 μm. (B) Anionic hemichannel assay data are expressed as the mean percentage of LY+/RD- per Hoechst+ nuclei per well. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. AF109 (6 μM), AF102 (6 μM), AF108 (6 μM), AF112 (6 μM), AF104 (6 μM), AF107 (6 μM) and AF101 (6 μM) caused a significantly reduction in anionic hemichannel activity in NT2/D1 cells. Scale bar = 100 μm. (C) Cationic hemichannel assay data are expressed as the mean percentage of PI+/FITCD- per Hoechst+ nuclei per well. Data represent the mean plus standard error of the mean (SEM) of 20 fields averaged to yield a single data point per well conducted in replicates of six wells and repeated in duplicate experiments, yielding n=12 per condition. AF102 (6 μM) significantly reduced cationic hemichannel activity in NT2/D1 cells. Scale bar = 100 μm. Statistics were one-way ANOVA, *post-hoc* Fisher's LSD test. * denotes $P < 0.05$ from DMSO control.


A
Fold change in GJIC
2.0
1.5
1.0
0.5
0.0
*
AF101 (6 μM)
AF109 (6 μM)
AF108 (6 μM)
AF107 (6 μM)
DMSO (0.05%)
AF102 (6 μM)
AF104 (6 μM)
AF112 (6 μM)
AF105 (6 μM)
AF113 (6 μM)
AF111 (6 μM)
AF110 (6 μM)
AF106 (6 μM)
Calcein
DMSO
AF101
B
LY+/RD- cells (%)
30
20
10
0
**
**
**
**
*
*
*
AF109 (6 μM)
AF102 (6 μM)
AF108 (6 μM)
AF112 (6 μM)
AF104 (6 μM)
AF107 (6 μM)
AF101 (6 μM)
AF110 (6 μM)
AF113 (6 μM)
AF105 (6 μM)
DMSO (0.05%)
AF106 (6 μM)
AF111 (6 μM)
LY
DMSO
AF109
C
PI+/FITCD- cells (%)
8
6
4
2
0
*
AF102 (6 μM)
AF109 (6 μM)
AF101 (6 μM)
AF105 (6 μM)
AF104 (6 μM)
AF107 (6 μM)
AF112 (6 μM)
DMSO (0.05%)
AF113 (6 μM)
AF108 (6 μM)
AF110 (6 μM)
AF106 (6 μM)
AF111 (6 μM)
PI
DMSO
AF102

inhibited anionic hemichannel activity (Figure 19B). AF102 was the only compound that significantly inhibited cationic hemichannel activity (Figure 19C).

4 DISCUSSION

4.1 Summary

In this thesis, I sought to use three complementary approaches to the targeted screening of a large, ethnobotanically-derived library to address my overarching objective of identifying compounds that selectively alter connexin and/or pannexin channel function. First, I characterized the repertoire of connexins and pannexins expressed at the mRNA level in NT2/D1 cells, post-mortem human hippocampus and gap junction-deficient HeLa cells. Second, I identified novel activity within the *Piperaceae* family for capacity to modulate gap junctions and hemichannels. Third, I examined the activity of compounds within the families of known connexin and pannexin inhibitors. Finally, I defined a novel class of β-amino sulfoxide gap junction and hemichannel blockers.

4.2 NT2/D1 cells are a suitable *in vitro* model of human hippocampus connexin and pannexin expression

This thesis presents the first extensive analysis of the connexin and pannexin expression profile of post-mortem human hippocampus at the mRNA level. Here, it was shown that this tissue expresses Cx26, Cx30, Cx32, Cx36, Cx37, Cx43, Cx45, Cx47, Panx1 and Panx2. Parallel analysis of NT2/D1 neural progenitor-like cells confirmed previous findings that these cells express Cx30, Cx36, Cx37 and Cx43 (25) and also, for the first time, demonstrated mRNA expression in these cells of

Cx26, Cx45, Cx47, Panx1 and Panx2. Connexin and pannexin expression is highly cell type-specific *in vivo* (13, 245); therefore, while these data demonstrate that human NT2/D1 cells closely model the connexin and pannexin transcripts expressed in human hippocampus tissue as a whole, they do not give information on which cell type(s) within the hippocampus NT2/D1 cells most closely model. Our lab has previously shown that murine postnatal Type 2a hippocampal neural progenitors express at least Cx26, Cx30, Cx37, Cx40, Cx43 and Cx45 (100). These data therefore suggest a phenotypic relation between NT2/D1 and Type 2a neural progenitor cells.

Surprisingly, RT-PCR profiling of HeLa cells indicated that these cells endogenously express Cx26, Cx37, Cx43, Cx45, Cx47, Panx1 and Panx2 at the mRNA level. This finding was unexpected, given that these cells are commonly used as an *in vitro* cellular model deficient in functional connexin channel activity (20, 35, 63, 135). This suggests that the mRNA expression of these connexins likely does not result in functional GJIC or connexin-mediated hemichannel activity in HeLa cells. Importantly, these data emphasize the inability to reliably equate mRNA expression with the presence of functional protein. Immunoblot and immunofluorescence studies were not performed in this thesis to assess protein expression. However, in the context of this study, this implies that caution should be exercised in ascribing the activity observed to all connexins and pannexins expressed at the mRNA level by NT2/D1 cells.

4.3 GJIC and dye uptake assays measure both connexin and pannexin channel activity

In this thesis, the flux of the anionic dye calcein (623 Da, charge 4-) between adjacent cells was quantified as a means of assessing GJIC. The intercellular diffusion of calcein is only observed in cells expressing connexins and is inhibited by the gold standard connexin and pannexin inhibitors CBX and glycyrrhetinic acid but not their inactive analog glycyrrhizic acid (83). When used at 10 μM, CBX effectively blocks Panx1 and Panx2, but not connexins, which require a concentration of 100 μM for inhibition (5, 29). Here, CBX at a concentration of 100 μM caused a nearly complete inhibition of GJIC in NT2/D1 cells, whereas 10 μM CBX had no significant effect relative to control. These results indicate that connexins, and not pannexins, are mediating the intercellular diffusion of calcein measured in this assay. Further, these data provide support for the general consensus in the field that endogenously-expressed pannexins function exclusively as hemichannels (197). This assay, therefore, enables the quantification of anionic GJIC.

To assess hemichannel activity, uptake of anionic LY (444 Da, charge 2-) and cationic PI (415 Da, charge 2+) from calcium-free medium was measured. As expected, 100 μM CBX significantly reduced uptake of both LY and PI relative to PBS control, indicating that pannexin and/or connexin hemichannels were the major facilitators of this process. Interestingly, and in contrast to what was observed in GJIC assays, a pannexin-specific concentration of CBX (10 μM) was also effective in reducing LY and PI uptake in the absence of extracellular calcium. Taken

together, these data indicate that hemichannels composed of Panx1 and/or Panx2 are the channels mediating the uptake of LY and PI measured in these assays. Since connexin and not pannexin hemichannels are directly gated by millimolar concentrations of extracellular calcium, calcium was added to dye uptake medium to the concentration present in normal growth medium (1.05 mM). Surprisingly, given what was observed using CBX, this led to a significant reduction in uptake of both LY and PI.

Initially, these results appear to be incompatible with pannexin-mediated dye uptake. However there are several potential explanations for this disconnect. First, while it is accepted that homomeric Panx1 and heteromeric Panx1/Panx2 hemichannel opening are not influenced by extracellular calcium (29), the mechanisms regulating Panx2 (and Panx3) gating have received little attention and an insensitivity to extracellular calcium has been neither confirmed nor refuted in the literature. While speculative, it is possible that Panx1 and Panx2 may respond differentially to changes in extracellular calcium and that Panx2 hemichannels are mediating the uptake of LY and PI measured in these assays. This is plausible given the differences in oligomeric number (Panx1 six, Panx2 eight) (5) and N-glycosylation (Panx1 Asn254 first extracellular loop, Panx2 Asn86 second extracellular loop) (23) that exist between these two channels. Second, there is evidence to suggest that reductions in extracellular calcium can indirectly activate pannexins. For example, the removal of extracellular calcium can elicit calcium waves in primary cortical astrocyte cultures that propagate intracellularly via gap junctions or extracellularly via ATP-mediated paracrine signaling (249). Panx1, coupled with the purinergic

P2X7 receptor, is now understood to be the key ATP-releasing pore involved in this pathway (168). Interestingly, the affinity of the P2X7 receptor for ATP is increased by low extracellular calcium (209, 233). Therefore, this represents a second mechanism by which pannexin hemichannel activation in response to reductions in extracellular calcium may be occurring in these assays. Future work in which Panx1 and/or Panx2 are transiently knocked down using RNA interference would help clarify which pannexin(s) are facilitating the dye uptake measured in these assays.

A notable difference between LY and PI uptake assays is the effect of simultaneous application of extracellular calcium ($CaCl_2$), which blocks hemichannels, and 100 μM CBX, which blocks both hemichannels and gap junctions. While $CaCl_2$ significantly (though not completely) reduced LY uptake below PBS control, a further, statistically significant decrease was observed when 100 μM CBX was additionally applied. This suggests that LY is diffusing to gap junction-coupled cells following uptake via pannexin hemichannels from the extracellular medium, as is clearly observed in the photomicrographs. In contrast, there is no significant difference in PI uptake when cells are treated with $CaCl_2$ alone or with 100 μM CBX, suggesting that that there is little, if any, intercellular PI diffusion occurring following pannexin-mediated uptake. These contrasting results may be the result of a greater permeability of anionic LY than cationic PI through the connexin channels expressed in NT2/D1 cells or, alternatively, by the cytoplasmic retention of PI, since it strongly binds nucleic acids (91).

Interestingly, it is evident from Figure 11A that the intercellular diffusion of LY following uptake was blocked at the pannexin-specific concentration of 10 μM CBX. This concentration resulted in maximal inhibition with both dyes since there was no significant increase in either LY+/RD- or PI+/FITCD- cells when CBX concentration was increased to 100 μM. Together, these data suggest connexin or pannexin hemichannels do not mediate dye uptake in these remaining LY+/RD- and PI+/FITCD- cells. Further, the lack of LY diffusion to gap junction-coupled cells at the pannexin-specific concentration of 10 μM CBX suggests that residual LY (and possibly PI) is not localized within the cytosol where it would be capable of diffusing through gap junctions. One possible explanation, given that LY is a well-established marker of fluid phase endocytosis (21, 211), is that this dye is sequestered in pinocytotic vesicles. Regardless of the mechanism responsible for CBX-insensitive LY and PI uptake, the significant reduction in LY+/RD- and PI+/FITCD- cells elicited by treatment with 10 μM CBX is consistent with LY and PI uptake through pannexin hemichannels.

The results discussed above are summarized in Figure 20A. The calcein-based parachute assay is sensitive to changes in connexin-mediated GJIC, while the PI uptake assay measures single-membrane pannexin hemichannel activity. Since LY passes freely though connexin gap junctions following pannexin-mediated uptake from the extracellular medium, the LY-based assay reflects both connexin and pannexin channel activity. It should be noted that the connexins and likely pannexins expressed in NT2/D1 are not equally permeable to calcein, LY and PI (Table 2). The implications of this, given that anionic tracers (calcein and LY) are poorly permeable

through Cx30, Cx36 and Cx45 channels, is that the methodology of this study may not be able to detect changes in the activity of these channels.

	Calcein (623 Da, 4-)	**LY (444 Da, 2-)**	**PI (415 Da, 2+)**
Cx26	Permeable (20, 248)	Permeable (20, 35, 63, 134, 248)	Permeable (35, 63, 210, 248)
Cx30	Impermeable (20, 248)	Impermeable (20, 134, 248)	Permeable (210, 248)
Cx36	Not shown; unlikely (91)	Poorly permeable (37)	Permeable (37)
Cx37	Permeable (229)	Permeable (63)	Permeable (63)
Cx43	Permeable (62)	Permeable (37, 62, 63, 139, 180, 205)	Permeable (37, 63, 180)
Cx45	Not shown; unlikely (91)	Poorly permeable (35, 63, 139, 180, 205)	Permeable (35, 63, 180)
Cx47	Permeable (3)	Permeable (162)	Not previously shown
Panx1	Permeable (218)	Permeable (60)	Not previously shown
Panx2	Not previously shown	Not previously shown	Not previously shown

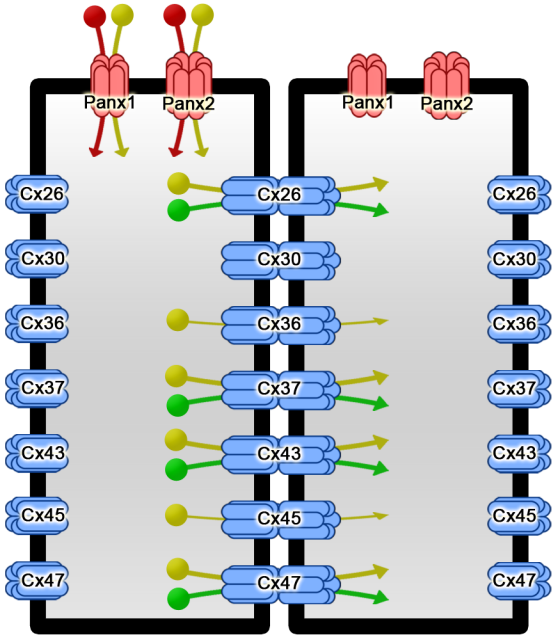
Table 2. Tracer permeability through connexin and pannexin channels.

