Modulation of the Junctional Conductance of Retinal AII Amacrine Cell Electrical Synapses

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Abstract

Retinal AII amacrine cells are extensively coupled together by electrical synapses. Changes to the strength of these synapses affect how signals are routed through rod and cone retinal pathways during scotopic and photopic vision. Plasticity at these electrical synapses have not, to date, been characterized using electrophysiological approaches. We investigated the effects of adenosine (AR) and N-methyl-D-aspartate receptor (NMDAR) activation on the electrical coupling between AII cells using dual whole-cell patch-clamp electrophysiology in mouse retinal slices. While neither AR activation nor inhibition affected junctional conductance, NMDAR activation substantially decreased junctional conductance between AII cells. Relieving the Mg^{2+} block of NMDARs through bath application of Mg^{2+}-free solution or by depolarizing AII cells to 0 mV reduced junctional conductance. Exogenous application of NMDA decreased conductance between cells, a decrease which was blocked by the non-selective NMDAR antagonist APV but not by Ro 25-6981, a selective GluN2B-NMDAR antagonist. Addition of either D-serine or glycine, both NMDAR coagonists, without NMDA, reduced the junctional conductance and addition of either coagonist to NMDA-treated retinas further decreased conductance. Experiments were conducted in inositol 1,4,5-trisphosphate receptor type 2 KO and serine racemase KO mice and in WT mice with D-amino acid oxidase to reduce retinal D-serine levels. Under these conditions, the NMDA-mediated conductance decrease was maintained, indicating that D-serine is not necessary for NMDAR-mediated plasticity. These results demonstrate that NMDAR activation results in a decrease in electrical coupling between AII amacrine cells and suggests that both D-serine and glycine can serve as NMDAR coagonists for this plasticity.

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Chapter 1: Introduction

Overview

In the nervous system, electrical synapses facilitate fast and synchronous communication, bi-directional transmission of information, coincidence detection of subthreshold potentials, signal amplification, and noise reduction through the flow of current via gap junctions (Szczupak, 2016; Nagy et al., 2018). Like chemical synapses, electrical synapses also undergo plasticity, or changes to synapse strength to allow more or less communication between cells. While all brain areas rely on instantaneous electrical synapse communication, many studies researching electrical synapse plasticity focus on sensorimotor systems. Sensorimotor systems in particular depend on these synapses to finely tune entering signals into and outgoing signals from the brain that dictate how we perceive and react to the environment around us (Nagy et al., 2018). One such system is the retina, which has the highest diversity of neuronal gap junction proteins (Nagy et al., 2018).

Several studies in the retina have claimed electrical synapse plasticity to be critical for specific visual processes, such as light adaptation. Neurons of the light adaptation circuitry in the retina have gap junctional-dependent mechanisms to facilitate the activation of either the rod or cone pathway, depending on the amount of ambient light present (Bloomfield and Völgyi, 2009). One such neuron in this circuitry is the well-studied AII amacrine cell, which forms a highly interconnected network with other AII cells through gap junctions in the inner plexiform layer. The degree of electrical coupling in this AII syncytium serves as a gateway between the facilitation of signals from either the rod and cone pathway in darkness and light, respectively (Smith and Vardi, 1995;

Bloomfield et al., 1997; Bloomfield and Völgyi, 2004; Demb and Singer, 2012; Hartveit and Veruki, 2012).

Though AII electrical synapses are relatively well-studied, research has been limited to using tracer techniques to evaluate their plasticity. Some labs have demonstrated robust evidence that neuromodulators and light levels alter the size of the AII amacrine network with neuronal tracing experiments using neurobiotin, a gap junction permeable molecule. Some of these studies have elaborated on downstream mechanisms responsible for this plasticity by staining for phosphorylation of gap junction proteins or using pharmacological agents targeting second messenger systems (Hampson et al., 1992; Bloomfield et al., 1997; Bloomfield and Völgyi, 2004; Urschel et al., 2006; Kothmann et al., 2009, 2012). No studies to date have reproduced this plasticity between AII cells using electrophysiological means. This thesis does so by testing the effect of neuromodulators on AII electrical synapse plasticity using dual whole-cell patch-clamp electrophysiology, according to the following hypotheses:

1) **Adenosine receptor activation alters gap junctional conductance between AII amacrine cells.**
2) **NMDA receptor activation alters gap junctional conductance between AII amacrine cells.**
3) **Gliotransmitter and NMDAR coagonist D-serine facilitates NMDA receptor-mediated modulation of gap junctional conductance between AII amacrine cells.**

Synaptic Communication: Chemical *and* Electrical

While organs depend on complex biological processes to serve the various and specific needs of organisms, the basis for these processes is simple and common to all:

cell-to-cell communication. The field of neuroscience has described two kinds of cell-to-cell, or synaptic, communication between neurons: chemical and electrical. Chemical synapses facilitate communication through a series of steps on the scale of mere milliseconds. The summation of signals in the presynaptic neuronal cell body triggers the canonical action potential, which leads to a massive depolarization and the opening of voltage-gated sodium channels in the axon hillock, which causes a domino effect of depolarization through a cascade of opening sodium channels down the axon. The subsequent depolarization of the axon terminal triggers opening of voltage-gated calcium channels, which activates the vesicular release of neurotransmitters that cross the synaptic cleft between two neurons. Depending on the nature of the presynaptic neurotransmitter and postsynaptic receptors, the interaction of the two will trigger a response, and the process starts all over again in the post-synaptic neuron (Bean, 2007; Wang et al., 2009).

Neurons also communicate with one another via electrical synapses. To date, no studies have measured the timescale for such a communication, and have described it as “instantaneous,” (Furshpan and Potter, 1957; Bennett and Zukin, 2004) as opposed to milliseconds at chemical synapses (Sabatini and Regehr, 1996; Katz and Miledi, 1997). Compared to chemical synapses, communication facilitated through electrical synapses appears simplistic. Two neurons converse through the apposition of cell processes connected by gap junctions, which allow the flow of current carried by ions. Electrical synapses play an integral role in fast and synchronous communication in many brain areas, especially those that facilitate sensorimotor processes. Though fast and synchronous communication is necessary for many central and peripheral brain functions, the mechanisms behind electrical synapse function and plasticity remain

understudied compared to its chemical counterpart. Underestimating the role of electrical synapses in neuronal communication is a disservice to the field's goal of understanding overall brain function. Thus, it is imperative to support research that expands our knowledge on electrical synapse plasticity, and its function within brain health and disease.

Electrical synapses

History

In the first half of the 20th century, neuroscientists relentlessly argued over synaptic communication being chemical or electrical, a debate otherwise known as 'soup or spark' (Bennett, 1997). As evidence for 'soup' accumulated in studies largely focusing on acetylcholine release at the neuromuscular junction, arguments for 'spark' fell to the wayside (Bennett, 1997; Cowan and Kandel, 2001; Nagy et al., 2018; Sotelo, 2020). Even so, studies demonstrating undeniable evidence for electrical synapses emerged soon after the field's resolution to the debate in the early 50s (Bennett, 1997). Akira Watanabe published on neuronal synchronization mediated by electrical coupling in lobster (Watanabe, 1958), and Furshpan and Potter reported the crayfish escape response to rely on both chemical and electrical synapses (Furshpan and Potter, 1959). These papers in invertebrates, along with other seminal experiments performed in fish (Bennett et al., 1959, 1963; Robertson, 1963), continually gave the impression that electrical synapse communication is simplistic, primitive, and less sophisticated compared to chemical synapse communication. However, an abundance of studies in mammalian CNS emerged shortly after in the 1970s that documented electrical synapses in various brain areas such as olfactory bulb (Pinching and Powell, 1971), sensory motor cortex (Sloper, 1972), inferior olive (Llinas et al., 1974), and cerebellum (Sotelo and Llinás, 1972; Bennett, 1997).

Recognition and prevalence of electrical synapses in mammalian CNS really took off with the discovery of connexin36 (Cx36), as well as the development of genetic, imaging, and electrophysiological techniques suited to studying gap junctional communication in mammals. Expression of Cx36, the most common neuronal gap junction protein, was identified in many types of neurons in various areas of the nervous system in the late 90s (Condorelli et al., 1998; Söhl et al., 1998). This discovery of a widely expressed neuronal connexin fueled the development of a Cx36 knockout mouse model in the early 2000s, which demonstrated that Cx36-containing electrical synapses are necessary for an array of nervous system functions, including memory, vision, and motor skills (Nagy et al., 2018). In addition, development of techniques such as freeze-fracture immunogold labeling and infrared differential interference contrast (IR-DIC) visualization of individual cells in dual whole-cell recordings enabled researchers to find many more neuronal gap junctions and record electrical synapse activity between neighboring neurons, respectively (Bennett and Zukin, 2004; Nagy et al., 2018).

Electrical synapse function

Compared to chemical synapses, electrical synapses facilitate faster communication through the gap junction channels that physically connect two cells together at the membrane. This speedy communication allowed by the immediate and direct flow of current between cells lends electrical synapse communication specific and unique properties, such as bi-directional transmission of information, synchronization of signals across a network, coincidence detection of subthreshold potentials, signal amplification, and noise reduction (Szczupak, 2016; Nagy et al., 2018). While electrical synapses are expressed in all areas of the nervous system, their function is mostly studied in sensorimotor systems, which in particular require fast, reliable, and

synchronous neuronal communication to process signals that control how we perceive and react to the environment around us (Nagy et al., 2018).