4.4 Patterns of activity in calcein, LY and PI assays reveal connexin and pannexin family selectivity

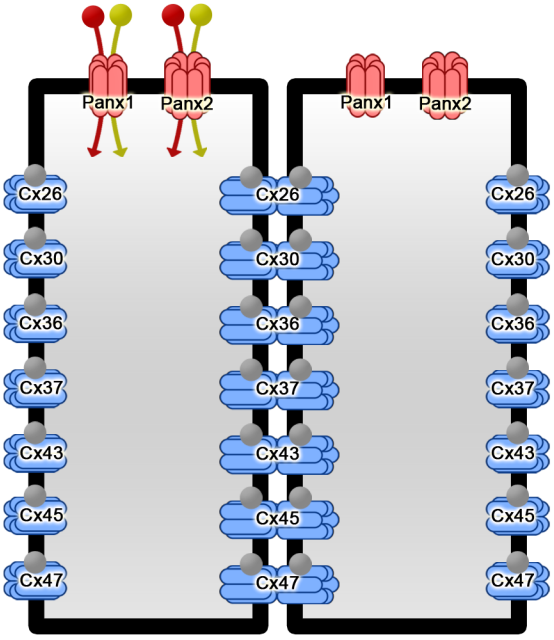
This thesis has described several compounds and extracts capable of altering connexin and pannexin channel activity. Based on their activity in the bioassays in this study, lead compounds and extracts may be grouped as 1) connexin-specific (Figure 20B), 2) pannexin-specific (Figure 20C), or 3) nonselective gap junction and hemichannel inhibitors (Figure 20D).

Figure 20. Mechanisms of compound action in GJIC and dye uptake assays. (A) Untreated or vehicle-treated cells freely take up LY (yellow) and PI (red) in the absence of extracellular calcium through Panx1 and/or Panx2 channels. Following uptake, LY efficiently diffuses to neighbouring cells coupled by gap junctions containing Cx26, Cx37, Cx43, or Cx47, or less efficiently through Cx36 or Cx45 gap junctions. Calcein-positive cells pass calcein (green) to neighbouring coupled cells through Cx26, Cx37, Cx43, or Cx47 gap junctions. (B) Connexin-specific inhibitors (grey) would abolish or reduce GJIC between neighbouring coupled cells, as assessed by intercellular calcein and LY diffusion. (C) Pannexin-specific inhibitors (grey) would abolish or reduce Panx1- and/or Panx2-mediated uptake of LY and PI while leaving connexin-mediated GJIC intact. (D) Non-selective inhibitors of pannexin and connexin channels (grey) would abolish both pannexin-mediated LY and PI uptake and connexin-mediated GJIC. Note: only homomeric hemichannel and homotypic gap junction formation is pictured for clarity.

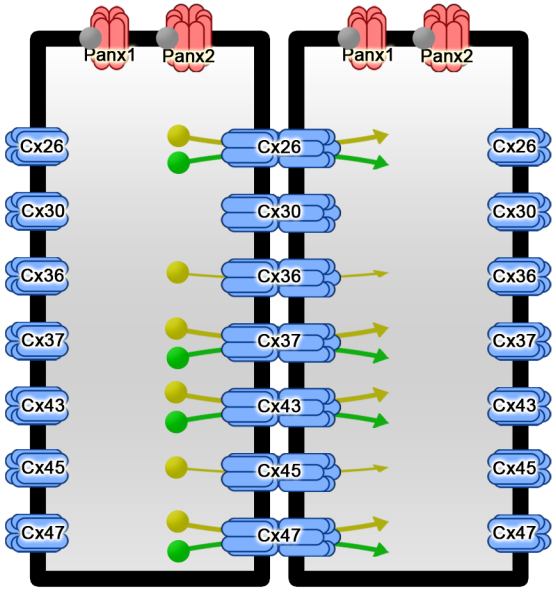
A - untreated



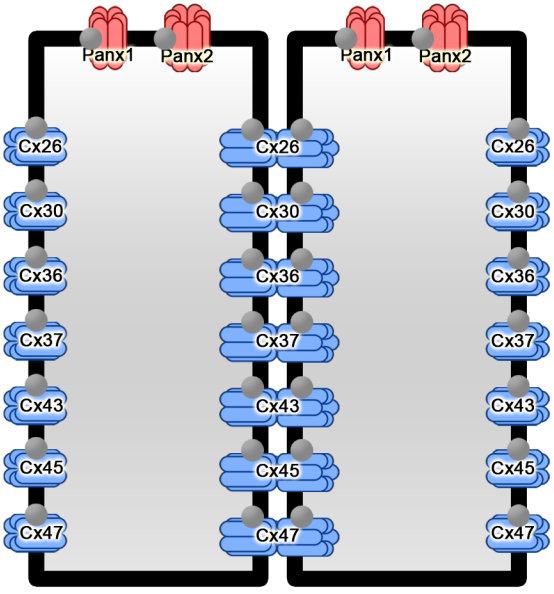
B - connexin-specific inhibition



C - pannexin-specific inhibition



D - non-selective inhibition



calcein (623 Da, 4-) lucifer yellow (444 Da, 2-) propidium (415 Da, 2+)

4.4.1 Connexin-specific modulators

Piper aduncum L. and *Piper quimirianum* Trel. treatment led to a significant increase in connexin-mediated GJIC. Both extracts were without effect in anionic hemichannel assays, while they led to a significant decrease in cationic hemichannel activity. Of note, these treatments were the only tested that were found capable of increasing GJIC, strongly hinting that these extracts may be enriched in compounds that either activate connexin channels or increase gap junctional stability.

In an effort to determine which compounds may be responsible, piperine, 4,5-dihydropiperlongumine and cubebin were selected for testing based on their predicted enrichment in the Piper extracts tested in this thesis. Piperine and 4,5-dihydropiperlongumine are typical piperamide alkaloids that the *Piperaceae* family is well known for (165). Cubebin, a lignan, also belongs to a class of compounds typically found in Pipers (165).

Piperine and 4,5-dihydropiperlongumine both strongly inhibited connexin-mediated GJIC, reducing the number of calcein-positive recipient cells to less than half of the vehicle treatment. At the same concentration, neither compound was active in LY assays, while 4,5-dihydropiperlongumine led to a modest but statistically significant increase in PI uptake. These data, along with the structural similarity between piperine and 4,5-dihydropiperlongumine, suggest these compounds may be acting in a similar mechanism to block connexin-mediated GJIC without blocking (and perhaps even enhancing) pannexin hemichannel activity.

In light of the activity of the extracts, and *Piper aduncum* L. in particular, these results are quite surprising given that piperine and 4,5-dihydropiperlongumine are having the opposite effect. This suggests that these compounds are not particularly enriched in these extracts and that the activity of *Piper aduncum* L. is the result of another compound or group of compounds not yet tested. The essential oil of Amazonian *Piper aduncum* L. is well known to be enriched in dillapiole, a phenylpropanoid (56, 133). Since the initial submission of this thesis, the extract of *Piper aduncum* L. has been analyzed by thin layer chromatography and the typical signals for a methylenedioxyphenyl group could not be detected, indicating that this particular extract is not enriched in dillapiole or piperamides (Dr. Tony Durst, personal communication, June 24, 2013). A bioassay-guided fractionation of *Piper aduncum* L. is underway in hopes of identifying the compound or compound(s) responsible for its activity. The relative scarcity of available compounds that activate connexin-mediated communication suggests that *Piper aduncum* L. holds great promise as the source of novel connexin-modulating compounds not previously reported in the literature.

Interestingly, piperine has documented use in Chinese traditional medicine to treat seizures (167) and biochemical evidence has been provided *in vivo* supporting its anticonvulsant properties (147). More recently, piperine has been shown to inhibit synchronized intercellular calcium oscillations in primary hippocampal neurons and protect cells from glutamate-induced apoptosis (73). This is the first report of an effect on connexins by piperine, opening the possibility that this may be a

mechanism by which it exerts its antiepileptic effects. It would be interesting to investigate this hypothesis in greater detail and establish whether piperine (and 4,5-dihydropiperlongumine) preferentially block channels formed by different connexins. This could be accomplished using primary cultures derived from connexin knockout mice readily available in the Bennett laboratory. Further, given the differential effects of piperine and 4,5-dihydropiperlongumine on pannexin-mediated PI uptake, it would be interesting to explore whether the structural differences between these compounds affect their affinity for pannexin channels and whether modifications to these compounds could yield even more connexin-specific compounds.

4.4.2 Pannexin-specific inhibitors

Asiaticoside, betulinic acid, quercetin, myricitrin, rutin, reserpine and 12 of the 15 *Piperaceae* extracts tested significantly reduced PI uptake without having a significant effect in calcein and LY assays. These results are consistent with blockage of Panx1 and/or Panx2 but not connexin channels by the compounds/extracts in question.

The activity of the majority of the *Piperaceae* extracts in cationic hemichannel assays suggests that there may be something common to the *Piperaceae* family that blocks pannexin channels. Although the constituents of these particular extracts have not been thoroughly examined to date, it is possible to speculate on what they may be. Given that quercetin, myricitrin and rutin had similar effects, it is possible that flavonoids may be contributing to the activity of the extracts. Supporting this,

flavonoids have been regularly isolated from a wide range of *Piperaceae* species (165). Another possibility is that fatty acid methyl esters typically present in leaf extracts are accounting for the activity. If they are present in significant concentrations, this is plausible, given that fatty acids and their derivatives have well documented effects on gap junctions and hemichannels (93). A bioassay-guided fractionation of the most active *Piperaceae* extracts identified in this thesis should be performed to clarify the source of the activity.

It is possible that these compounds are directly inhibiting Panx1 and or Panx2 channels. It should be noted, however, that there are also pannexin-independent and indirect mechanisms by which a reduction in PI uptake could occur. In PI assays, approximately 50% of PI-positive cells are insensitive to CBX. Therefore, the reduction in PI-positive cells observed following treatment with these compounds could be achieved independently of pannexin hemichannels by targeting this CBX-insensitive pathway. Whether a synergistic effect following simultaneous application of CBX and a particular compound or extract is observed would clarify which pathway the compound/extract is acting on. If no synergistic effect is observed, it implies that the compound or extract is altering activity of the pannexin-dependent pathway. While a direct interaction with Panx1 and/or Panx2 is possible, inhibition of P2X7 receptors could also potentially explain these results. This mechanism could be ruled out if compound activity is retained when applied simultaneously with a P2X7 receptor antagonist, such as KN-62 (98).

Given that the LY assay also measure pannexin hemichannel activity, it is surprising that none of these treatments had a significant effect in this assay. Since this assay is sensitive to changes in both pannexin hemichannel and connexin gap junction activity, it is possible that any modest effects elicited by these compounds on pannexins are masked. To address this, additional experiments could be conducted at low cell density to minimize the presence of GJIC or by treating cells simultaneously with a connexin inhibitor that does not significantly alter pannexin channel function, such as flufenamic acid. Future work should explore this possibility. Nonetheless, these data represent an important first step in identifying novel pannexin-specific pharmacological agents.

4.4.3 Nonselective gap junction and hemichannel blockers

In dye uptake assays, cinchonidine significantly reduced the number of LY+/RD- and PI+/FITCD- cells. In GJIC assays, cinchonidine also reduced the number of calcein-positive recipient cells to nearly half of vehicle, though this did not reach statistical significance (P value = 0.15). Cinchonidine is closely related to quinine and mefloquine, potent inhibitors of Panx1, Cx36 and, to a lesser extent, other connexins (47, 203). These results suggest that cinchonidine retains the activity of these compounds to some extent. Since it is well known that quinine and mefloquine are able to discriminate to varying degrees between different connexin channels, it would be interesting to determine if the same is true for cinchonidine. Given that NT2/D1 cells express multiple connexins, it is tempting to speculate that the modest

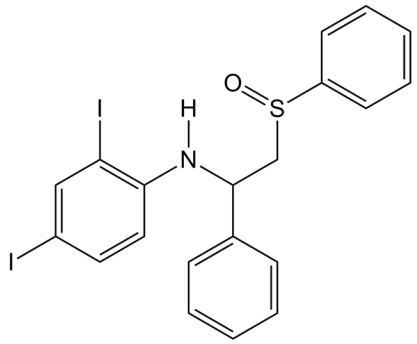
effect of cinchonidine in GJIC assays is the result of a selective block of a single connexin family member.

AF101 and its 11 derivatives had differential effects in GJIC and dye uptake assays. None of the derivatives retained the activity of AF101 in GJIC assays, however AF101 and six analogs were active in anionic hemichannel assays. AF102 was the only analog that inhibited cationic hemichannel activity. Collectively, these data imply that this class of compounds is capable of interacting with both connexin and pannexin channels. Given the preliminary state of the deconvolution, the mechanisms by which these compounds are modulating connexin and pannexin channel activity are still unknown. However, some conclusions can be drawn regarding the functional groups that are important for activity.

Three of the four analogs that are directly related to AF101 contained different substituents on the N-linked phenyl group. The impact of the substitutions on the N-linked phenyl group may be to reduce the hydrophobicity and bulk of this ring. In the case of AF108, the methoxy group would also serve to increase the hydrogen bonding capacity of the compound. The inactivity of AF102, AF107 and AF108 suggest that reducing the hydrophobicity of this ring is not desirable. It would be interesting to examine the effects of increasing the hydrophobicity of the N-linked phenyl group, potentially by replacing bromine with iodine (Figure 21A) or trifluoromethyl groups (Figure 21B). The fourth analog directly related to AF101, AF109, replaced the sulfoxide group in AF101 with a carbonyl. The molecular orientation of AF101 obtained from X-ray crystallography reveals that the S- and N-

Figure 21. Proposed analogs of AF101 to be tested in future experiments.

A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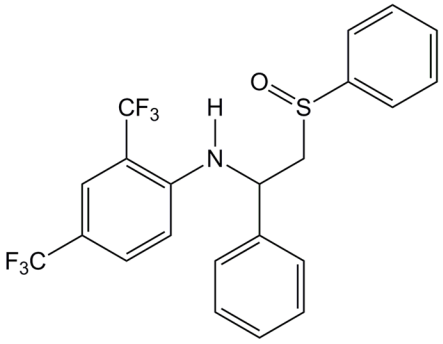
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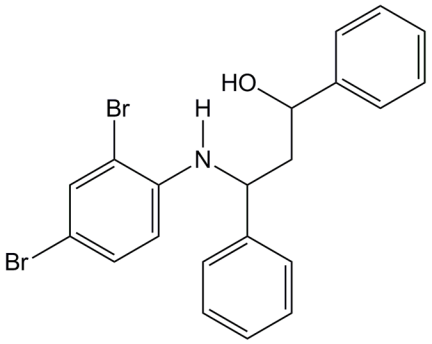
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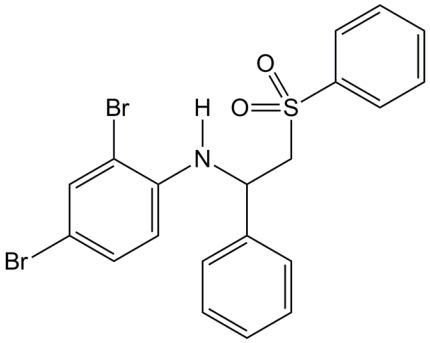
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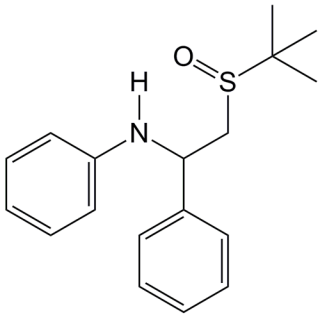
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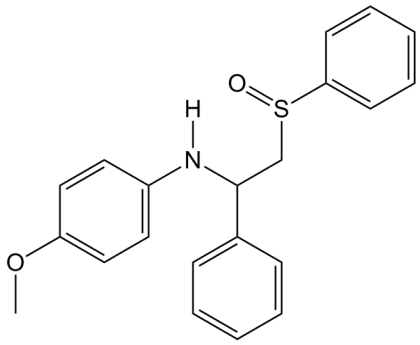
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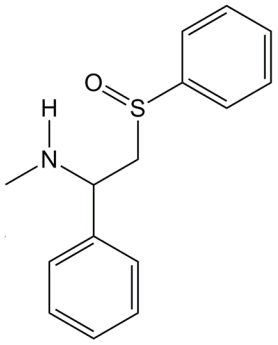
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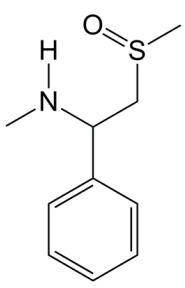
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linked phenyl groups exist on the same plane, while the C-linked phenyl group is oriented perpendicularly (see Appendix, section 7.2). This may facilitate hydrophobic interactions with a flat, hydrophobic transmembrane domain on a connexin channel. This planar orientation is made possible by the tetrahedral geometry of the sulfur atom. The inactivity of AF109, therefore, may possibly be explained by the trigonal planar geometry of the carbonyl carbon that could conceivably prevent the compound from interacting with its binding site. Future analogs that retain the tetrahedral geometry of the sulfur atom should be synthesized to test this hypothesis. For example, the sulfoxide could be replaced with a secondary alcohol (Figure 21C) or with a sulfone (Figure 21D).

The larger number of analogs that were active in hemichannel assays gives fewer clues as to which functional groups are important for activity. One possibility is that the flexible link between the amine and sulfoxide or carbonyl groups is important, since the two analogs that had this link fixed with a benzene ring, AF105 and AF106, were inactive. Additionally, the replacement of the S-linked phenyl group with a methyl group did not abolish activity, suggesting that this ring is not crucial for activity. This hypothesis should be explored, since this group may potentially be a useful area to modify in future analogs to increase specificity. For example, a tert-butyl group would be a useful substitution to make to add steric bulk (Figure 21E). It would also to interesting to further explore the role of the N-linked phenyl group on activity, since substitutions to this ring did not significantly alter activity. An analog with a single methoxy group substituted para to the amine could be tested to see what effect a decrease in ring hydrophobicity has (Figure 21F). Additionally, analogs

in which this ring is removed completely should be tested to clarify whether this ring is necessary for activity (Figure 21 G-H).

An additional detail that should not be overlooked is that these synthetic compounds contain stereochemistry. AF109, AF110 and AF111 each contain a chiral tertiary carbon attached to nitrogen, while AF101, AF102, AF104, AF107, AF108, AF112 and AF113 contain both a chiral tertiary carbon and a chiral sulfoxide. Since NMR data suggests that all of the sulfoxide compounds were produced as a single diasteriomer (Dr. Tony Durst, personal communication, June 24, 2013), all chiral compounds were tested as a pair of enantiomers. If AF101 and its analogs are interacting with stereospecific binding sites, it is possible that activity could be improved by purifying compounds into single, optically active enantiomers. It would be worthwhile to produce dose-response curves on each enantiomer of the most active compounds identified in this thesis to clarify whether stereochemistry is involved in compound activity.

4.5 Spatial and temporal delivery of connexin- and pannexin-specific modulators to the injured spinal cord may be a viable therapeutic strategy

This thesis has identified several lead compounds and extracts capable of discriminating between connexin and pannexin families. The future directions of this study should focus on establishing whether any selectivity within families exists, and if so, whether compounds are capable of therapeutically altering connexin and/or

pannexin function in an *in vivo* model of neural injury. Within the CNS, the injured spinal cord represents the best starting point for the pre-clinical testing of the lead compounds and extracts identified in this study for several reasons. First, it would be possible to use lead compounds in complement to currently proposed treatments for spinal cord injury. This approach has the advantages of being minimally invasive, highly spatially targetable and, by bypassing systemic delivery, minimizing non-desirable effects to other tissues. Second, changes in connexin function and expression have been documented following spinal cord injury and, as discussed in the introduction to this thesis, existing research suggests that they represent a potential therapeutic target to both minimize the propagation of secondary cell death and direct progenitor cell fate decisions.

The current standard treatment for spinal cord injuries is administration of methylprednisolone as soon as possible following the injury to reduce inflammation (28). However, the neurological improvements possible with methylprednisolone are only modest and strategies to improve upon this treatment are actively being investigated (27). The overall trend in spinal cord injury research suggests that a combinatorial approach involving cell transplantation, biomaterial engineering and molecular therapy is most likely to succeed in effectively treating these injuries (207, 223). Many different combinations of cell types (e.g. neural progenitor cells, embryonic stem cells, inducible pluripotent stem cells), degradable bioengineered scaffolds (e.g. chitosan, collagen, poly(α-hydroxyacids), poly(β-hydroxybutyrate)), injectable hydrogels (e.g. chitosan, collagen, fibrin, hyaluronic acid, Matrigel) and encapsulated biomolecules (neurotrophic factors, antagonists for neurite growth

inhibitors) have been studied extensively (207), though to date none have successfully translated to the clinic (244).

Selective connexin and pannexin modulators would be highly useful pharmacological agents that could be combined with this type of strategy. In the hours following injury, astrocytes in the peritraumatic zone release high levels of ATP into the extracellular space (78, 237). In turn, this leads to ATP-gated P2X7 receptor activation on vulnerable neurons adjacent to the injury epicenter, excessive calcium influx through activated P2X7 receptors and irreversible neuronal degeneration (237). Panx1 channels are important mediators of this pathological ATP during the first hours, after which Cx43 hemichannels also contribute (78). Pharmacological blockade using Cx43 mimetic peptides (158, 159) and genetic knockout of Cx43 (97) are both effective means of reducing inflammation and improving locomotor recovery, suggesting Cx43 and likely Panx1 are viable therapeutic targets. Together, these data suggest that reagents capable of selectively blocking Cx43 and/or Panx1 channels would likely be helpful in mitigating neuronal death and promoting functional recovery if delivered in a timely manner following spinal cord injury. Their identification, therefore, should be prioritized in future work.

4.6 Conclusion

Using three complementary approaches to the targeted screening of a large, ethnobotanically-derived compound and extract library, this thesis has identified

several lead compounds and extracts that differentially alter connexin and pannexin channel function. Notably, I have revealed for the first time an effect of two compounds derived from the *Piperaceae* family, piperine and 4,5-dihydropiperlongumine, on connexin channel activity and I have described how this family may also serve as a source for pannexin-specific compounds. Further, I have identified a new class of connexin and pannexin inhibitors that may serve as the basis for the development of more selective reagents. While further work is necessary to investigate whether selectivity of lead compounds and extracts exists within the connexin and pannexin families, this work represents an important first step in identifying novel non-toxic, selective modulators of connexin and pannexin channels that are useful in a therapeutic context.

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6 CONTRIBUTIONS OF COLLABORATORS

All *Piperaceae* plants tested were collected and extracted by Gabriel Picard (laboratory of Dr. John Thor Arnason, University of Ottawa).

AF101 and analogs were synthesized by Ana Francis Carballo Arce (laboratory of Dr. Tony Durst, University of Ottawa).