Structure and localization

In the nervous system, electrical synapses facilitate communication through the flow of current via gap junctions (Szczupak, 2016; Nagy et al., 2018). Gap junctions between two cells form ion channels that allow the exchange of ions and second messengers (Shimizu and Stopfer, 2013). In vertebrates, these gap junction channels are comprised of a connexon, or hemichannel, from each cell which are made of six connexin proteins (Nielsen et al., 2012). In invertebrates, these protein aggregates are called innexons and are made of eight innexin proteins (Oshima et al., 2016). The mammalian nervous system expresses five types of neuronal connexin (Cx) proteins: Cxs 36, 45, 50, 57, and 30.2 (O'Brien, 2019). The numbers represent the protein molecular weight in kilodalton, and slightly vary by species (Nielsen et al., 2012). The type of connexin expressed at an electrical synapse confers specific properties to the gap junctions such as degree of conductance, open probability, ion permeability, and voltage-dependence (Shimizu and Stopfer, 2013). For example, gap junctions comprised of Cx36 are weakly sensitive to voltage differences between two cells and have a small unitary conductance of 10-15 pS (Srinivas et al., 1999), whereas those comprised of Cx45 are more sensitive to voltage differences and have a larger unitary conductance of 30 pS (Moreno et al., 1995). Additionally, gap junctions may form homotypic electrical synapses, where the same connexin type forms each hemichannel, or heterotypic synapses, where two different connexin types form each hemichannel (Nielsen et al., 2012).

Like their chemical counterparts, electrical synapses are found in a variety of subcellular structures, neuronal types, and brain areas. Previous research has described axo-axonic (Smedowski et al., 2020), somato-somatic (Curti et al., 2012), and dendro-dendritic (Hidaka et al., 2004) electrical synapses. The field largely focuses on describing synapses between inhibitory neurons (Nagy et al., 2018), but many studies also describe gap junctional communication between excitatory neurons in brain areas such as spinal cord (Logan et al., 1996), inferior olive (Llinas et al., 1974), dorsal cochlear nucleus (Apostolides and Trussell, 2013), retina (Pan et al., 2010), hippocampus and cortex (Mercer et al., 2006).

While ‘electrical synapses’ and ‘gap junctions’ are often referred to interchangeably in the electrical synapse field, it should be noted that gap junctional communication is not exclusive to the nervous system, and is prevalent in other tissues and organs such as the heart, liver, gastrointestinal tract, and pancreas, just to name a few (Nielsen et al., 2012). Furthermore, within the nervous system, gap junctions also facilitate communication between different types of glial cells, and are often studied in astrocytes (Nielsen et al., 2012). For the purpose of this thesis, any mention of gap junctions refers to electrical synapse communication between neurons.

Electrical synapse plasticity

Like chemical synapses, electrical synapses are capable of modification, or plasticity, in response to the needs of the nervous system. The plasticity of the synapses allows for more or less current flow across the electrical synapses, which alters the amount of communication across a coupled network of cells. The time scale and mechanisms behind these changes vary. Electrical synapses may undergo intermediate-term plasticity, which represents changes that occur over seconds to minutes, and are

the result of post-translational modifications, such as phosphorylation or dephosphorylation of connexin proteins. Long-term plasticity may occur over minutes to days, and can be mediated by insertion or deletion of gap junction channels, or changes in gene expression (O'Brien, 2019). Both intermediate- and long-term plasticity are carried out via intracellular second messenger signaling systems.

The general second messenger signaling systems that lead to intermediate- and long-term plasticity mirror those observed with chemical synapse plasticity. Changes in G-protein coupled receptor (GPCR) activity or intracellular calcium levels lead to differential activity of kinases such as PKA, PKC, PKG, and CamKII and phosphatases such as PP2 and PP1 (Nielsen et al., 2012; O'Brien, 2014; Haas et al., 2016; Cachope and Pereda, 2021). The balance between kinase and phosphatase activity changes the phosphorylation state of gap junction proteins or CREB activity, which may prompt modulation of gene transcription and subsequent insertion or deletion of gap junction proteins (Pereda, 2014; O'Brien, 2019). Insertion of gap junction proteins correlates with increased communication between cells, and deletion of gap junctions correlates with decreased communication. Typically, enhanced kinase activity and subsequent phosphorylation of connexins correlates with enhanced gap junctional communication (O'Brien, 2019), but sometimes an anti-correlation is reported (Urschel et al., 2006). While the electrical synapse field generally accepts phosphorylation state as a broad mechanism that predicts modulation of neuronal gap junction communication, curiously, this has only been demonstrated experimentally in synapses expressing Cx50, and especially, Cx36 (O'Brien, 2019). This is likely due to the lack of genetic and molecular techniques readily available for targeting neuronal connexins other than Cx36.

Techniques to measure electrical synapse plasticity

Researchers of the field have developed several techniques to explore the electrical synapse function and plasticity. These methods evaluate gap junctional communication between individual cells and across networks using various imaging, molecular, and electrophysiological techniques.

After Furshpan and Potter demonstrated electrophysiological evidence for electrical synapses (Furshpan and Potter, 1959), investigators used electron microscopy (EM) to identify other neuronal gap junctions. Early on, the limitations of EM regarding magnification and fixation led the field to overlook the prevalence of electrical synapses in mammals, which express smaller gap junctions. The development of freeze-fracture immunogold labeling uncovered an increased prevalence of mammalian connexin expression, which enabled the development of genetic and molecular techniques to not only identify electrical synapses, but evaluate mechanisms mediating plasticity at these synapses (Nagy et al., 2018). Current approaches evaluate phosphorylation state or insertion and deletion of specific connexin proteins by quantifying protein or RNA expression levels using immunohistochemistry, immunoblot, and RT-PCR (Dong et al., 2018).

Another early imaging technique that continues to be a mainstay in the field is tracer or dye coupling, which visualizes the spatial extent of networks coupled by gap junctions. Researchers inject cells or bathe wounded tissue with a gap junction permeable molecule, and detect the spread of the tracer through a network of coupled cells. In response to a given manipulation, an increase in cells labeled with tracer correlates to a strengthening of the coupled network, while a decrease correlates to a weakened network. Ideally, investigators visualize the changes in the coupled network in real time using a fluorescent molecule such as Lucifer Yellow; however, fluorescent

molecules are too large to diffuse through many types of gap junctions. To circumvent this issue, many studies employ smaller, biotin-derived molecules such as biocytin or neurobiotin, and analyze the coupled network using an immunohistochemical protocol taking advantage of the streptavidin-biotin conjugation system. While this solution also accomplishes spatial visualization of the coupled network, it comes at the cost of temporal precision (Dong et al., 2018).

While molecular techniques provide valuable information about electrical synapse dynamics, they lack temporal precision. Some investigators take advantage of electrophysiological techniques to gauge electrical synapse plasticity in real time. A subset of these methods indirectly measures electrical synapse strength by analyzing the corresponding properties that electrical synapses are known to have. For example, gap junctional communication increases signal-to-noise ratios through the summation of signals across a coupled cell network. Noise analysis through electrophysiological means may indicate an increase or decrease in coupling across a network (Szczupak, 2016). Evaluating the synchrony of signals between cells is another proxy for evaluating electrical synapse strength. A higher coincidence of signals between cells may correlate to a stronger electrical synapse network (Bennett and Zukin, 2004). While these methods may provide valuable insight into the role of electrical synapses within neural circuitry, the results provide indirect evidence regarding plasticity and must be interpreted with caution.

While the aforementioned electrophysiological techniques assess electrical synapse strength through correlated properties thought to be associated with their function, dual whole-cell patch-clamp electrophysiology evaluates the degree in which coupled cells communicate with one another. Dual patch-clamp involves simultaneous,

paired recordings from two cells coupled by electrical synapses. Stimulation of Cell 1 yields a response Cell 2, and the degree of that response indicates the degree of electrical synapse strength. Investigators have the option of recording the pairs in current clamp or voltage clamp mode, which yield the coupling coefficient or gap junctional conductance, respectively. In response to an injection of current in Cell 1, the coupling coefficient measures the ratio between the change in voltage between Cell 1 and Cell 2. Because changes in membrane potential between the cells may reflect changes in non-junctional membrane activity, the coupling coefficient does not directly measure the strength of gap junctional communication (Curti and O'Brien, 2016). On the other hand, gap junctional conductance as measured in voltage clamp mode directly evaluates the ability of gap junctions to pass current. In response to a voltage step in Cell 1, the amount of current in Cell 2 divided by the difference in voltage between Cells 1 and 2 yields gap junctional conductance. Monitoring of series resistance during recordings allows the experimenter to account for both error in voltage commands, as well as membrane resistances of Cells 1 and 2, which results in a direct and accurate representation of conductance at electrical synapses (Hartveit and Veruki, 2010). While electrophysiological techniques provide temporally precise information about electrical synapses, these methods lack the spatial resolution of tracer coupling and immunohistochemistry protocols.

In addition to the invasive and technically demanding methods described above, researchers examining gap junctions in the brain, as well as other organs, have developed non-invasive imaging methods to investigate communication between coupled cells. For example, through tissue incubation with membrane-permeant dyes, the gap junction fluorescence recovery after photobleaching (gap-FRAP) and local

activation of molecular fluorescent probes (LAMP) techniques track dye movement with temporal precision using photobleaching and UV-uncaging, respectively. These methods yield both spatial and temporal information about electrical synapses, but lack cell specificity (Wade et al., 1986; Dakin et al., 2005). Other studies combine imaging with genetic tools to achieve cell specificity, at the cost of temporal resolution. The incubation of tissue expressing a genetically encoded esterase or transporter with a gap junction-permeable fluorescent substrate allows for diffusion of fluorescent molecules in a cell-specific manner; but, incubation with the substrate occurs on the scale of hours (Tian et al., 2012; Qiao and Sanes, 2016). A newer approach that seemingly combines the best of all worlds is using genetically encoded voltage indicators. These genetically-encoded sensors allow for cell specificity, temporal resolution, and spatial resolution, and can be combined with optogenetics to assess coupling dynamics in neighboring cells (Tian et al., 2021).