7 APPENDIX

7.1 RT-PCR product sequencing

7.1.1 Cx26 sense strand, reference RNA

Homo sapiens gap junction protein, beta 2, 26kDa (GJB2), mRNA
Sequence ID: ref|NM_004004.5| Length: 2347 Number of Matches: 1 Range 1: 487 to 757

```
Query  13   TGG-CATGCACGTGGCCTACCGGAGACATGAGAAGAAGAGGAAGTTCATCAAGGGGGAGA  71
            ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  487  TGGCCATGCACGTGGCCTACCGGAGACATGAGAAGAAGAGGAAGTTCATCAAGGGGGAGA  546
Query  72   TAAAGAGTGAATTTAAGGACATCGAGGAGATCAAAACCCAGAAGGTCCGCATCGAAGGCT  131
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547  TAAAGAGTGAATTTAAGGACATCGAGGAGATCAAAACCCAGAAGGTCCGCATCGAAGGCT  606
Query  132  CCCTGTGGTGGACCTACACAAGCAGCATCTTCTTCCGGGTCATCTTCGAAGCCGCCTTCA  191
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  607  CCCTGTGGTGGACCTACACAAGCAGCATCTTCTTCCGGGTCATCTTCGAAGCCGCCTTCA  666
Query  192  TGTACGTCTTCTATGTCATGTACGACGGCTTCTCCATGCAGCGGCTGGTGAAGTGCAACG  251
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  667  TGTACGTCTTCTATGTCATGTACGACGGCTTCTCCATGCAGCGGCTGGTGAAGTGCAACG  726
Query  252  CCTGGCCTTGTCCCAACACTGTGGACTGCTT  282
            ||||||||||||||||||||||||||||||
Sbjct  727  CCTGGCCTTGTCCCAACACTGTGGACTGCTT  757
```

7.1.2 Cx26 antisense strand, reference RNA

Homo sapiens gap junction protein, beta 2, 26kDa (GJB2), mRNA
Sequence ID: ref|NM_004004.5| Length: 2347 Number of Matches: 1 Range 1: 450 to 714

```
Query  15   CCAGCCGCTGCATGGAGAAGCCGTCGTACATGACATAGAAGACGTACATGAAGGCGGCTT  74
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  714  CCAGCCGCTGCATGGAGAAGCCGTCGTACATGACATAGAAGACGTACATGAAGGCGGCTT  655
Query  75   CGAAGATGACCCGGAAGAAGATGCTGCTTGTGTAGGTCCACCACAGGGAGCCTTCGATGC  134
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  654  CGAAGATGACCCGGAAGAAGATGCTGCTTGTGTAGGTCCACCACAGGGAGCCTTCGATGC  595
Query  135  GGACCTTCTGGGTTTTGATCTCCTCGATGTCCTTAAATTCACTCTTTATCTCCCCCTTGA  194
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  594  GGACCTTCTGGGTTTTGATCTCCTCGATGTCCTTAAATTCACTCTTTATCTCCCCCTTGA  535
Query  195  TGAACTTCCTCTTCTTCTCATGTCTCCGGTAGGCCACGTGCATGGCCACTAGGAGCGCTG  254
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  534  TGAACTTCCTCTTCTTCTCATGTCTCCGGTAGGCCACGTGCATGGCCACTAGGAGCGCTG  475
Query  255  GCGTGGACACGAAG-TCAGCTGCAG  278
            |||||||||||||| ||||||||||
Sbjct  474  GCGTGGACACGAAGATCAGCTGCAG  450
```

7.1.3 Cx36 sense strand, NT2/D1

Homo sapiens gap junction protein, delta 2, 36kDa (GJD2), mRNA
Sequence ID: ref|NM_020660.2| Length: 1096 Number of Matches: 1 Range 1: 446 to 1005

```
Query  12    GAGA-CCCCCTGAGT-CATAGGAGGTCCTGGAGGAACTGGGGGTGGGGGCAGTGGTGGGG  69
             ||||  ||||||||||  ||||||||||||||||||||||||||||||||||||||||||
Sbjct  446   GAGACCCCCCTGAGTCCATAGGAGGTCCTGGAGGAACTGGGGGTGGGGGCAGTGGTGGGG  505
Query  70    GCAAACGAGAAGATAAGAAGTTGCAAAATGCTATTGTGAATGGGGTGCTGCAGAACACAG  129
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  506   GCAAACGAGAAGATAAGAAGTTGCAAAATGCTATTGTGAATGGGGTGCTGCAGAACACAG  565
Query  130   AGAACACCAGTAAGGAGACAGAGCCAGATTGTTTAGAGGTTAAGGAGCTGACTCCACACC  189
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  566   AGAACACCAGTAAGGAGACAGAGCCAGATTGTTTAGAGGTTAAGGAGCTGACTCCACACC  625
Query  190   CATCAGGTCTACGCACTGCATCAAAATCCAAGCTCAGAAGGCAGGAAGGCATCTCCCGCT  249
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  626   CATCAGGTCTACGCACTGCATCAAAATCCAAGCTCAGAAGGCAGGAAGGCATCTCCCGCT  685
Query  250   TCTACATTATCCAAGTGGTGTTCCGAAATGCCCTGGAAATTGGGTTCCTGGTTGGCCAAT  309
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  686   TCTACATTATCCAAGTGGTGTTCCGAAATGCCCTGGAAATTGGGTTCCTGGTTGGCCAAT  745
Query  310   ATTTTCTCTATGGCTTTAGTGTCCCAGGGTTGTATGAGTGTAACCGCTACCCCTGCATCA  369
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  746   ATTTTCTCTATGGCTTTAGTGTCCCAGGGTTGTATGAGTGTAACCGCTACCCCTGCATCA  805
Query  370   AGGAGGTGGAATGTTATGTGTCCCGGCCAACTGAGAAGACTGTCTTTCTAGTGTTCATGT  429
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  806   AGGAGGTGGAATGTTATGTGTCCCGGCCAACTGAGAAGACTGTCTTTCTAGTGTTCATGT  865
Query  430   TTGCTGTAAGTGGCATCTGTGTTGTGCTCAACCTGGCTGAACTCAACCACCTGGGATGGC  489
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  866   TTGCTGTAAGTGGCATCTGTGTTGTGCTCAACCTGGCTGAACTCAACCACCTGGGATGGC  925
Query  490   GCAAGATCAAGCTGGCTGTGCGAGGGGCTCAGGCCAAGAGAAAGTCAATCTATGAGATTC  549
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  926   GCAAGATCAAGCTGGCTGTGCGAGGGGCTCAGGCCAAGAGAAAGTCAATCTATGAGATTC  985
Query  550   GTAACAAGGACCTGCCAAGG  569
             ||||||||||||||||||||
Sbjct  986   GTAACAAGGACCTGCCAAGG  1005
```

7.1.4 Cx36 antisense strand, NT2/D1

Homo sapiens gap junction protein, delta 2, 36kDa (GJD2), mRNA
Sequence ID: ref|NM_020660.2| Length: 1096 Number of Matches: 1 Range 1: 410 to 974

```
Query  8    ATTGAACTTTCTCTTGGCCTGAGCCCTTCGCACAGCCAGCTTGATCTTGCGCCATCCCAG  67
            ||||  |||||||||||||||||||  ||||||||||||||||||||||||||||||||
Sbjct  974  ATTG-ACTTTCTCTTGGCCTGAGCCCCTCGCACAGCCAGCTTGATCTTGCGCCATCCCAG  916
Query  68   GTGGTTGAGTTCAGTCAGGTTGAGCACAACACAGATGCCACTTACAGCAAACATGAACAC  127
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  915  GTGGTTGAGTTCAGCCAGGTTGAGCACAACACAGATGCCACTTACAGCAAACATGAACAC  856
Query  128  TAGAAAGACAGTCTTCTCAGTTGGCCGGGACACATAACATTCCACCTCCTTGATGCAGGG  187
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  855  TAGAAAGACAGTCTTCTCAGTTGGCCGGGACACATAACATTCCACCTCCTTGATGCAGGG  796
Query  188  GTAGCGGTTACACTCATACAACCCTGGGACACTAAAGCCATAGAGAAAATATTGGCCAAC  247
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  795  GTAGCGGTTACACTCATACAACCCTGGGACACTAAAGCCATAGAGAAAATATTGGCCAAC  736
Query  248  CAGGAACCCAATTTCCAGGGCATTTCGGAACACCACTTGGATAATGTAGAAGCGGGAGAT  307
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  735  CAGGAACCCAATTTCCAGGGCATTTCGGAACACCACTTGGATAATGTAGAAGCGGGAGAT  676
Query  308  GCCTTCCTGCCTTCTGAGCTTGGATTTTGATGCAGTGCGTAGACCTGATGGGTGTGGAGT  367
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  675  GCCTTCCTGCCTTCTGAGCTTGGATTTTGATGCAGTGCGTAGACCTGATGGGTGTGGAGT  616
Query  368  CAGCTCCTTAACCTCTAAACAATCTGGCTCTGTCTCCTTACTGGTGTTCTCTGTGTTCTG  427
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  615  CAGCTCCTTAACCTCTAAACAATCTGGCTCTGTCTCCTTACTGGTGTTCTCTGTGTTCTG  556
Query  428  CAGCACCCCATTCACAATAGCATTTTGCAACTTCTTATCTTCTCGTTTGCCCCCACCACT  487
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  555  CAGCACCCCATTCACAATAGCATTTTGCAACTTCTTATCTTCTCGTTTGCCCCCACCACT  496
Query  488  GCCCCCACCCCCAGTTCCTCCAGGACCTCCTATGGACTCAGGGGGGTCTCTGTCCAGGGC  547
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  495  GCCCCCACCCCCAGTTCCTCCAGGACCTCCTATGGACTCAGGGGGGTCTCTGTCCAGGGC  436
Query  548  TAGGAAGACTGTAAAGTAGCGGCGTT  573
            |||||||||||| ||||||||||||
Sbjct  435  TAGGAAGACTGTAGAGTAGCGGCGTT  410
```

7.1.5 Cx45 sense strand, NT2/D1

Homo sapiens gap junction protein, gamma 1, 45kDa (GJC1), transcript variant 2, mRNA
Sequence ID: ref|NM_001080383.1| Length: 7640 Number of Matches: 1 Range 1: 271 to 643

```
Query  9    ATGAGTTGGAGCTTCCTGACTCGCCTGCTAGAGGAGATTCACAACCATTCCACATTTGTG  68
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  271  ATGAGTTGGAGCTTCCTGACTCGCCTGCTAGAGGAGATTCACAACCATTCCACATTTGTG  330
Query  69   GGGAAGATCTGGCTCACTGTTCTGATTGTCTTCCGGATCGTCCTTACAGCTGTAGGAGGA  128
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  331  GGGAAGATCTGGCTCACTGTTCTGATTGTCTTCCGGATCGTCCTTACAGCTGTAGGAGGA  390
Query  129  GAATCCATCTATTACGATGAGCAAAGCAAATTTGTGTGCAACACAGAACAGCCGGGCTGT  188
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  391  GAATCCATCTATTACGATGAGCAAAGCAAATTTGTGTGCAACACAGAACAGCCGGGCTGT  450
Query  189  GAGAATGTCTGTTATGATGCGTTTGCACCTCTCTCCCATGTACGCTTCTGGGTGTTCCAG  248
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  451  GAGAATGTCTGTTATGATGCGTTTGCACCTCTCTCCCATGTACGCTTCTGGGTGTTCCAG  510
Query  249  ATCATCCTGGTGGCAACTCCCTCTGTGATGTACCTGGGCTATGCTATCCACAAGATTGCC  308
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  511  ATCATCCTGGTGGCAACTCCCTCTGTGATGTACCTGGGCTATGCTATCCACAAGATTGCC  570
Query  309  AAAATGGAGCACGGTGAAGCAGACAAGAAGGCAGCTCGGAGCAAGCCCTATGCAATGCGC  368
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  571  AAAATGGAGCACGGTGAAGCAGACAAGAAGGCAGCTCGGAGCAAGCCCTATGCAATGCGC  630
Query  369  TGGAAACAACACC  381
            |||||||||||||
Sbjct  631  TGGAAACAACACC  643
```

Homo sapiens gap junction protein, gamma 1, 45kDa (GJC1), transcript variant 1, mRNA
Sequence ID: ref|NM_005497.3| Length: 7644 Number of Matches: 1 Range 1: 275 to 647

```
Query  9    ATGAGTTGGAGCTTCCTGACTCGCCTGCTAGAGGAGATTCACAACCATTCCACATTTGTG  68
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  275  ATGAGTTGGAGCTTCCTGACTCGCCTGCTAGAGGAGATTCACAACCATTCCACATTTGTG  334
Query  69   GGGAAGATCTGGCTCACTGTTCTGATTGTCTTCCGGATCGTCCTTACAGCTGTAGGAGGA  128
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  335  GGGAAGATCTGGCTCACTGTTCTGATTGTCTTCCGGATCGTCCTTACAGCTGTAGGAGGA  394
Query  129  GAATCCATCTATTACGATGAGCAAAGCAAATTTGTGTGCAACACAGAACAGCCGGGCTGT  188
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  395  GAATCCATCTATTACGATGAGCAAAGCAAATTTGTGTGCAACACAGAACAGCCGGGCTGT  454
Query  189  GAGAATGTCTGTTATGATGCGTTTGCACCTCTCTCCCATGTACGCTTCTGGGTGTTCCAG  248
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  455  GAGAATGTCTGTTATGATGCGTTTGCACCTCTCTCCCATGTACGCTTCTGGGTGTTCCAG  514
Query  249  ATCATCCTGGTGGCAACTCCCTCTGTGATGTACCTGGGCTATGCTATCCACAAGATTGCC  308
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  515  ATCATCCTGGTGGCAACTCCCTCTGTGATGTACCTGGGCTATGCTATCCACAAGATTGCC  574
Query  309  AAAATGGAGCACGGTGAAGCAGACAAGAAGGCAGCTCGGAGCAAGCCCTATGCAATGCGC  368
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  575  AAAATGGAGCACGGTGAAGCAGACAAGAAGGCAGCTCGGAGCAAGCCCTATGCAATGCGC  634
Query  369  TGGAAACAACACC  381
            |||||||||||||
Sbjct  635  TGGAAACAACACC  647
```

7.1.6 Cx45 antisense strand, NT2/D1

Homo sapiens gap junction protein, gamma 1, 45kDa (GJC1), transcript variant 2, mRNA
Sequence ID: ref|NM_001080383.1| Length: 7640 Number of Matches: 1 Range 1: 236 to 612

```
Query  4    GCTCTCGA-CTG-CTTCTTGTCTGCTTC-CCGTGCTCCATTTTGGCAATCTTGTGGATAG  60
            ||||  |||  |||  ||||||||||||||  |||||||||||||||||||||||||||||
Sbjct  612  GCTC-CGAGCTGCCTTCTTGTCTGCTTCACCGTGCTCCATTTTGGCAATCTTGTGGATAG  554
Query  61   CATAGCCCAGGTACATCACAGAGGGAGTTGCCACCAGGATGATCTGGAACACCCAGAAGC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  553  CATAGCCCAGGTACATCACAGAGGGAGTTGCCACCAGGATGATCTGGAACACCCAGAAGC  494
Query  121  GTACATGGGAGAGAGGTGCAAACGCATCATAACAGACATTCTCACAGCCCGGCTGTTCTG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  493  GTACATGGGAGAGAGGTGCAAACGCATCATAACAGACATTCTCACAGCCCGGCTGTTCTG  434
Query  181  TGTTGCACACAAATTTGCTTTGCTCATCGTAATAGATGGATTCTCCTCCTACAGCTGTAA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  433  TGTTGCACACAAATTTGCTTTGCTCATCGTAATAGATGGATTCTCCTCCTACAGCTGTAA  374
Query  241  GGACGATCCGGAAGACAATCAGAACAGTGAGCCAGATCTTCCCCACAAATGTGGAATGGT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  373  GGACGATCCGGAAGACAATCAGAACAGTGAGCCAGATCTTCCCCACAAATGTGGAATGGT  314
Query  301  TGTGAATCTCCTCTAGCAGGCGAGTCAGGAAGCTCCAACTCATGGTGATTGAATTGGTAT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  313  TGTGAATCTCCTCTAGCAGGCGAGTCAGGAAGCTCCAACTCATGGTGATTGAATTGGTAT  254
Query  361  GCCCTGTTGACCAGAACT  378
            |||||||| ||||||||
Sbjct  253  GCCCTGTTGTCCAGAACT  236
```

Homo sapiens gap junction protein, gamma 1, 45kDa (GJC1), transcript variant 1, mRNA
Sequence ID: ref|NM_005497.3| Length: 7644 Number of Matches: 1 Range 1: 240 to 616

```
Query  4    GCTCTCGA-CTG-CTTCTTGTCTGCTTC-CCGTGCTCCATTTTGGCAATCTTGTGGATAG  60
            |||| ||| ||| ||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  616  GCTC-CGAGCTGCCTTCTTGTCTGCTTCACCGTGCTCCATTTTGGCAATCTTGTGGATAG  558
Query  61   CATAGCCCAGGTACATCACAGAGGGAGTTGCCACCAGGATGATCTGGAACACCCAGAAGC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  557  CATAGCCCAGGTACATCACAGAGGGAGTTGCCACCAGGATGATCTGGAACACCCAGAAGC  498
Query  121  GTACATGGGAGAGAGGTGCAAACGCATCATAACAGACATTCTCACAGCCCGGCTGTTCTG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  497  GTACATGGGAGAGAGGTGCAAACGCATCATAACAGACATTCTCACAGCCCGGCTGTTCTG  438
Query  181  TGTTGCACACAAATTTGCTTTGCTCATCGTAATAGATGGATTCTCCTCCTACAGCTGTAA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  437  TGTTGCACACAAATTTGCTTTGCTCATCGTAATAGATGGATTCTCCTCCTACAGCTGTAA  378
Query  241  GGACGATCCGGAAGACAATCAGAACAGTGAGCCAGATCTTCCCCACAAATGTGGAATGGT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  377  GGACGATCCGGAAGACAATCAGAACAGTGAGCCAGATCTTCCCCACAAATGTGGAATGGT  318
Query  301  TGTGAATCTCCTCTAGCAGGCGAGTCAGGAAGCTCCAACTCATGGTGATTGAATTGGTAT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  317  TGTGAATCTCCTCTAGCAGGCGAGTCAGGAAGCTCCAACTCATGGTGATTGAATTGGTAT  258
Query  361  GCCCTGTTGACCAGAACT  378
            ||||||||| ||||||||
Sbjct  257  GCCCTGTTGTCCAGAACT  240
```

7.1.7 Panx1 sense strand, reference RNA

Homo sapiens pannexin 1 (PANX1), mRNA
Sequence ID: ref|NM_015368.3| Length: 2782 Number of Matches: 1 Range 1: 775 to 1593

```
Query  12    AGCTGCT-CTC-TATTTGCTCAGACTTGAAGTTTATCATGGAAGAACTTGACAAAGTTTA  69
            ||||||| ||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  775   AGCTGCTCCTCATATTTGCTCAGACTTGAAGTTTATCATGGAAGAACTTGACAAAGTTTA  834
Query  70    CAACCGTGCAATTAAGGCTGCAAAGAGTGCGCGTGACCTTGACATGAGAGATGGAGCCTG  129
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  835   CAACCGTGCAATTAAGGCTGCAAAGAGTGCGCGTGACCTTGACATGAGAGATGGAGCCTG  894
Query  130   CTCAGTTCCAGGTGTTACCGAGAACTTAGGGCAAAGTTTGTGGGAGGTATCTGAAAGCCA  189
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  895   CTCAGTTCCAGGTGTTACCGAGAACTTAGGGCAAAGTTTGTGGGAGGTATCTGAAAGCCA  954
Query  190   CTTCAAGTACCCAATTGTGGAGCAGTACTTGAAGACAAAGAAAAATTCTAATAATTTAAT  249
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  955   CTTCAAGTACCCAATTGTGGAGCAGTACTTGAAGACAAAGAAAAATTCTAATAATTTAAT
1014
Query  250   CATCAAGTACATTAGCTGCCGCCTGCTGACACTCATCATTATACTGTTAGCGTGTATCTA  309
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1015  CATCAAGTACATTAGCTGCCGCCTGCTGACACTCATCATTATACTGTTAGCGTGTATCTA
1074
Query  310   CCTGGGCTATTACTTCAGCCTCTCCTCACTCTCAGACGAGTTTGTGTGCAGCATCAAATC  369
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1075  CCTGGGCTATTACTTCAGCCTCTCCTCACTCTCAGACGAGTTTGTGTGCAGCATCAAATC
1134
Query  370   AGGGATCCTGAGAAACGACAGCACCGTGCCCGATCAGTTTCAGTGCAAACTCATTGCCGT  429
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1135  AGGGATCCTGAGAAACGACAGCACCGTGCCCGATCAGTTTCAGTGCAAACTCATTGCCGT
1194
Query  430   GGGCATCTTCCAGTTGCTCAGTGTCATTAACCTTGTGGTTTATGTCCTGCTGGCTCCCGT  489
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1195  GGGCATCTTCCAGTTGCTCAGTGTCATTAACCTTGTGGTTTATGTCCTGCTGGCTCCCGT
1254
Query  490   GGTTGTCTACACGCTGTTTGTTCCATTCCGACAGAAGACAGATGTTCTCAAAGTGTACGA  549
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1255  GGTTGTCTACACGCTGTTTGTTCCATTCCGACAGAAGACAGATGTTCTCAAAGTGTACGA
1314
Query  550   AATCCTCCCCACTTTTGATGTTCTGCATTTCAAATCTGAAGGGTACAACGATTTGAGCCT  609
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1315  AATCCTCCCCACTTTTGATGTTCTGCATTTCAAATCTGAAGGGTACAACGATTTGAGCCT
1374
Query  610   CTACAATCTCTTCTTGGAGGAAAATATAAGTGAGGTCAAGTCATACAAGTGTCTTAAGGT  669
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1375  CTACAATCTCTTCTTGGAGGAAAATATAAGTGAGGTCAAGTCATACAAGTGTCTTAAGGT
1434
Query  670   ACTGGAGAATATTAAGAGCAGTGGTCAGGGGATCGACCCAATGCTACTCCTGACAAACCT  729
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1435  ACTGGAGAATATTAAGAGCAGTGGTCAGGGGATCGACCCAATGCTACTCCTGACAAACCT
1494
Query  730   TGGCATGATCAAGATGGATGTTGTTGATGGCAAAACTCCCATGTCTGCAGAGATGAGAGA  789
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1495  TGGCATGATCAAGATGGATGTTGTTGATGGCAAAACTCCCATGTCTGCAGAGATGAGAGA
1554
Query  790   GGAGCATGGGAACCAGACGGCAGAGCTCCAAGGGTATGAA  829
            |||||| ||||||||||||||||||||||| ||||||
Sbjct  1555  GGAGCAGGGGAACCAGACGGCAGAGCTCCAAGG-TATGAA  1593
```

7.1.8 Panx1 antisense strand, reference RNA

Homo sapiens pannexin 1 (PANX1), mRNA
Sequence ID: ref|NM_015368.3| Length: 2782 Number of Matches: 1 Range 1: 740 to 1555

```
Query  16    CTCTCT-ATCTCTGCAGACATGGGAGTTTTGCCATCAACAACATCCATCTTGATCATGCC  74
             ||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1555  CTCTCTCATCTCTGCAGACATGGGAGTTTTGCCATCAACAACATCCATCTTGATCATGCC
1496
Query  75    AAGGTTTGACAGGAGTAGCATTGGGTCGATCCCCTGACCACTGCTCTTAATATTCTCCAG  134
             ||||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1495  AAGGTTTGTCAGGAGTAGCATTGGGTCGATCCCCTGACCACTGCTCTTAATATTCTCCAG
1436
Query  135   TACCTTAAGACACTTGTATGACTTGACCTCACTTATATTTTCCTCCAAGAAGAGATTGTA  194
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1435  TACCTTAAGACACTTGTATGACTTGACCTCACTTATATTTTCCTCCAAGAAGAGATTGTA
1376
Query  195   GAGGCTCAAATCGTTGTACCCTTCAGATTTGAAATGCAGAACATCAAAAGTGGGGAGGAT  254
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1375  GAGGCTCAAATCGTTGTACCCTTCAGATTTGAAATGCAGAACATCAAAAGTGGGGAGGAT
1316
Query  255   TTCGTACACTTTGAGAACATCTGTCTTCTGTCGGAATGGAACAAACAGCGTGTAGACAAC  314
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1315  TTCGTACACTTTGAGAACATCTGTCTTCTGTCGGAATGGAACAAACAGCGTGTAGACAAC
1256
Query  315   CACGGGAGCCAGCAGGACATAAACCACAAGGTTAATGACACTGAGCAACTGGAAGATGCC  374
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1255  CACGGGAGCCAGCAGGACATAAACCACAAGGTTAATGACACTGAGCAACTGGAAGATGCC
1196
Query  375   CACGGCAATGAGTTTGCACTGAAACTGATCGGGCACGGTGCTGTCGTTTCTCAGGATCCC  434
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1195  CACGGCAATGAGTTTGCACTGAAACTGATCGGGCACGGTGCTGTCGTTTCTCAGGATCCC
1136
Query  435   TGATTTGATGCTGCACACAAACTCGTCTGAGAGTGAGGAGAGGCTGAAGTAATAGCCCAG  494
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1135  TGATTTGATGCTGCACACAAACTCGTCTGAGAGTGAGGAGAGGCTGAAGTAATAGCCCAG
1076
Query  495   GTAGATACACGCTAACAGTATAATGATGAGTGTCAGCAGGCGGCAGCTAATGTACTTGAT  554
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1075  GTAGATACACGCTAACAGTATAATGATGAGTGTCAGCAGGCGGCAGCTAATGTACTTGAT
1016
Query  555   GATTAAATTATTAGAATTTTTCTTTGTCTTCAAGTACTGCTCCACAATTGGGTACTTGAA  614
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1015  GATTAAATTATTAGAATTTTTCTTTGTCTTCAAGTACTGCTCCACAATTGGGTACTTGAA  956
Query  615   GTGGCTTTCAGATACCTCCCACAAACTTTGCCCTAAGTTCTCGGTAACACCTGGAACTGA  674
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  955   GTGGCTTTCAGATACCTCCCACAAACTTTGCCCTAAGTTCTCGGTAACACCTGGAACTGA  896
Query  675   GCAGGCTCCATCTCTCATGTCAAGGTCACGCGCACTCTTTGCAGCCTTAATTGCACGGTT  734
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  895   GCAGGCTCCATCTCTCATGTCAAGGTCACGCGCACTCTTTGCAGCCTTAATTGCACGGTT  836
Query  735   GTAAACTTTGTCAAGTTCTTCCATGATAAACTTCAAGTCTGAGCAAATATGAGGAGCAGC  794
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  835   GTAAACTTTGTCAAGTTCTTCCATGATAAACTTCAAGTCTGAGCAAATATGAGGAGCAGC  776
Query  795   TGCGAAACGCCAGAACAGCGGGGGCAGGGTACAGGAG  831
             |||||||||||||||||||||||||| ||||||||
Sbjct  775   TGCGAAACGCCAGAACAGCGGGGGCAGG-TACAGGAG  740
```