The role of electrical synapses in disease

Even with the development of the techniques described above, few studies have investigated the role of electrical synapses in disease, let alone their potential as a therapeutic target. Other fields document gap junction involvement in numerous disease pathologies in various human tissues and organs, including the brain, but those studies focus on gap junction loss in glia or vasculature, rather than neurons (Nielsen et al., 2012). In addition, many studies propose that abnormal neuronal synchrony contributes to a number of diseases such as schizophrenia, epilepsy, autism, Alzheimer's disease, and Parkinson's disease. Yet, few consider how electrical synapses, which are known to facilitate this synchrony, may contribute to this pathology (Uhlhaas and Singer, 2006; Chen et al., 2018; Lapato and Tiwari-Woodruff, 2018). Nevertheless, an increasing

number of publications document the effect of neuronal gap junction loss on broader brain function through genetic knockout of neuronal connexins. Knockout of Cx36, the most commonly expressed neuronal connexin (Belousov and Fontes, 2013), impairs function such as long-term potentiation and learning and memory in the hippocampus (Bissiere et al., 2011; Wang and Belousov, 2011), transmission of the rod pathway signals in the retina (Güldenagel et al., 2001; Deans et al., 2002), and neuronal synchrony in the cortex, olfactory bulb, and inferior olive (Deans et al., 2001; Long et al., 2002; Pouille et al., 2017). Furthermore, studies investigating the role of electrical synapses in disease have reported changes in neuronal connexin expression, phosphorylation, or dye coupling in disease models such as amyotrophic lateral sclerosis (Kobayakawa et al., 2018), retinal degeneration (Ivanova et al., 2015), epilepsy (Perez-Velazquez et al., 1994), nerve injury (Chang et al., 2000), and ischemia (Oguro et al., 2001). Regarding therapeutic interventions, a few investigations in the retina have considered the role of electrical synapses in the 'bystander' effect, whereby cells undergoing cell death propagate apoptotic signals to neighboring cells, which then also undergo cell death (Freeman et al., 1993). Gap junctional communication between neurons is well suited for propagation of such signals, and genetic deletion of Cx36 reduced cell death in response to excitotoxicity and glaucoma (Akopian et al., 2014, 2017).

Electrical synapses of the retina

Role of electrical synapses in the retina

The retina is tissue of the central nervous system that detects light patterns using vertical and horizontal processing facilitated by chemical and electrical synapses.

Vertical processing entails chemical synapse communication of light patterns that traverses each of layer of the retina in this order: photoreceptor, outer nuclear, outer plexiform (synaptic), inner nuclear, inner plexiform, ganglion. This translates to the transmission of light-mediated signals through activation of photoreceptors, bipolar cells, and ganglion cells, in that order, which is then relayed to the lateral geniculate nucleus and the visual cortex and other brain regions. On the other hand, horizontal processing occurs within each layer of the retina through both chemical and electrical synapses, and facilitates fine tuning of the signals that allows for contrast detection, temporal coding, and light adaptation (Kolb, 1994).

The field of electrical synapses has learned much about the structure, function, and plasticity of gap junctional communication using the retina as a model. The retina expresses an abundance of electrical synapses, and also the highest diversity of connexin proteins compared to other brain areas (Nagy et al., 2018). While not every retinal neuron expresses connexin proteins, electrical synapses have been observed in all categories of major neuronal cell types of the retina: photoreceptors, horizontal cells, bipolar cells, amacrine cells, and ganglion cells. These electrical synapses may be homozygous, or between cells of the same type, or heterozygous, between two different cell types (O'Brien and Bloomfield, 2018). The permutation of electrical synapse combinations, along with it being a readily accessible area of the central nervous system, makes the retina an obvious tool for the electrical synapse researcher.

Besides the sheer abundance of retinal electrical synapses, an even more important advantage to using the retina as a model is contextualizing the role of electrical synapses with well-studied retinal functions within visual processing. Through genetic deletion of connexins, researchers have found that electrical synapses are

necessary for broad retinal functions. For example, Deans et al. found that Cx36 expression is necessary for rod-mediated, or night time, vision using a Cx36 knockout model and electrical recordings from ON-center ganglion cells (Deans et al., 2002). Furthermore, investigation into communication between specific cell types has uncovered mechanisms by which electrical synapses facilitate these functions. While Deans et al demonstrated Cx36 expression to be critical for rod-mediated vision in 2002, earlier studies detected rod signals in cones, and attributed this finding to gap junctional communication between photoreceptors (Nelson, 1977; Schneeweis and Schnapf, 1995). Other studies have identified electrical synapses between cones to be integral for improving signal-to-noise in the retina by the summation of signals across gap junctions. This summation both increases signal and decreases noise through combining visually-evoked signals while averaging out noise from individual cones due to random photon absorption and fluctuation (Bloomfield and Völgyi, 2009). Often, electrical coupling combined with chemical synaptic transmission lends the retina the capability of complex visual computations in response to diverse stimuli at a circuit-specific level. For example, direction-selective ganglion cells detect leading edges through antagonistic surround receptive fields mediated by excitatory and inhibitory chemical synapses; however, responses by chemical synapses are slow and create a lag time. Through lateral excitation of electrically coupled direction-selective ganglion cells, the circuitry normalizes for lag time by spreading current to a cell prior to the edge reaching the cell's dendritic field (Trenholm et al., 2013a, 2013b). The sharing of signals through gap junctions confers the retina with specialized computations that become even more complex when considering how the plasticity of gap junctional communication facilitates vision.

Plasticity of electrical synapses in the retina

Some retinal functions are computed through dynamic changes in electrical synapse communication. On a broad mechanistic level, electrical synapses between retinal cells may dictate the balance between sensitivity and spatial resolution of signal detection. An increase in electrical coupling, resulting from phosphorylation of gap junction proteins or insertion of channels between cells, leads to increased communication and lateral excitation across a retinal layer. This lateral excitation increases sensitivity through the summation of visual signals, at the cost of spatial resolution (Bloomfield and Völgyi, 2009; Szczupak, 2016). On the other hand, a decrease in electrical coupling decreases the reach of cell receptive fields, and thereby increases specificity at the cost of sensitivity (Bloomfield and Völgyi, 2009; Szczupak, 2016). This mechanism balancing specificity and sensitivity is often observed in functions relating to light adaptation and circadian rhythms.

A broad retinal function that is attributed to electrical synapse plasticity is adaptation to illumination levels and circadian rhythms. While research has documented the effect of various neurotransmitters on electrical synapse plasticity, many studies have focused on the role of dopamine in particular. Retinal dopamine levels vary with light adaptation and circadian rhythms (Wirz-Justice et al., 1984; Godley and Wurtman, 1988), and thus in theory, may mediate these light-induced changes. Importantly, dopamine receptor (DR) modulation of gap junctional coupling has been reported between photoreceptor, horizontal, amacrine, and ganglion cells through the activation of D1R, D2R, and D4R in rabbits, rodents, and fish (Hampson et al., 1992; McMahon, 1994; Mills et al., 2007; Hu et al., 2010; Li et al., 2013; Arroyo et al., 2016). DR-mediated electrical synapse plasticity plays a role in various visual functions related to light

adaptation. For example, some studies attribute contrast detection of the inner retina to outer retina horizontal cell receptive field size, which is dictated by changes in electrical synapse plasticity in response to various light levels. In bright light, increased dopamine release uncouples horizontal cells, which leads to smaller horizontal cell receptive field sizes that detect light patterns from a specific area of retina. In turn, those cells provide more concentrated areas of lateral inhibition to cone bipolar cells, which limits the size of bipolar cell center surround receptive fields. These smaller receptive fields facilitate finer contrast detection and edge discrimination in bright light (Goel and Mangel, 2021). Other dopamine-mediated changes in gap junction coupling preferentially allow signal transmission from either the cone or rod pathways, depending on the amount of ambient light present. For example, in darkness, retinal dopamine levels are decreased, which allows for increased coupling between rods and cones through decreased D4R activation. This increase in electrical synapse strength facilitates transmission of the secondary rod pathway, as mentioned previously (Goel and Mangel, 2021). Light and dopamine-mediated changes in coupling are also observed in the inner retina, between AII amacrine cells.

AII amacrine cells

Structure and function

The AII amacrine cell is a well-characterized, glycinergic, narrow-field interneuron that plays a role in transmission of signals in both rod and cone pathways through mixed chemical and electrical synapses. While this cell type integrates information from as many as 28 distinct classes of neurons (Marc et al., 2014), it is most well-known for its role in the primary rod pathway, which facilitates night time vision.

Light activated hyperpolarization of one rod photoreceptor decreases release of glutamate onto inhibitory metabotropic glutamate receptors on two rod bipolar cells (Sterling et al., 1988). Unlike cone bipolar cells, rod bipolar cells do not communicate directly with ganglion cells, which facilitate the transmission of rod signals to the brain through the optic nerve. The primary rod pathway co-opts the cone circuitry through the intermediate AII amacrine cell (Figure 1.1). Depolarized rod bipolar cells release glutamate which binds to AMPA receptors on approximately 5 postsynaptic AII amacrine cells, which electrically synapse onto about eight ON cone bipolar cells. These eight ON cone bipolar cells transmit the information from the rod pathway to two ON ganglion cells (Sterling et al., 1988). Through this pathway, the heterologous AII amacrine-ON cone bipolar electrical synapse serves as a necessary intermediate for the transmission of rod-mediated vision. In addition to this role, the AII cell also facilitates contrast detection of rod-mediated signals through its glycinergic inhibition of OFF cone bipolar and OFF ganglion cells through chemical synapses (Murphy and Rieke, 2008; Liang and Freed, 2010, 2012; Graydon et al., 2018).

The bi-stratified structure of the AII confers the cell with its specific properties in the rod and cone pathways. The pear-shaped AII soma sits at the borders of the retinal inner plexiform (IPL) and nuclear (INL) layers, with a primary neurite that bi-stratifies into proximal, lobular appendages terminating in the IPL OFF-sublamina and distal, arboreal dendritic trees terminating in the IPL ON-sublamina. The lobular appendages form the aforementioned

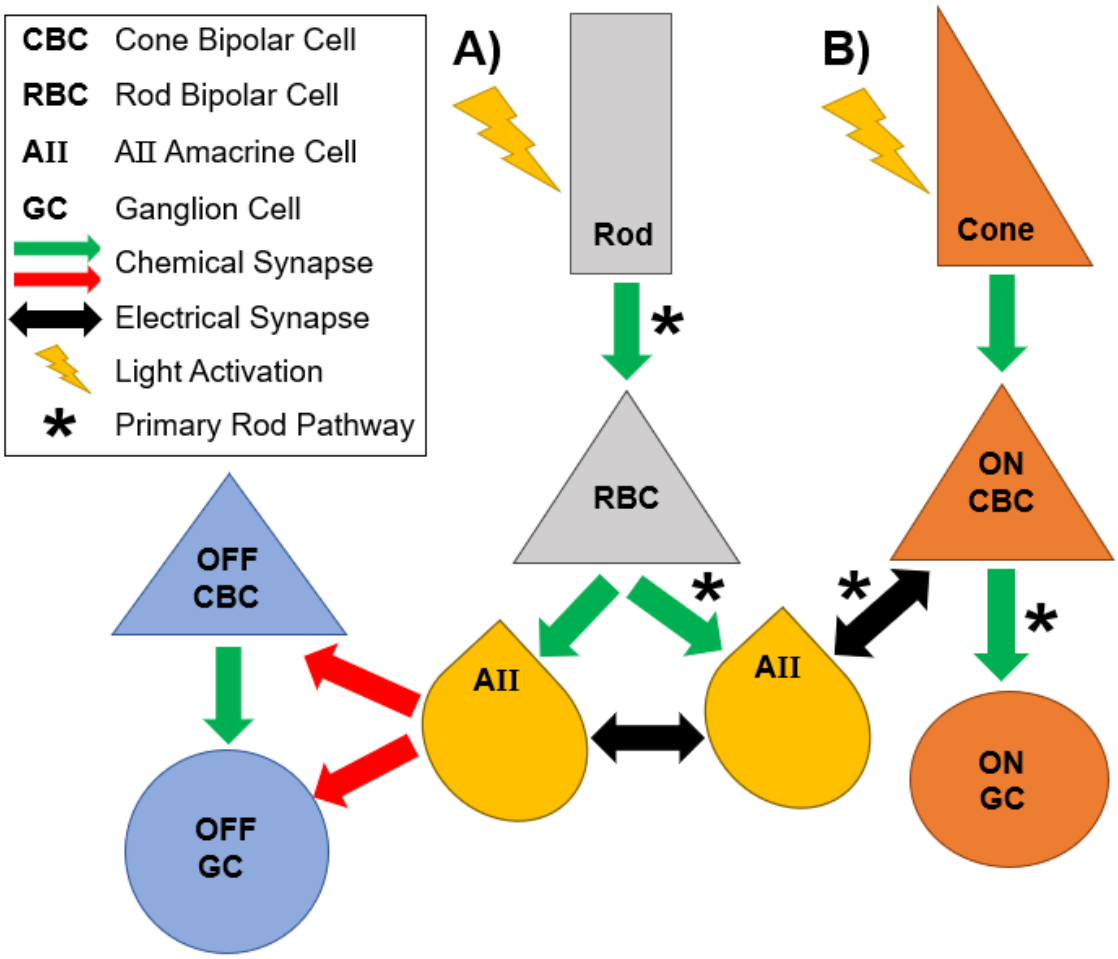

Figure 1.1 Diagram depicting the circuitry of the primary rod pathway. **A)** In the primary rod pathway, light activation of rods depolarizes rod bipolar cells (RBC), which synapse onto AII amacrine cells, and not directly onto ganglion cells (GC). AIIs pass on rod signals through electrical synapses with ON cone bipolar cells (CBC), which activate GCs that transmit the information to the brain through the optic nerve. In addition, AIIs facilitate contrast detection in this pathway by inhibiting OFF CBCs and GCs at glycinergic synapses. **B)** Unlike the rod pathway, the light activation of the cone pathway directly results in activation of ON CBCs and GCs. Green arrows represent excitatory chemical synapses, whereas red arrows signify inhibitory chemical synapses. Asterisks depict transmission of the primary rod pathway.

chemical, glycinergic synapses with OFF-cone bipolar and OFF-ganglion cells, while the arboreal dendrites house glutamatergic synapses with rod bipolar cells and electrical synapses with other AII cells as well as ON-cone bipolar cells (Kolb and Famiglietti, 1974; Famiglietti and Kolb, 1975).

In addition to the lobular and arboreal appendages, more recent studies have described the AII cell as having another process expressing properties similar to an axon initial segment (AIS). Previous to these reports, the retinal field considered the AII to be 'axonless' (Strettoi et al., 1992), even though electrical recordings demonstrated the cell to be capable of small, TTX-sensitive action potentials (Boos et al., 1993; Tamalu and Watanabe, 2007). Follow-up investigations into the mechanisms behind such action potentials revealed an AIS-like segment originating from AII lobular appendages expressing AIS characteristic proteins, as well as clustering of $Na_v1.1$ voltage-gated sodium channels (Kaneko and Watanabe, 2007; Wu et al., 2011; Liu et al., 2021). Viral-mediated replacement of AIS $Na_v1.1$ channels with ChR2-GFP-Na_vII-III eliminated AII action potentials (Wu et al., 2011). Excision or application of TTX to the AIS-like process also eliminates action potentials (Cembrowski et al., 2012). Some have published evidence that scotopic rod bipolar cell signaling is encoded by AIS spiking activity, implicating the AII AIS to be integral in nighttime visual processing (Tamalu and Watanabe, 2007; Tian et al., 2010).

Homologous AII amacrine cell electrical synapse plasticity

Many studies and various techniques have painted a picture of the complex circuitry surrounding the AII amacrine cell and its role in rod- and cone-mediated vision.

A subset of these studies has addressed the specific role of AII electrical synapse plasticity in these visual processes. AII cells form homologous gap junctions (Famiglietti and Kolb, 1975) composed of Cx36 proteins with other AII cells (Feigenspan et al., 2001), and form heterologous gap junctions with ON cone bipolar cells composed of Cx36 and Cx45 proteins (Demb and Singer, 2012). A combination of single cell electrophysiological recording, neurobiotin tracer, and computer modeling experiments have revealed that the dynamic plasticity of AII homologous junctions in response to background lighting serves as a “light switch” between the rod and cone pathways (Demb and Singer, 2012). In darkness, tracer coupling between cells, as well as single cell receptive field sizes, are small. In theory, the limited coupling between cells preserves the sensitivity and fidelity of rod-mediated signals from single photons in complete darkness. Additionally, weak electrical coupling between AII cells allows current to spread preferentially through heterologous gap junctions between AII and ON cone bipolar cells, resulting in activation of ON cone bipolar cells and downstream ganglion cells. With increasing background illumination into the range of dim light levels, or scotopic vision, tracer coupling and receptive field sizes increase as much as tenfold, in theory to increase signal-to-noise ratios while summating rod signals over a wide area of retina. As background lighting increases into the mesopic and photopic range, past the range of rod-mediated vision, tracer coupling and receptive field sizes decrease in order to preserve cone-mediated signaling by preventing leakage of the signal into the AII cell network (Smith and Vardi, 1995; Bloomfield et al., 1997; Bloomfield and Völgyi, 2004). These seminal studies cemented the importance of homologous AII electrical

synapses in both rod- and cone-mediated vision; however, the cellular signaling mechanisms that carry out this light-mediated plasticity remain elusive.

Neuromodulators of homologous AII amacrine cell electrical synapse plasticity

Previous to the aforementioned background light modulation studies, Hampson et. al was the first to demonstrate AII cell electrical synapses to be plastic. Activation of dopamine receptors, specifically, D1Rs and subsequent cAMP stimulation, decreased tracer coupling in the AII cell network as shown by neurobiotin experiments (Hampson et al., 1992). This finding corresponds with AII cells decoupling in photopic conditions, as retinal dopamine release increases with light exposure and during the subjective day of the circadian rhythm (Wirz-Justice et al., 1984; Godley and Wurtman, 1988). Follow up studies using neurobiotin agree with Hampson et. al, but disagree on whether or not phosphorylation or dephosphorylation of Cx36 leads to decoupling of AII cells (Urschel et al., 2006; Kothmann et al., 2009). While evidence of dopamine-mediated plasticity in tracer coupling and IHC phosphorylation experiments is robust, it has not been reproduced using other techniques. Two groups report that neither dopamine, nor D1R agonists, affect conductance between AII cells using dual whole-cell patch-clamp electrophysiology (Demb and Singer, 2012; Hartveit and Veruki, 2012). This discrepancy remains mysterious, and calls into question what these two techniques are actually measuring. It is possible that the tracer coupling experiments documented decreased AII electrical synapse strength in response to D1R activation on neurons upstream of AII cells. These studies do not take measures to block chemical synapse transmission in their preparations (Hampson et al., 1992; Urschel et al., 2006; Kothmann et al., 2009). Furthermore, other publications claim there being a lack of evidence of D1R expression

in AIIs (Demb and Singer, 2012). Even with this discrepancy, a strong argument for the importance of dopaminergic input to AII electrical synapses has been revealed in Parkinsonian retinas of human and non-human primates, which have decreased dopaminergic signaling resulting in scotopic vision impairments, decreased expression of AII Cx36, and deteriorated AII electrical synapses (Cuenca et al., 2005; Ortuño-Lizarán et al., 2020).