7.1.9 Panx2 sense strand, hippocampus

Homo sapiens pannexin 2 (PANX2), transcript variant 3, non-coding RNA
Sequence ID: ref|NR_027691.1| Length: 3035 Number of Matches: 1 Range 1: 299 to 1011

```
Query  14    CCCCGCACAACTTTACGCGCGACCAGGCGCTGTACGCCCGCGGCTACTGCTGGACGGAGC  73
             ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  299   CCCCGCACAACTTCACGCGCGACCAGGCGCTGTACGCCCGCGGCTACTGCTGGACGGAGC  358
Query  74    TGCGGGACGCGCTGCCCGGCGTGGACGCCAGCCTGTGGCCGTCGCTGTTTGAGCACAAGT  133
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  359   TGCGGGACGCGCTGCCCGGCGTGGACGCCAGCCTGTGGCCGTCGCTGTTTGAGCACAAGT  418
Query  134   TCCTGCCCTACGCGCTGCTGGCCTTCGCCGCCATCATGTACGTGCCCGCGCTGGGCTGGG  193
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  419   TCCTGCCCTACGCGCTGCTGGCCTTCGCCGCCATCATGTACGTGCCCGCGCTGGGCTGGG  478
Query  194   AGTTCCTGGCCTCCACGCGCCTCACCTCCGAGCTCAACTTCCTGCTGCAGGAGATCGACA  253
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  479   AGTTCCTGGCCTCCACGCGCCTCACCTCCGAGCTCAACTTCCTGCTGCAGGAGATCGACA  538
Query  254   ACTGTTACCACCGGGCGGCCGAGGGCCGCGCGCCCAAGATCGAGAAGCAGATCCAGTCCA  313
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  539   ACTGTTACCACCGGGCGGCCGAGGGCCGCGCGCCCAAGATCGAGAAGCAGATCCAGTCCA  598
Query  314   AGGGCCCGGGCATCACGGAGCGCGAGAAGCGCGAGATCATCGAGAACGCGGAGAAGGAGA  373
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  599   AGGGCCCGGGCATCACGGAGCGCGAGAAGCGCGAGATCATCGAGAACGCGGAGAAGGAGA  658
Query  374   AGAGCCCGGAGCAGAACCTGTTCGAGAAGTACCTGGAGCGCCGCGGCCGCAGCAACTTCC  433
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  659   AGAGCCCGGAGCAGAACCTGTTCGAGAAGTACCTGGAGCGCCGCGGCCGCAGCAACTTCC  718
Query  434   TGGCCAAGCTGTACCTGGCGCGGCACGTGCTGATCCTGCTGCTGAGCGCCGTGCCCATCT  493
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  719   TGGCCAAGCTGTACCTGGCGCGGCACGTGCTGATCCTGCTGCTGAGCGCCGTGCCCATCT  778
Query  494   CCTACCTGTGCACCTACTACGCCACGCAGAAGCAGAACGAGTTCACCTGCGCGCTGGGCG  553
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  779   CCTACCTGTGCACCTACTACGCCACGCAGAAGCAGAACGAGTTCACCTGCGCGCTGGGCG  838
Query  554   CGTCCCCGGACGGGGCGGCAGGTGCGGGGCCCGCGGTGCGCGTGAGCTGCAAGCTCCCGT  613
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  839   CGTCCCCGGACGGGGCGGCAGGTGCGGGGCCCGCGGTGCGCGTGAGCTGCAAGCTCCCGT  898
Query  614   CCGTGCAACTGCAGCGCATCATCGCGGGCGTGGACATCGTGCTGCTGTGCGTCATGAACC  673
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  899   CCGTGCAACTGCAGCGCATCATCGCGGGCGTGGACATCGTGCTGCTGTGCGTCATGAACC  958
Query  674   TCATCATCCTCGTCAACCTCATCCACCTCTTCATCTTCCGCAAGAGCAACTTC  726
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  959   TCATCATCCTCGTCAACCTCATCCACCTCTTCATCTTCCGCAAGAGCAACTTC  1011
```

Homo sapiens pannexin 2 (PANX2), transcript variant 2, mRNA
Sequence ID: ref|NM_001160300.1| Length: 2984 Number of Matches: 1 Range 1: 248 to 960

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Query  14   CCCCGCACAACTTTACGCGCGACCAGGCGCTGTACGCCCGCGGCTACTGCTGGACGGAGC  73
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  248  CCCCGCACAACTTCACGCGCGACCAGGCGCTGTACGCCCGCGGCTACTGCTGGACGGAGC  307
Query  74   TGCGGGACGCGCTGCCCGGCGTGGACGCCAGCCTGTGGCCGTCGCTGTTTGAGCACAAGT  133
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  308  TGCGGGACGCGCTGCCCGGCGTGGACGCCAGCCTGTGGCCGTCGCTGTTTGAGCACAAGT  367
Query  134  TCCTGCCCTACGCGCTGCTGGCCTTCGCCGCCATCATGTACGTGCCCGCGCTGGGCTGGG  193
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  368  TCCTGCCCTACGCGCTGCTGGCCTTCGCCGCCATCATGTACGTGCCCGCGCTGGGCTGGG  427
Query  194  AGTTCCTGGCCTCCACGCGCCTCACCTCCGAGCTCAACTTCCTGCTGCAGGAGATCGACA  253
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  428  AGTTCCTGGCCTCCACGCGCCTCACCTCCGAGCTCAACTTCCTGCTGCAGGAGATCGACA  487
Query  254  ACTGTTACCACCGGGCGGCCGAGGGCCGCGCGCCCAAGATCGAGAAGCAGATCCAGTCCA  313
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  488  ACTGTTACCACCGGGCGGCCGAGGGCCGCGCGCCCAAGATCGAGAAGCAGATCCAGTCCA  547
Query  314  AGGGCCCGGGCATCACGGAGCGCGAGAAGCGCGAGATCATCGAGAACGCGGAGAAGGAGA  373
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  548  AGGGCCCGGGCATCACGGAGCGCGAGAAGCGCGAGATCATCGAGAACGCGGAGAAGGAGA  607
Query  374  AGAGCCCGGAGCAGAACCTGTTCGAGAAGTACCTGGAGCGCCGCGGCCGCAGCAACTTCC  433
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  608  AGAGCCCGGAGCAGAACCTGTTCGAGAAGTACCTGGAGCGCCGCGGCCGCAGCAACTTCC  667
Query  434  TGGCCAAGCTGTACCTGGCGCGGCACGTGCTGATCCTGCTGCTGAGCGCCGTGCCCATCT  493
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  668  TGGCCAAGCTGTACCTGGCGCGGCACGTGCTGATCCTGCTGCTGAGCGCCGTGCCCATCT  727
Query  494  CCTACCTGTGCACCTACTACGCCACGCAGAAGCAGAACGAGTTCACCTGCGCGCTGGGCG  553
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  728  CCTACCTGTGCACCTACTACGCCACGCAGAAGCAGAACGAGTTCACCTGCGCGCTGGGCG  787
Query  554  CGTCCCCGGACGGGGCGGCAGGTGCGGGGCCCGCGGTGCGCGTGAGCTGCAAGCTCCCGT  613
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  788  CGTCCCCGGACGGGGCGGCAGGTGCGGGGCCCGCGGTGCGCGTGAGCTGCAAGCTCCCGT  847
Query  614  CCGTGCAACTGCAGCGCATCATCGCGGGCGTGGACATCGTGCTGCTGTGCGTCATGAACC  673
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  848  CCGTGCAACTGCAGCGCATCATCGCGGGCGTGGACATCGTGCTGCTGTGCGTCATGAACC  907
Query  674  TCATCATCCTCGTCAACCTCATCCACCTCTTCATCTTCCGCAAGAGCAACTTC  726
            |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  908  TCATCATCCTCGTCAACCTCATCCACCTCTTCATCTTCCGCAAGAGCAACTTC  960
```

Homo sapiens pannexin 2 (PANX2), transcript variant 1, mRNA
Sequence ID: ref|NM_052839.3| Length: 3069 Number of Matches: 1 Range 1: 248 to 960

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Query  14   CCCCGCACAACTTTACGCGCGACCAGGCGCTGTACGCCCGCGGCTACTGCTGGACGGAGC  73
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  248  CCCCGCACAACTTCACGCGCGACCAGGCGCTGTACGCCCGCGGCTACTGCTGGACGGAGC  307
Query  74   TGCGGGACGCGCTGCCCGGCGTGGACGCCAGCCTGTGGCCGTCGCTGTTTGAGCACAAGT  133
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  308  TGCGGGACGCGCTGCCCGGCGTGGACGCCAGCCTGTGGCCGTCGCTGTTTGAGCACAAGT  367
Query  134  TCCTGCCCTACGCGCTGCTGGCCTTCGCCGCCATCATGTACGTGCCCGCGCTGGGCTGGG  193
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  368  TCCTGCCCTACGCGCTGCTGGCCTTCGCCGCCATCATGTACGTGCCCGCGCTGGGCTGGG  427
Query  194  AGTTCCTGGCCTCCACGCGCCTCACCTCCGAGCTCAACTTCCTGCTGCAGGAGATCGACA  253
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  428  AGTTCCTGGCCTCCACGCGCCTCACCTCCGAGCTCAACTTCCTGCTGCAGGAGATCGACA  487
Query  254  ACTGTTACCACCGGGCGGCCGAGGGCCGCGCGCCCAAGATCGAGAAGCAGATCCAGTCCA  313
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  488  ACTGTTACCACCGGGCGGCCGAGGGCCGCGCGCCCAAGATCGAGAAGCAGATCCAGTCCA  547
Query  314  AGGGCCCGGGCATCACGGAGCGCGAGAAGCGCGAGATCATCGAGAACGCGGAGAAGGAGA  373
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  548  AGGGCCCGGGCATCACGGAGCGCGAGAAGCGCGAGATCATCGAGAACGCGGAGAAGGAGA  607
Query  374  AGAGCCCGGAGCAGAACCTGTTCGAGAAGTACCTGGAGCGCCGCGGCCGCAGCAACTTCC  433
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  608  AGAGCCCGGAGCAGAACCTGTTCGAGAAGTACCTGGAGCGCCGCGGCCGCAGCAACTTCC  667
Query  434  TGGCCAAGCTGTACCTGGCGCGGCACGTGCTGATCCTGCTGCTGAGCGCCGTGCCCATCT  493
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  668  TGGCCAAGCTGTACCTGGCGCGGCACGTGCTGATCCTGCTGCTGAGCGCCGTGCCCATCT  727
Query  494  CCTACCTGTGCACCTACTACGCCACGCAGAAGCAGAACGAGTTCACCTGCGCGCTGGGCG  553
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  728  CCTACCTGTGCACCTACTACGCCACGCAGAAGCAGAACGAGTTCACCTGCGCGCTGGGCG  787
Query  554  CGTCCCCGGACGGGGCGGCAGGTGCGGGGCCCGCGGTGCGCGTGAGCTGCAAGCTCCCGT  613
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  788  CGTCCCCGGACGGGGCGGCAGGTGCGGGGCCCGCGGTGCGCGTGAGCTGCAAGCTCCCGT  847
Query  614  CCGTGCAACTGCAGCGCATCATCGCGGGCGTGGACATCGTGCTGCTGTGCGTCATGAACC  673
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  848  CCGTGCAACTGCAGCGCATCATCGCGGGCGTGGACATCGTGCTGCTGTGCGTCATGAACC  907
Query  674  TCATCATCCTCGTCAACCTCATCCACCTCTTCATCTTCCGCAAGAGCAACTTC  726
            |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  908  TCATCATCCTCGTCAACCTCATCCACCTCTTCATCTTCCGCAAGAGCAACTTC  960
```