More recently, another neuromodulator of AII electrical synapses has been documented: NMDA receptors (NMDAR). Kothmann et. al published evidence that NMDA receptor activation increases neurobiotin tracer coupling and CamKII-mediated Cx36 phosphorylation. Furthermore, they postulated that light-driven ON bipolar cell glutamate release causes this plasticity, which is mediated by extrasynaptic NMDARs located near Cx36 proteins expressed on AII cells (Kothmann et al., 2012). To date, no other techniques have been used to corroborate these findings.

Adenosine receptors: Potential role in homologous AII amacrine cell electrical synapse plasticity?

While the retina and electrical synapse fields largely acknowledge dopaminergic modulation of AII electrical synapses, no studies to date have addressed the potential for adenosine receptor-mediated modulation at these synapses. While dopamine levels in the retina increase during subjective day and light adaptation (Wirz-Justice et al., 1984; Godley and Wurtman, 1988), in a complementary fashion, adenosine levels increase during subjective night and dark adaptation (Ribelayga and Mangel, 2005). Furthermore, this complementary dynamic has been studied in tracer coupling studies of

photoreceptors, which are also coupled by electrical synapses expressing Cx36. Increased dopamine levels during the day and bright light activate D4Rs and uncouple photoreceptors (Goel and Mangel, 2021), while increased adenosine levels during the night and darkness activate A2aRs and increase coupling between photoreceptors (Li et al., 2013; Cao et al., 2021). These results suggest that Cx36 gap junctional communication between AIIs, which are critical for processing night vision, could also be modulated by a signaling molecule like adenosine, the levels of which increase in darkness.

Heterologous AII amacrine-ON cone bipolar cell electrical synapses

As mentioned previously, the heterologous AII amacrine-ON cone bipolar cell electrical synapses serve an integral role in transmission of the rod primary pathway. While earlier studies investigating AII cell physiology and tracer coupling report their role to be limited to unidirectional, rod-mediated signaling from AIIs to ON cone bipolar cells (Nelson, 1982; Dacheux and Raviola, 1986; Vaney, 1997), later studies report otherwise. AIIs also process cone-mediated signaling through these heterologous electrical synapses capable of bidirectional communication with ON cone bipolar cells. Light activation of cone photoreceptors leads to depolarization of ON cone bipolar cells, and thus AII cells through gap junctional communication. Depolarized AII cells then inhibit OFF cone bipolar and OFF ganglion cells through glycinergic chemical synapses (Xin and Bloomfield, 1999; Trexler et al., 2001; Pang et al., 2004, 2007; Manookin et al., 2008). In addition, one study claims that AII and ON cone bipolar cells are capable of

neurotransmitter coupling, where ON cone bipolar cells express glycine originating from AII cells (Vaney et al., 1998).

Neuromodulation and plasticity at these synapses are less studied compared to their homologous counterpart. Dopamine decreases tracer coupling at both AII homologous and heterologous synapses, but does so preferentially to homologous synapses. Yet, a membrane-permeant cAMP agonist decreases tracer coupling in both networks equally (Xia and Mills, 2004). Nitric oxide and cGMP decrease tracer coupling at heterologous synapses, but not at homologous synapses (Mills and Massey, 1995; Xia and Mills, 2004).

AII electrical synapses and disease

More recent work has emerged describing the involvement of AII electrical synapses in diseases, especially Parkinson's (described above) and retinal degeneration. Borowska et al. were one of the first to do so by postulating that intrinsic oscillatory activity propagated by AII-ON cone bipolar electrical synapses is responsible for ganglion cell hyperactivity observed in a mouse model of retinitis pigmentosa (RP, or often referred to as retinal degeneration, RD), an umbrella term for diseases involving photoreceptor cell death (Borowska et al., 2011). In addition, Ivanova et al. report increased Cx36 expression and phosphorylation in RD retina (Ivanova et al., 2015, 2016). Curiously, a study investigating early stages of RD found aberrant electrical coupling between AII amacrine cells and *rod bipolar cells*, a cell type that doesn't express connexins whatsoever in healthy retinas (Pfeiffer et al., 2020). One study, a bioRxiv preprint, reports restored vision in RD retinas with optogenetic targeting of AIIs

(Khabou et al., 2022). Perhaps targeting of AII electrical synapses for vision restoration in retinal pathologies is on the horizon.

NMDA receptors and synaptic plasticity

Role of NMDARs in electrical synapse plasticity

Many studies and various electrophysiological, genetic, imaging, and behavioral techniques have demonstrated NMDARs to play a pivotal role in chemical synapse plasticity, especially in long term potentiation (Hunt and Castillo, 2012). While research on chemical synapse plasticity largely outnumbers them, an increasing number of studies address the role of NMDARs in electrical synapse plasticity as well. Using tracer coupling, paired recordings, and immunoblot, several groups provide evidence that NMDARs mediate plasticity of various time scales in the inferior olive, locus coeruleus, hypothalamus, spinal cord, and retina. A subset of these publications point to NMDARs as the mechanism to facilitate activity-dependent plasticity at electrical synapses (Pereda and Faber, 1996; Mentis et al., 2002; Arumugam et al., 2005; Kothmann et al., 2012; Mathy et al., 2014; Turecek et al., 2014; Kourosh-Arami et al., 2023).

NMDAR receptor function and subtypes

The primary agonist of NMDARs is glutamate, but unlike other glutamate receptors, NMDAR activation requires more than that. It also requires binding of a coagonist, either glycine or D-serine, as well as removal of the magnesium (Mg^{2+}) molecule occupying the pore of the receptor. Depolarization of the cell forces the Mg^{2+} molecule out of the pore and allows for activation of the receptor (Nowak et al., 1984).

Activation of the receptor and opening of the pore allows for the influx of sodium and calcium ions into the cell, which leads to further depolarization and activation of second messenger signals. The activation of second messenger signals, along with the relief of the Mg^{2+} block, reinforces the multitude of research that points to NMDARs as a lynchpin for triggering potentiation of synapses, chemical or electrical. Repeated release of glutamate and activation of AMPA receptors leads to the depolarization necessary to relieve the magnesium block, which leads to calcium influx and a triggering of second messenger signaling cascades that lead to the strengthening of the synapse (Hunt and Castillo, 2012).

Investigation into NMDAR subtypes has also expanded our knowledge on the role of these receptors in synaptic plasticity. NMDARs are heterotetramers composed of two GluN1 subunits and either two GluN2 or GluN3 subunits (Sanz-Clemente et al., 2013). Most studies concerning synaptic plasticity focus on the diverging roles of receptors containing either GluN2A or GluN2B subunits. Canonically, researchers consider GluN2A-containing receptors to localize synaptically and preferentially bind coagonist D-serine, as opposed to glycine (Papouin et al., 2012). On the other hand, GluN2B-containing receptors localize extrasynaptically, preferentially bind coagonist glycine, and bind glutamate in response to repeated synaptic activation and resulting spillover of glutamate from the synapse (Zhou et al., 2013). While this theory largely prevails in the field, other studies claim that coagonist binding is more variable based on brain region (Mothet et al., 2015). The cell type, subunit composition, localization in relation to the synapse, as well as the availability of either of the two coagonists to bind and activate the receptor confers specific properties and functions to a region's NMDARs (Papouin et al., 2012). In particular, the circuitry's release and uptake

mechanisms of D-serine and glycine will dictate NMDAR function, as the channel pore of the receptor will not open without either binding to the NMDAR coagonist binding site.

Source of D-serine: Glia or Neurons?

Compared to glycine, some studies suggest that D-serine is considered the dominant, or preferred, coagonist of NMDARs in the retina as well as other areas of the brain (Matsui et al., 1995; Mothet et al., 2000; Ren et al., 2006; Gustafson et al., 2007). Even so, the role of D-serine at a circuitry level remains clouded due to the continued conflict over its release mechanisms and cellular origin. When the concept of D-serine as a potent neuromodulator was initially gaining traction, there was strong support for evidence suggesting a glial origin of D-serine release. Through immunoreactivity and functional experiments in neuro-glial cell cultures, studies claimed that D-serine and serine racemase, the enzyme that converts L-serine to D-serine, are localized to glia and that D-serine is released vesicularly through calcium-dependent mechanisms (Matsui et al., 1995; Wolosker et al., 1999a, 1999b; Mothet et al., 2005). Later findings indicated that serine racemase and D-serine are actually localized to neurons, and not astroglial cells, and claimed that the serine racemase antibodies and fixation techniques used in immunoreactivity experiments lacked specificity (Kartvelishvily et al., 2006; Miya et al., 2008; Ding et al., 2011; Benneyworth et al., 2012; Ishiwata et al., 2015). Some also pointed out that functional experiments performed in culture lacked physiological relevance, and that astrocytes take on an inflammatory phenotype when isolated to a dish, further confounding any evidence of glial D-serine release (Zhang and Barres, 2010; Benneyworth et al., 2012). These opposing points of view sparked a debate of back-and-forth arguments reviewing the data and claiming why the opposing camp was erroneous in their conclusions, with Herman Wolosker, one of the first to propose glial

derivation of D-serine, arguing for neurons to be the true source of D-serine (Wolosker et al., 2016, 2017; Papouin et al., 2017). The argument for glial D-serine is cast further into doubt as the debate described here is also mirrored in that regarding the physiological relevance of gliotransmission, or the active role of glia in synaptic transmission through the release of glial molecules (Fiacco and McCarthy, 2018; Savtchouk and Volterra, 2018).

While this contention continues to be polarizing, Wolosker and others also support theories that take a middle ground betwixt the two camps. Wolosker has proposed a serine shuttle hypothesis, whereby astrocytic L-serine is transported to neurons, where it's converted to D-serine by serine racemase and released. In this context, astrocytes modulate synaptic transmission through activity-dependent production of L-serine (Wolosker, 2011). In addition, recent studies have considered early experiments detecting serine racemase in cultured astrocytes as evidence for D-serine release in reactive astrocytes, and have further investigated the role of astrocytic D-serine release in traumatic brain injury and Alzheimer's disease (Perez et al., 2017; Balu et al., 2019; Tapanes et al., 2022; Folorunso et al., 2023).

NMDA receptors in the retina

Through mediating light-evoked responses and plasticity in the retina (Diamond and Copenhagen, 1993; Jones et al., 2012), NMDARs contribute to various visual computations, including temporal resolution, contrast sensitivity, direction-selectivity in healthy retinas (Manookin et al., 2010; Stafford et al., 2014; Poleg-Polsky and Diamond, 2016). On the other hand, in diseases such as glaucoma and diabetic retinopathy, a number of studies attribute degeneration of retinal tissue to NMDAR dysfunction and excitotoxicity. Though the retina expresses various NMDAR subtypes in all five neuronal

cell types, as well as in Müller glial cells, the vast majority of these studies focuses on those expressed in ganglion cells (Boccuni and Fairless, 2022).

AII amacrine cell NMDARs

AIIs are one of many retinal cell types to express NMDARs. The first study to analyze voltage- and neurotransmitter-gated currents in the AII determined that these cells do not express NMDARs, as treatment of retinal slice with NMDA did not alter whole-cell currents (Boos et al., 1993). Later publications, primarily from the Harveit laboratory, demonstrated expression and function of extrasynaptic NMDARs on AII cells (Hartveit and Veruki, 1997; Zhou and Dacheux, 2004; Kothmann et al., 2012; Zhou et al., 2016; Veruki et al., 2019; Beltrán-Matas et al., 2021). Extrasynaptic, Glu-N2B containing NMDARs on AII cells co-localize with Cx36 gap junctions on arboreal dendrites, and are activated by vesicular glutamate released by other neurons (Kothmann et al., 2012; Veruki et al., 2019; Beltrán-Matas et al., 2021). Furthermore, enzymatic degradation of D-serine in retinal slice decreases the activity of Glu-N2B, extrasynaptic NMDARs, indicating that D-serine is the endogenous coagonist of AII cell NMDARs (Beltrán-Matas et al., 2021). This finding contradicts the consensus that D-serine acts as the endogenous coagonist to synaptic, Glu-N2A containing NMDARs rather than Glu-N2B containing receptors (Papouin et al., 2012).

Release mechanisms of D-serine and glycine in the retina

Both D-serine and glycine contribute to NMDAR signaling in the retina, though some postulate D-serine to be the favored coagonist (Gustafson et al., 2007). Regardless of the cellular origin of D-serine, several publications from Robert Miller's group indicate

that D-serine release is evoked with light stimulation. Specifically, D-serine release increases with stronger contrast and light stimulation intensity, and that enzymatic breakdown or blocking of the synthesis of D-serine reduces NMDAR-mediated light responses in ganglion cells (Gustafson et al., 2007, 2015; Stevens et al., 2010b; Sullivan et al., 2011; Sullivan and Miller, 2012). Furthermore, the role of D-serine as the primary coagonist of retinal NMDARs is maintained through the high-affinity uptake of glycine through neuronal glycine transporter GlyT1 (Stevens et al., 2010a). On the other hand, glycine release originates from narrow-field amacrine cells of the inner nuclear layer, including AII cells (Menger et al., 1998) and largely fine tunes local interactions between sublaminas of the inner plexiform layer (Hsueh et al., 2008). Marc et al. claims that ON pathway glycinergic amacrine cells synapse onto AII arboreal dendrites (Marc et al., 2013, 2014). Light activation may trigger glycine release and activate NMDARs located on AII arboreal dendrites (Kothmann et al., 2012; Veruki et al., 2019). Currently, the specific subtypes of amacrine cells that are presynaptic to AII amacrine cells is unknown.

Summary

Electrical synapses facilitate fast, synchronous neuronal communication in all areas of the nervous system, especially in sensorimotor areas such as the retina. While studies of chemical synapse function and plasticity have dominated the neuroscience field since the 1950s, electrical synapse research and technique development began to pick up speed at the turn of the current century. This surge in understanding of gap junctional communication between neurons has revealed that electrical synapses are necessary for a variety of brain functions and that plasticity of such synapses manifests

similarly to that of chemical synapses. Yet, unresolved discrepancies regarding electrical synapse plasticity and mechanisms persist, likely due to differences in species and approaches. Both the progress and hindrances of the field are well exemplified in the studies focused on AII amacrine cell electrical synapses of the retina.

After the publication of Furshpan and Potter's seminal work in crayfish (Furshpan and Potter, 1959), ultrastructural studies of the retina strongly suggested electrical synapses of AII amacrine cells to be necessary for night vision of rod-mediated signals. It was observed that rod bipolar cells make no direct contact with ganglion cells, and only made indirect contact through an AII-ON cone bipolar electrical synapse intermediate (Famiglietti and Kolb, 1975; Strettoi et al., 1992). Later, studies using neurobiotin and single cell AII recordings indicated that background light levels modulate coupling at homologous electrical synapses between AIIs, rather than the heterologous synapses between AIIs and ON cone bipolar cells (Bloomfield et al., 1997; Bloomfield and Völgyi, 2004). This finding was significant for both the retina and electrical synapse fields, as a specific visual function was linked to a specific electrical synapse. This launched further inquiry as to how these homologous synapses mediate rod and cone signaling pathways. While other tracer coupling studies have identified dopamine and glutamate as modulators of AII electrical synapses (Hampson et al., 1992; Urschel et al., 2006; Kothmann et al., 2012), no other approaches have validated these findings. In fact, two other groups reported negative results when evaluating the effect of dopamine using dual whole-cell patch-clamp electrophysiology (Demb and Singer, 2012; Hartveit and Veruki, 2012).

Here, we investigate the role of adenosine and NMDA receptors in AII homologous electrical synapse plasticity using dual whole-cell patch-clamp electrophysiology, a technique that directly measures gap junctional conductance and yields temporally precise information on junctional conductance between two cells. Adenosine is a known neuromodulator of both chemical and electrical synapses of the retina (Stella et al., 2003; Li et al., 2013), and adenosine levels increase in darkness (Ribelayga and Mangel, 2005), making it a strong candidate as a modulator of AII electrical synapses. NMDA receptors are also identified as modulators of chemical and electrical synapses in all areas of the brain (Hunt and Castillo, 2012; Pereda, 2014). However, neither of these receptor types have been confirmed to mediate electrical synapse plasticity in the retina as determined by dual whole-cell patch-clamp electrophysiology. We hypothesized that junctional conductance at AII electrical synapses would change in response to adenosine and NMDA receptor activity as determined by dual recordings. By testing mechanisms behind AII electrical synapse plasticity, we aim to further our understanding of how electrical synapse modulation influences rod-mediated signaling of the retina as well as neuronal communication as a whole.

Chapter 2: Junctional Conductance of Retinal AII Amacrine Cell Electrical Synapses is Modulated by NMDA, but not Adenosine, Receptors

Introduction

Electrical synapses in the CNS facilitate fast, synchronous communication, bi-directional transmission of information, coincidence detection of subthreshold potentials, signal amplification, and noise reduction through the flow of current across gap junctions (Szczupak, 2016; Nagy et al., 2018). Like chemical synapses, electrical synapses, undergo plasticity, which manifests as increases or decreases in electrical coupling between neurons (Shimizu and Stopfer, 2013). Although electrical synapses are expressed throughout the brain, most research has focused on sensorimotor systems such as the retina, which expresses an abundance of electrical synapses that facilitate the processing of visual information. Previous studies have demonstrated plasticity of electrical synapses in the retina through neuromodulators such as adenosine and dopamine, the levels of which vary with background illumination and circadian rhythms (Wirz-Justice et al., 1984; Godley and Wurtman, 1988; Hampson et al., 1992; Ribelayga and Mangel, 2005; Li et al., 2013).

Many studies have focused on electrical synapses of the retinal AII amacrine cell, a well-characterized subclass of interneuron that plays a role in transmission of signals in both rod and cone pathways through chemical and electrical synapses (Famiglietti and Kolb, 1975; Strettoi et al., 1992; Graydon et al., 2018). Tracer coupling experiments testing the effect of background illumination have implicated AII amacrine cell electrical synapses in serving as a “light switch” between the rod and cone pathways (Demb and Singer, 2012). In darkness, electrically coupled AII cells facilitate the transfer

of primary rod pathway signals through a cone pathway intermediary using electrical synapses between AII and ON cone bipolar cells (Demb and Singer, 2012). In bright light, electrical synapses between AII cells uncouple, preventing signal attenuation of the cone pathway through leakage into the AII cell network (Bloomfield and Völgyi, 2004). Additional tracer coupling experiments have observed that both dopamine and NMDA receptors (NMDARs) modulate AII cell coupling (Hampson et al., 1992; Kothmann et al., 2012).

To date, all studies documenting neurotransmitter-induced plasticity in the AII electrical synapse network have utilized the tracer coupling technique. Modulation of AII electrical synapses has not been characterized using dual whole-cell patch-clamp electrophysiology, which provides a direct measure of the strength of electrical coupling. While tracer coupling experiments yield a spatial representation of an electrically coupled network, this electrophysiological technique directly measures the strength of the junctional conductance between two electrically coupled cells.

Here, we report the effect of adenosine receptor and NMDA receptor activation on AII electrical synapse plasticity using dual whole-cell patch-clamp electrophysiology. We find that neither activation nor antagonism of adenosine receptors (AR) alters conductance at AII electrical synapses. In contrast, NMDAR activation decreases conductance, and the addition of the NMDAR coagonists D-serine or glycine potentiates this NMDAR-mediated decrease in conductance.