7.1.10 Panx2 antisense strand, hippocampus

Homo sapiens pannexin 2 (PANX2), transcript variant 2, mRNA
Sequence ID: ref|NM_001160300.1| Length: 2984 Number of Matches: 1 Range 1: 211 to 1107

```
Query 6       GTTGGGTGA-GGAGT-CAGCCGGTTGAGCGACTTGATGTGGTCGCGGTTCTCGTTGCAGA  63
              ||| ||||| | ||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1107    GTT-GGTGATGAAGTCCAGCCGGTTGAGCGACTTGATGTGGTCGCGGTTCTCGTTGCAGA
1049
Query  64     ACATGGCCAGGATGTTGATGTCGCAGAACTGCGAGCGGCGCCACTGCCGGCGCGTCTTGA  123
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1048   ACATGGCCAGGATGTTGATGTCGCAGAACTGCGAGCGGCGCCACTGCCGGCGCGTCTTGA  989
Query  124    TGCCCACCTTGTGCAGCTTGTCGAAGATGAAGTTGCTCTTGCGGAAGATGAAGAGGTGGA  183
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  988    TGCCCACCTTGTGCAGCTTGTCGAAGATGAAGTTGCTCTTGCGGAAGATGAAGAGGTGGA  929
Query  184    TGAGGTTGACGAGGATGATGAGGTTCATGACGCACAGCAGCACGATGTCCACGCCCGCGA  243
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  928    TGAGGTTGACGAGGATGATGAGGTTCATGACGCACAGCAGCACGATGTCCACGCCCGCGA  869
Query  244    TGATGCGCTGCAGTTGCACGGACGGGAGCTTGCAGCTCACGCGCACCGCGGGCCCCGCAC  303
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  868    TGATGCGCTGCAGTTGCACGGACGGGAGCTTGCAGCTCACGCGCACCGCGGGCCCCGCAC  809
Query  304    CTGCCGCCCCGTCCGGGGACGCGCCCAGCGCGCAGGTGAACTCGTTCTGCTTCTGCGTGG  363
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  808    CTGCCGCCCCGTCCGGGGACGCGCCCAGCGCGCAGGTGAACTCGTTCTGCTTCTGCGTGG  749
Query  364    CGTAGTAGGTGCACAGGTAGGAGATGGGCACGGCGCTCAGCAGCAGGATCAGCACGTGCC  423
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  748    CGTAGTAGGTGCACAGGTAGGAGATGGGCACGGCGCTCAGCAGCAGGATCAGCACGTGCC  689
Query  424    GCGCCAGGTACAGCTTGGCCAGGAAGTTGCTGCGGCCGCGGCGCTCCAGGTACTTCTCGA  483
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  688    GCGCCAGGTACAGCTTGGCCAGGAAGTTGCTGCGGCCGCGGCGCTCCAGGTACTTCTCGA  629
Query  484    ACAGGTTCTGCTCCGGGCTCTTCTCCTTCTCCGCGTTCTCGATGATCTCGCGCTTCTCGC  543
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  628    ACAGGTTCTGCTCCGGGCTCTTCTCCTTCTCCGCGTTCTCGATGATCTCGCGCTTCTCGC  569
Query  544    GCTCCGTGATGCCCGGGCCCTTGGACTGGATCTGCTTCTCGATCTTGGGCGCGCGGCCCT  603
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  568    GCTCCGTGATGCCCGGGCCCTTGGACTGGATCTGCTTCTCGATCTTGGGCGCGCGGCCCT  509
Query  604    CGGCCGCCCGGTGGTAACAGTTGTCGATCTCCTGCAGCAGGAAGTTGAGCTCGGAGGTGA  663
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  508    CGGCCGCCCGGTGGTAACAGTTGTCGATCTCCTGCAGCAGGAAGTTGAGCTCGGAGGTGA  449
Query  664    GGCGCGTGGAGGCCAGGAACTCCCAGCCCAGCGCGGGCACGTACATGATGGCGGCGAAGG  723
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  448    GGCGCGTGGAGGCCAGGAACTCCCAGCCCAGCGCGGGCACGTACATGATGGCGGCGAAGG  389
Query  724    CCAGCAGCGCGTAGGGCAGGAACTTGTGCTCAAACAGCGACGGCCACAGGCTGGCGTCCA  783
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  388    CCAGCAGCGCGTAGGGCAGGAACTTGTGCTCAAACAGCGACGGCCACAGGCTGGCGTCCA  329
Query  784    CGCCGGGCAGCGCGTCCCGCAGCTCCGTCCAGCAGTAGCCGCGGGCGTACAGCGCCTGGT  843
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  328    CGCCGGGCAGCGCGTCCCGCAGCTCCGTCCAGCAGTAGCCGCGGGCGTACAGCGCCTGGT  269
Query  844    CGCGCGTGAAGTTGTGCGGGGTGTAACAGTAAATGGGTTCCTCTGCGAAGTTTCTTGGT  902
              |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  268    CGCGCGTGAAGTTGTGCGGGGTGTAACAGTAAATGGGTTCCTCTGCGAAGTT-CTTGGT  211
```

Homo sapiens pannexin 2 (PANX2), transcript variant 1, mRNA
Sequence ID: ref|NM_052839.3| Length: 3069 Number of Matches: 1 Range 1: 211 to 1107

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Query 6       GTTGGGTGA-GGAGTCCAGCCGGTTGAGCGACTTGATGTGGTCGCGGTTCTCGTTGCAGA  63
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Sbjct  1107   GTT-GGTGATGAAGTCCAGCCGGTTGAGCGACTTGATGTGGTCGCGGTTCTCGTTGCAGA
1049
Query  64     ACATGGCCAGGATGTTGATGTCGCAGAACTGCGAGCGGCGCCACTGCCGGCGCGTCTTGA  123
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Sbjct  1048   ACATGGCCAGGATGTTGATGTCGCAGAACTGCGAGCGGCGCCACTGCCGGCGCGTCTTGA  989
Query  124    TGCCCACCTTGTGCAGCTTGTCGAAGATGAAGTTGCTCTTGCGGAAGATGAAGAGGTGGA  183
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Query  184    TGAGGTTGACGAGGATGATGAGGTTCATGACGCACAGCAGCACGATGTCCACGCCCGCGA  243
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Sbjct  928    TGAGGTTGACGAGGATGATGAGGTTCATGACGCACAGCAGCACGATGTCCACGCCCGCGA  869
Query  244    TGATGCGCTGCAGTTGCACGGACGGGAGCTTGCAGCTCACGCGCACCGCGGGCCCCGCAC  303
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Sbjct  868    TGATGCGCTGCAGTTGCACGGACGGGAGCTTGCAGCTCACGCGCACCGCGGGCCCCGCAC  809
Query  304    CTGCCGCCCCGTCCGGGGACGCGCCCAGCGCGCAGGTGAACTCGTTCTGCTTCTGCGTGG  363
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Sbjct  808    CTGCCGCCCCGTCCGGGGACGCGCCCAGCGCGCAGGTGAACTCGTTCTGCTTCTGCGTGG  749
Query  364    CGTAGTAGGTGCACAGGTAGGAGATGGGCACGGCGCTCAGCAGCAGGATCAGCACGTGCC  423
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Sbjct  748    CGTAGTAGGTGCACAGGTAGGAGATGGGCACGGCGCTCAGCAGCAGGATCAGCACGTGCC  689
Query  424    GCGCCAGGTACAGCTTGGCCAGGAAGTTGCTGCGGCCGCGGCGCTCCAGGTACTTCTCGA  483
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Sbjct  688    GCGCCAGGTACAGCTTGGCCAGGAAGTTGCTGCGGCCGCGGCGCTCCAGGTACTTCTCGA  629
Query  484    ACAGGTTCTGCTCCGGGCTCTTCTCCTTCTCCGCGTTCTCGATGATCTCGCGCTTCTCGC  543
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Sbjct  628    ACAGGTTCTGCTCCGGGCTCTTCTCCTTCTCCGCGTTCTCGATGATCTCGCGCTTCTCGC  569
Query  544    GCTCCGTGATGCCCGGGCCCTTGGACTGGATCTGCTTCTCGATCTTGGGCGCGCGGCCCT  603
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Sbjct  568    GCTCCGTGATGCCCGGGCCCTTGGACTGGATCTGCTTCTCGATCTTGGGCGCGCGGCCCT  509
Query  604    CGGCCGCCCGGTGGTAACAGTTGTCGATCTCCTGCAGCAGGAAGTTGAGCTCGGAGGTGA  663
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Sbjct  508    CGGCCGCCCGGTGGTAACAGTTGTCGATCTCCTGCAGCAGGAAGTTGAGCTCGGAGGTGA  449
Query  664    GGCGCGTGGAGGCCAGGAACTCCCAGCCCAGCGCGGGCACGTACATGATGGCGGCGAAGG  723
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Sbjct  448    GGCGCGTGGAGGCCAGGAACTCCCAGCCCAGCGCGGGCACGTACATGATGGCGGCGAAGG  389
Query  724    CCAGCAGCGCGTAGGGCAGGAACTTGTGCTCAAACAGCGACGGCCACAGGCTGGCGTCCA  783
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Sbjct  388    CCAGCAGCGCGTAGGGCAGGAACTTGTGCTCAAACAGCGACGGCCACAGGCTGGCGTCCA  329
Query  784    CGCCGGGCAGCGCGTCCCGCAGCTCCGTCCAGCAGTAGCCGCGGGCGTACAGCGCCTGGT  843
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Sbjct  328    CGCCGGGCAGCGCGTCCCGCAGCTCCGTCCAGCAGTAGCCGCGGGCGTACAGCGCCTGGT  269
Query  844    CGCGCGTGAAGTTGTGCGGGGTGTAACAGTAAATGGGTTCCTCTGCGAAGTTTCTTGGT  902
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Sbjct  268    CGCGCGTGAAGTTGTGCGGGGTGTAACAGTAAATGGGTTCCTCTGCGAAGTT-CTTGGT  211
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Homo sapiens pannexin 2 (PANX2), transcript variant 3, non-coding RNA
Sequence ID: ref|NR_027691.1| Length: 3035 Number of Matches: 1 Range 1: 278 to 1158