Methods

Ethics Statement

All experimental procedures were approved by and adhered to the guidelines of the Institutional Animal Care and Use Committee of the University of Minnesota.

Retinal slice preparation

Experiments were conducted on 200 µm thick retinal slices from 63 C57BL/6J wildtype (WT; 34 male, 29 female), 6 IP3R2 KO (1 male, 5 female), and 7 serine racemase KO (SRKO; 3 male, 4 female) mice between the ages of 5 and 10 weeks. Light-adapted mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Eyes were removed and submerged in oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (aCSF). Following cornea, lens, and vitreous removal, eyecups were hemisected and the retina gently removed. Isolated retinae were embedded in low temperature-gelling 4% agarose (Sigma-Aldrich, A0701) dissolved in aCSF, and sliced in oxygenated aCSF with a vibratome. Individual slices were placed in a perfusion chamber cut surface facing upwards, held in place with a platinum harp, and perfused with oxygenated room temperature aCSF at 2.5 ml/min.

Identification of AII amacrine cells

Slices were visualized with infrared differential interference contrast microscopy and an Olympus FV1000 confocal microscope. AII amacrine cells were identified by the position of pear-shaped somata at the border between the inner nuclear and inner plexiform layers. Cells were also dye-filled in initial experiments to confirm their identity. Figure 1A shows an example of a pair of AII cells that were patched and dye-filled with Alexa Fluor 488 hydrazide (Sigma-Aldrich, A10436). AII amacrine cell axons have a

characteristic primary stalk with proximal lobular appendages that form chemical synapses onto OFF cone bipolar and OFF ganglion cells. Distal, arboreal appendages that reach the ganglion cell layer contain electrical synapses that contact other AII amacrine cells and ON cone bipolar cells, as well as chemical synapses that contact rod bipolar cells (Fig. 1A) (Strettoi et al., 1992).

Electrophysiology

Electrical coupling was assessed by dual whole-cell patch-clamp measurements of junctional conductance using a MultiClamp 700a amplifier (Molecular Devices) and MATLAB based, open-source Symphony software (https://symphony-das.github.io/). 5-8 MΩ borosilicate micropipettes filled with a potassium-based internal solution were used to patch onto pairs of AII cells within 30 □m of each other. Both cells of the pair were voltage clamped to -60 mV. Applying depolarizing steps resulted in characteristic outward currents to the depolarized cell and inward currents to the coupled cell (Fig. 1B; Veruki et al., 2003), and confirmation that the two cells were electrically coupled.

Data were collected by applying a family of voltage step pulses between -80 and -20 mV at 10 mV intervals to one of the pair of voltage-clamped cells (Fig. 1B), followed by a -20 mV step applied to both cells simultaneously to monitor series resistance. Each voltage step consisted of a 500 ms baseline at -60 mV, a 500 ms voltage step, and a 500 ms post stimulus baseline. Voltage-clamp currents were sampled at 20 kHz and filtered at 3 kHz. 0.5 s voltage steps were presented at 1.5 s intervals and families of steps repeated every 20 s. In preliminary experiments, recordings were collected by applying the family of voltage steps to just one of the pair of cells throughout the entire experiment. In later experiments, recordings were collected by alternately applying a

family of voltage steps to each cell of the pair. The two approaches yielded similar values of calculated junctional conductance and all data were combined.

Analysis

The effect of drug treatment on electrical synapse strength was evaluated using dual whole-cell patch-clamp measurements of conductance during the time period when the drug was administered. Conductance was calculated (Fig. 1C) and plotted for the duration of an experiment (Fig. 1D). The health and stability of electrical recordings were monitored using holding current and series resistance measurements (Fig 1D). Junctional conductance (g_j) of the electrical synapses between pairs of AII amacrine cells can be calculated as the current change in Cell 2 divided by the voltage difference between Cell 1 and Cell 2 following a voltage step in Cell 1, according to equation (1) (Hartveit and Veruki, 2010),

(1)

$$g_j = \frac{I_\mathrm{j}}{V_\mathrm{j}} = \frac{I_2}{V_1 - V_2}$$

where g_j is junctional conductance, I_j is junctional current, V_j is junctional voltage, I_2 is current in Cell 2, V_1 is voltage in Cell 1, and V_2 is voltage in Cell 2.

The series resistance of the pipettes used in recording from Cells 1 and 2 can cause discrepancies between the voltage of the command step and the resulting voltage step induced in the cells. This discrepancy was compensated for by calculating the series resistance of each pipette and using equation (2) (Hartveit and Veruki, 2010),

(2)

$$g_j = \frac{I_\mathrm{j}}{V_\mathrm{j}} = \frac{-I_b + ((V_b - I_b \times R_{s2})/R_{m2})}{(V_a - I_a \times R_{s1}) - (V_b - I_b \times R_{s2})}$$

where I_b is the current recorded in Cell 2, V_b is the voltage command given to Cell 2, R_{s2} is the series resistance of Cell 2 and Pipette 2, R_{m2} is the membrane resistance of Cell 2, V_a is the voltage command given to Cell 1, I_a is the current recorded in Cell 1, and R_{s1} is the series resistance of Cell 1 and Pipette 1. R_s was estimated using Ohm's law by measuring the peak amplitude of the transient current response to a -20 mV voltage step commanded to Cells 1 and 2 simultaneously. R_m was calculated by subtracting R_s from the total resistance as defined by the steady state current response to the -20 mV step.

Solutions and drugs

Retinas were dissected, incubated, sliced, and perfused in room temperature aCSF containing (mM): 125 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 1.5 $MgCl_2$, 20 glucose, 1.2 $CaCl_2$, 0.5 L-glutamine, 0.4 sodium ascorbate, bubbled with 95% O_2/5% CO_2, (pH 7.35; 305–310 mOsm). For experiments performed in Mg^{2+}-free aCSF, $MgCl_2$ was omitted from the

aCSF while maintaining the same pH and osmolality as aCSF containing Mg^{2+}. The patch pipette solution contained (in mM): 120 K-gluconate, 4.5 $MgCl_2$, 9 HEPES, 0.1 EGTA, 14 $tris_2$-phosphocreatine, 4 Na_2-ATP, 0.3 tris-GTP, sucrose to bring the solution to 280 – 290 mOsm, pH 7.25. In preliminary experiments, the patch pipette solution contained 0.1% Alexa Fluor 488 Hydrazide to visualize AII amacrine cells. All drugs were applied by addition to the bath solution.

The following experimental drugs were purchased from:

Sigma Aldrich - meclofenamic acid (MFA), gabazine, strychnine hydrochloride, adenosine, 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), D-serine, D-amino acid oxidase (DAAO), glycine.

Tocris - CNQX disodium salt (CNQX), N-Methyl-D-aspartic acid (NMDA), Ro 25-6981 maleate, D-(-)-2-amino-5-phosphonopentanoic acid (APV), ZM 241385. Alomone Labs - tetrodotoxin citrate (TTX). Cayman Chemical Company - aminophylline.

Statistics

For statistical analysis of conductance measurements, Student's paired t-tests, one-way repeated measures ANOVAs, and two-way mixed model ANOVAs were used, as appropriate. ANOVAs were used in conjunction with Tukey-Kramer multiple comparisons post hoc tests to make direct comparisons between two conditions. All p values in the text are considered significant when $p < 0.05$ and were calculated using Student's paired t-test or Tukey-Kramer post hoc tests unless otherwise stated. Data are expressed as mean ± SD in the text and figures. Sample size (n) represents the number of paired cell recordings for each group and are reported throughout the text. Data analysis and statistics were carried out using custom MATLAB scripts. The data, images, and MATLAB scripts for analysis and statistics will be made available upon request.

Results

Dual whole-cell patch-clamp measurement of retinal AII amacrine cell electrical synapse strength

We have measured the junctional conductance between pairs of AII amacrine cells in mouse retinal slices using dual whole-cell patch-clamp electrophysiology. Previous studies have demonstrated that AII cells are electrically coupled to one another

via distal, arboreal appendages in the proximal inner plexiform layer (Strettoi et al., 1992; Veruki and Hartveit, 2002). An example of a pair of dye-filled, electrically-coupled AII amacrine cells is shown in Fig. 1A, with arrows indicating the location of the homologous AII electrical synapses.

Previous studies have reported that junctional conductance measured with dual whole-cell patch-clamp drifts upward during recording, perhaps due to intracellular washout and changes in second messenger signaling (Veruki et al., 2008). We observed a similar phenomenon in our recordings, with the degree of drift varying between experiments (Fig. 2A, B). We adopted the following procedure to minimize the effect of this drift when calculating changes in the junctional conductance in response to pharmacological manipulations (Fig. 2C). For each experimental session, a running average of all conductance measurements was calculated using a sliding window of 3 min width (Fig. 2C, black line). The running average was used to identify the maximal change in conductance due to a pharmacological manipulation. The effect of a given manipulation was calculated as the difference between the average of all measurements during the last 3 min before the manipulation (Fig. 2C, green dots) and the average of all measurements during the 3 min period bracketing the minimum peak in conductance, identified by the running average (Fig. 2C, red dots). All junctional conductance values and percent changes in conductance reported in this work were calculated using this method. (It was not possible to correct for drift by extrapolating the drift from the control baseline as the rate of drift sometimes increased during experiments.) Since the drift (an increase in junctional conductance with time) was in the opposite direction to the pharmacologically-induced changes in conductance (a decrease in junctional

conductance) the estimates of pharmacologically-induced conductance changes using this correction method possibly underestimates actual changes.