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Query  6     GTTGGGTGA-GGAGT-CAGCCGGTTGAGCGACTTGATGTGGTCGCGGTTCTCGTTGCAGA  63
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1100
Query  64    ACATGGCCAGGATGTTGATGTCGCAGAACTGCGAGCGGCGCCACTGCCGGCGCGTCTTGA  123
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Sbjct  1099  ACATGGCCAGGATGTTGATGTCGCAGAACTGCGAGCGGCGCCACTGCCGGCGCGTCTTGA
1040
Query  124   TGCCCACCTTGTGCAGCTTGTCGAAGATGAAGTTGCTCTTGCGGAAGATGAAGAGGTGGA  183
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Query  184   TGAGGTTGACGAGGATGATGAGGTTCATGACGCACAGCAGCACGATGTCCACGCCCGCGA  243
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Query  244   TGATGCGCTGCAGTTGCACGGACGGGAGCTTGCAGCTCACGCGCACCGCGGGCCCCGCAC  303
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Sbjct  919   TGATGCGCTGCAGTTGCACGGACGGGAGCTTGCAGCTCACGCGCACCGCGGGCCCCGCAC  860
Query  304   CTGCCGCCCCGTCCGGGGACGCGCCCAGCGCGCAGGTGAACTCGTTCTGCTTCTGCGTGG  363
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Sbjct  859   CTGCCGCCCCGTCCGGGGACGCGCCCAGCGCGCAGGTGAACTCGTTCTGCTTCTGCGTGG  800
Query  364   CGTAGTAGGTGCACAGGTAGGAGATGGGCACGGCGCTCAGCAGCAGGATCAGCACGTGCC  423
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Sbjct  799   CGTAGTAGGTGCACAGGTAGGAGATGGGCACGGCGCTCAGCAGCAGGATCAGCACGTGCC  740
Query  424   GCGCCAGGTACAGCTTGGCCAGGAAGTTGCTGCGGCCGCGGCGCTCCAGGTACTTCTCGA  483
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  484   ACAGGTTCTGCTCCGGGCTCTTCTCCTTCTCCGCGTTCTCGATGATCTCGCGCTTCTCGC  543
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Sbjct  679   ACAGGTTCTGCTCCGGGCTCTTCTCCTTCTCCGCGTTCTCGATGATCTCGCGCTTCTCGC  620
Query  544   GCTCCGTGATGCCCGGGCCCTTGGACTGGATCTGCTTCTCGATCTTGGGCGCGCGGCCCT  603
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Sbjct  619   GCTCCGTGATGCCCGGGCCCTTGGACTGGATCTGCTTCTCGATCTTGGGCGCGCGGCCCT  560
Query  604   CGGCCGCCCGGTGGTAACAGTTGTCGATCTCCTGCAGCAGGAAGTTGAGCTCGGAGGTGA  663
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Sbjct  559   CGGCCGCCCGGTGGTAACAGTTGTCGATCTCCTGCAGCAGGAAGTTGAGCTCGGAGGTGA  500
Query  664   GGCGCGTGGAGGCCAGGAACTCCCAGCCCAGCGCGGGCACGTACATGATGGCGGCGAAGG  723
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Query  724   CCAGCAGCGCGTAGGGCAGGAACTTGTGCTCAAACAGCGACGGCCACAGGCTGGCGTCCA  783
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Sbjct  439   CCAGCAGCGCGTAGGGCAGGAACTTGTGCTCAAACAGCGACGGCCACAGGCTGGCGTCCA  380
Query  784   CGCCGGGCAGCGCGTCCCGCAGCTCCGTCCAGCAGTAGCCGCGGGCGTACAGCGCCTGGT  843
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Sbjct  379   CGCCGGGCAGCGCGTCCCGCAGCTCCGTCCAGCAGTAGCCGCGGGCGTACAGCGCCTGGT  320
Query  844   CGCGCGTGAAGTTGTGCGGGGTGTAACAGTAAATGGGTTCCT  885
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Sbjct  319   CGCGCGTGAAGTTGTGCGGGGTGTAACAGTAAATGGGTTCCT  278
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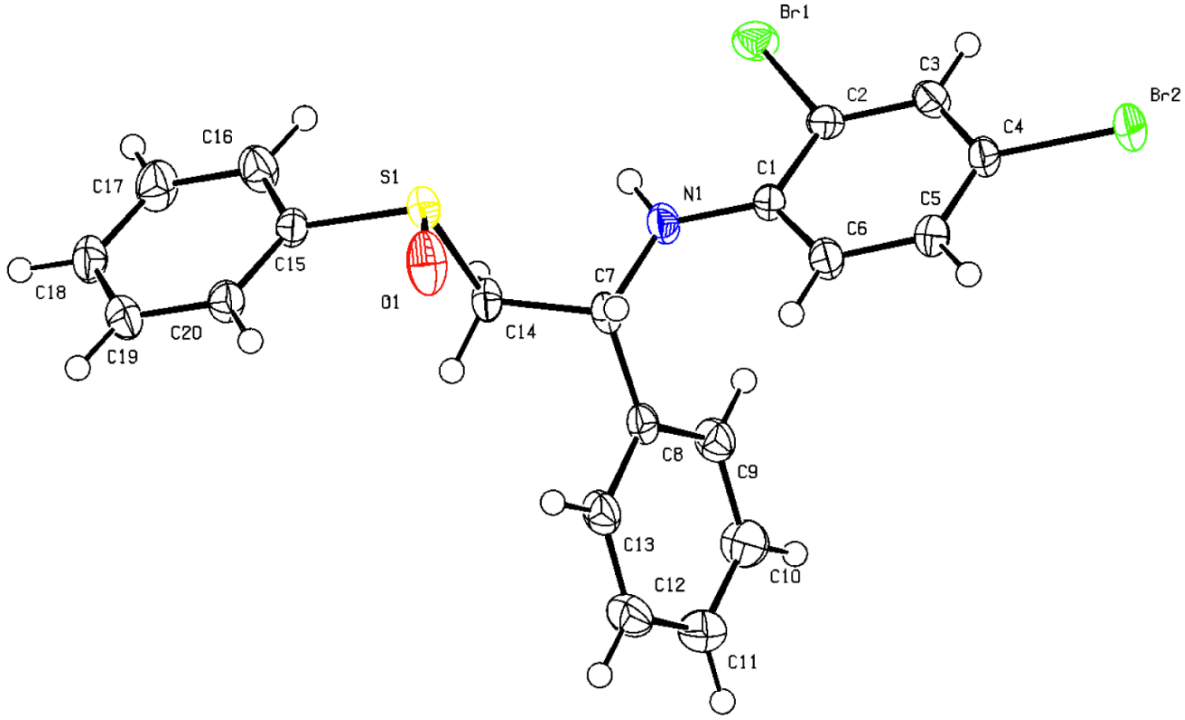
7.2 Structure of AF101 obtained from X-ray crystallography



8 CURRICULUM VITAE

Donald Matthew Cooke, B.Sc.

Publications

Blanchard AP*, McDowell GSV*, Valenzuela N*, Xu H, Gelbard S, Bertrand M, Slater GW, Figeys D*, Fai S†, Bennett SAL† (2012) Visualization and Phospholipid Identification (VaLID): An online integrated search engine capable of identifying and visualizing glycerophospholipids with given mass. Bioinformatics, doi: 10.1093/bioinformatics/bts662. *Authors contributed equally. † Joint corresponding authors.

The critical comments I provided while the program was under development are acknowledged in this publication.

Imbeault S*, Valenzuela N*, Fai S†, Bennett SAL† (2010) Localizing protein in 3D neural stem cell culture: A hybrid visualization methodology. J Vis Exp, 2010 Dec 19;(46). *Authors contributed equally. †Joint corresponding authors.

My assistance in data collection performed while an undergraduate student under the mentorship of Dr. Sophie Imbeault upon joining the Bennett laboratory in May 2010 is acknowledged in this publication.

Presentations

16/10/2012	Cooke DM, Taylor GP, Cao X, Tsai EC, Arnason JT, Bennett SAL (2012) Targeting connexins to promote functional neural repair and regeneration. Neuroscience 2012 (New Orleans, USA, poster presentation by Cooke DM)
16/10/2012	Taylor GP, Cooke DM, McLean AC, Cieniak C, Foster BC, Arnason JT, Bennett SAL (2012) Identification of housekeeping gene calibrators for real-time quantitative reverse transcription PCR (qRT-PCR) analysis of gene expression during neurogenesis and gliogenesis. Neuroscience 2012 (New Orleans, USA, poster presentation by Taylor GP)
22/02/2012	Cooke DM, Bennett SAL (2012) Targeting connexins to promote neural repair and regeneration. BMI Seminar Day (Ottawa, Canada, oral presentation by Cooke DM)

17/08/2011 Cooke DM, Hanley C, Fai S, Cao X, Tsai EC, Arnason JT, Bennett SAL (2011) Phenolic connexin modulators: Identification of new pharmacological tools to manipulate connexin function and expression. International Gap Junction Conference 2011 (Ghent, Belgium, poster presentation by Cooke DM)

08/06/2011 Cooke DM, Hanley C, Fai S, Arnason JT, Bennett SAL (2011) Bioassay guided validation of a systems ethnobotanical approach to the discovery of novel connexin modulators. Ottawa Institute of Systems Biology Research Retreat (Montebello, Canada, poster presentation by Cooke DM)

08/06/2011 Valenzuela N, Cooke DM, Fai S, Bennett SAL (2011) Representing the Alzheimer Disease Lipidome. Ottawa Institute of Systems Biology Retreat (Montebello, Canada, poster presentation by Valenzuela N)

19/05/2011 Cooke DM, Hanley C, Fai S, Bennett SAL (2011) Targeting connexins to promote stroke recovery. University of Ottawa BMI Poster Day (Ottawa, Canada, poster presentation by Cooke DM)

12/05/2011 Cooke DM, Hanley C, Fai S, Bennett SAL (2011) Targeting connexins to promote stroke recovery. Heart and Stroke Foundation Centre for Stroke Recovery Annual Scientific Meeting (Toronto, Canada, poster presentation by Cooke DM)

22/11/2010 Cooke DM*, Hanley C*, Fai S, Bennett SAL (2010) Spatial and temporal modeling of the entry of lipid second messengers into neural stem cells over the course of specification: step one. Neurolipidomics 2010 Annual Meeting and Research Symposium (Ottawa, Canada, oral presentation by Hanley C and Cooke DM) *Joint first authors

Published Abstracts

16/10/2012 Cooke DM, Taylor GP, Cao X, Tsai EC, Arnason JT, Bennett SAL (2012) Targeting connexins to promote functional neural repair and regeneration. Neuroscience 2012 (New Orleans, USA, poster presentation by Cooke DM)

16/10/2012 Taylor GP, Cooke DM, McLean AC, Cieniak C, Foster BC, Arnason JT, Bennett SAL (2012) Identification of housekeeping gene calibrators for real-time quantitative reverse transcription PCR (qRT-PCR) analysis of gene expression during neurogenesis and gliogenesis. Neuroscience 2012 (New Orleans, USA, poster presentation by Taylor GP)

17/08/2011 Cooke DM, Hanley C, Fai S, Cao X, Tsai EC, Arnason JT, Bennett SAL (2011) Phenolic connexin modulators: Identification of new pharmacological tools to manipulate connexin function and expression. International Gap Junction Conference 2011 (Ghent, Belgium, poster presentation by Cooke DM)

12/05/2011 Cooke DM, Hanley C, Fai S, Bennett SAL (2011) Targeting connexins to promote stroke recovery. Heart and Stroke Foundation Centre for Stroke Recovery Annual Scientific Meeting (Toronto, Canada, poster presentation by Cooke DM)

22/11/2010 Cooke DM*, Hanley C*, Fai S, Bennett SAL (2010) Spatial and temporal modeling of the entry of lipid second messengers into neural stem cells over the course of specification: step one. Neurolipidomics 2010 Annual Meeting and Research Symposium (Ottawa, Canada, oral presentation by Hanley C and Cooke DM) *Joint first authors

Academic Awards/Funding

2012-2015 CIHR Banting & Best Doctoral Award (declined)

2012-2013 Ontario Graduate Scholarship (declined)

2012 CIHR Institute of Aging Travel Award

2012 CIHR Training Program in Neurodegenerative Lipidomics Conference Travel Award

2011-2012 CIHR Banting & Best Master's Award

2011-2012 Ontario Graduate Scholarship (declined)

2011 CIHR Training Program in Neurodegenerative Lipidomics Conference Travel Award

2011	University of Ottawa Faculty of Graduate & Postdoctoral Studies Travel Grant
2011	University of Ottawa Biochemistry Graduate Program Travel Award
2011	CIHR Training Program in Neurodegenerative Lipidomics Short-Term Research Award
2010-2011	Ontario Graduate Scholarship in Science & Technology
2010-2011	CIHR Training Program in Neurodegenerative Lipidomics Graduate Student Award
2010	CIHR Training Program in Neurodegenerative Lipidomics Short-Term Research Award
2010	CIHR Training Program in Neurodegenerative Lipidomics Undergraduate Research Award
2006-2010	University of Ottawa Admission Scholarship
2010	University of Ottawa Dean's Honour List
2008	University of Ottawa Dean's Honour List
2007	University of Ottawa Dean's Honour List

Research Experience

2010-2013	**M.Sc. Candidate, Biochemistry Program, University of Ottawa, Ottawa, Canada** Project title: Targeting connexins to promote functional neural repair and regeneration Supervisors: Dr. Steffany Bennett, Dr. Xudong Cao, Dr. Eve Tsai Co-mentor: Dr. John Arnason
2010	**Undergraduate Summer Researcher, University of Ottawa, Ottawa, Canada** Project title: Targeting gap junction proteins to inhibit release of neurotoxic lipid second messengers and promote functional spinal cord repair Supervisor: Dr. John Arnason Co-mentor: Dr. Steffany Bennett

2009-2010	**Biomedical Science Honours Research Project, National Research Council of Canada, University of Ottawa, Ottawa, Canada** Project title: The function of CRMP3 during cerebral ischemia Supervisor: Dr. Sheng Hou
2009	**Undergraduate Summer Researcher, National Research Council of Canada, University of Ottawa, Ottawa, Canada** Project title: The function of CRMP3 during cerebral ischemia Supervisor: Dr. Sheng Hou

Selected Extracurricular Activities

2012-2013	Vice President Finance, Student Society for Alzheimer's Disease and Dementia, University of Ottawa
2012-2013	Awards & Communications Officer, CIHR Training Program in Neurodegenerative Lipidomics
2011-2013	Webmaster, CIHR Training Program in Neurodegenerative Lipidomics
2011-2013	Webmaster, Neural Regeneration Laboratory
2010-2011	Instructor, Teacher's Science & Technology Outreach Program
2009-2013	Volunteer, Ottawa Hospital