We validated our approach of measuring electrical synapse conductance with dual whole-cell patch-clamp using two pharmacological manipulations. We first evaluated the effect of meclofenamic acid (MFA) on the junctional conductance. 100 μM MFA blocks gap junctions and therefore should eliminate electrical synapse conductance. Indeed, addition of MFA reduced the junctional conductance to 0.6% of control, with the mean conductance dropping from 904 ± 271 pS (mean ± SD) in control solution to 5.20 ± 8.69 pS in MFA ($n = 5$, $p = 1.9 \times 10^{-3}$; Fig. 3A, B).

Additionally, we evaluated the effect of blocking chemical synapses on junctional conductance by addition of a cocktail of drugs: 10 μM CNQX to block AMPA receptors, 10 μM gabazine to block $GABA_A$ receptors, 1 μM strychnine to block glycine receptors, and 0.3 μM TTX to block voltage-gated Na^+ channels (Fig. 3C - F). The drug cocktail effectively silenced chemical synaptic currents recorded from the AII cells (Fig. 3C, D) without altering the junctional conductance between the cells (Fig. 3E, F). Remaining experiments reported here were conducted in the presence of this chemical synapse antagonist cocktail in order to isolate the effect of our manipulations to electrical synapses.

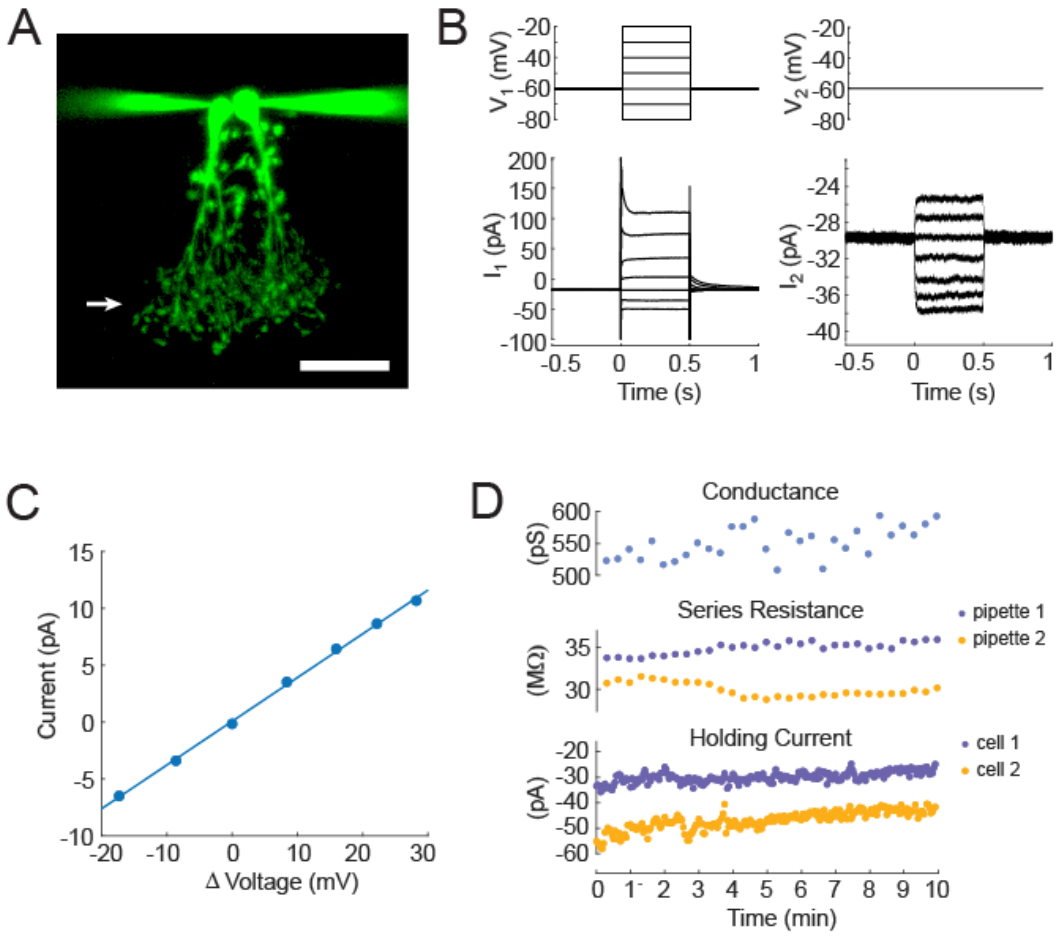

Figure 2.1 Dual whole-cell patch-clamp measurement of retinal AII amacrine cell electrical synapse strength. **A,** Confocal image of a pair of coupled AII amacrine cells, dye-filled with the gap junction-impermeable Alexa Fluor 488 hydrazide. The two patch pipettes are seen to the left and right of the cell somata. Arrow indicates the location of homologous AII electrical synapses. Both cells are voltage clamped to a holding potential of -60 mV. Scale bar, 20 μm. **B**, Measurement of junctional conductance. Cell 1 is given a family of voltage steps (V_1) while Cell 2 remains voltage-clamped at -60 mV (V_2). Both cells respond to V_1 with changes in current (I_1 and I_2). **C,** For each voltage step, the change in current I_2 is plotted against the difference between V_1 and V_2. Corrections are made for series resistance errors (see Methods for details). The

junctional conductance is calculated as the slope of the least squares fit for each family of voltage steps. **D,** Junctional conductance is monitored during the course of an experiment at 20 s intervals. Series resistance and holding current recorded from each pipette and cell are also monitored to track the integrity of the recordings (see Methods for details)

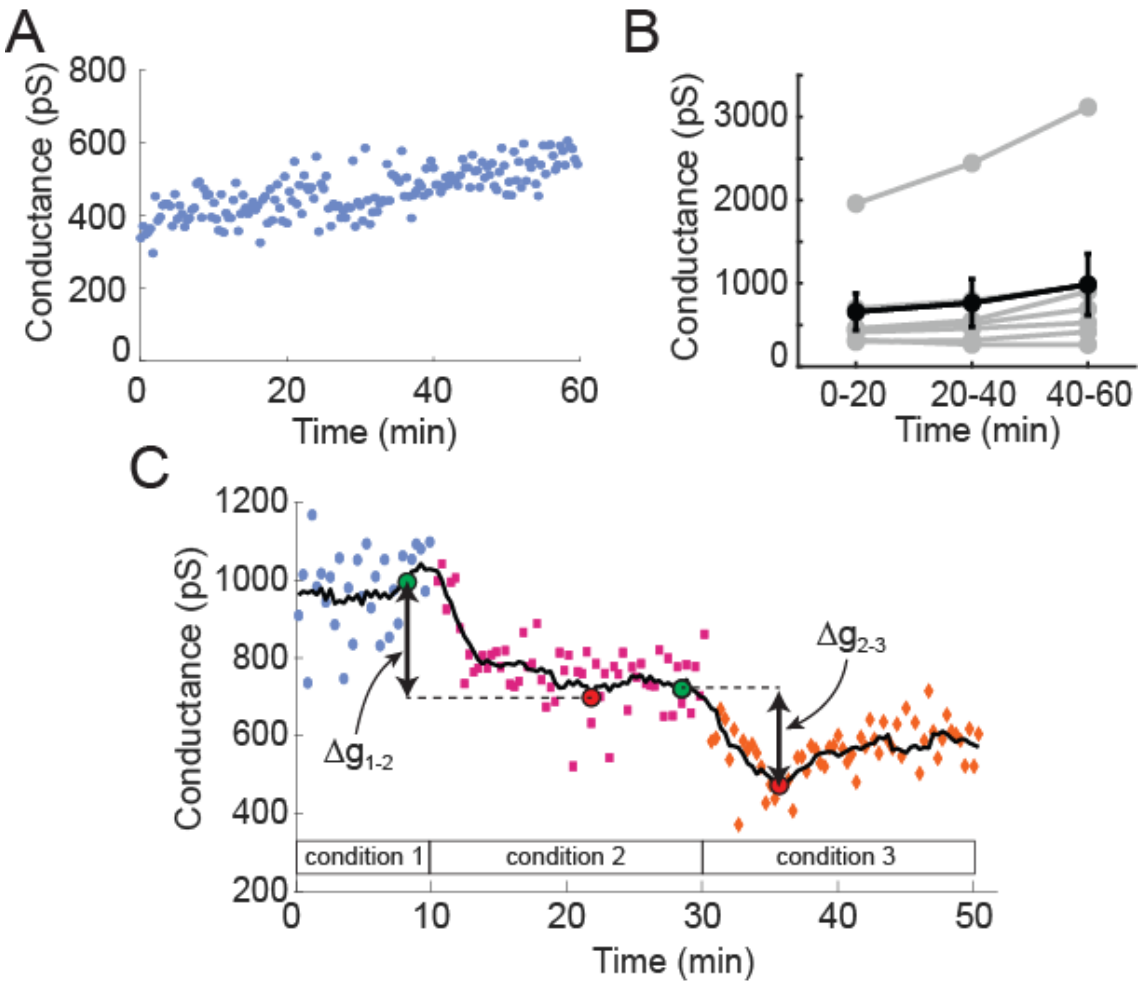

Figure 2.2 AII amacrine cell junctional conductance determined by dual whole-cell patch-clamp drifts upwards during experiments. **A,** Conductance measurements from a single experiment in control aCSF. **B**, Summary data showing the slow upward drift of conductance over time. Conductance was recorded for 60 minutes and averaged within 20-minute time windows for comparison. Individual experiments (gray lines) and mean ± SD (black lines; n=7). **C,** Diagram depicting the calculation of conductance changes between experimental conditions, used to minimize the effects of conductance drift. A running average of all conductance measurements is first calculated (black line, 3 min

time window). The change in conductance between two conditions caused by addition of a pharmacological agent (e.g., $\Delta g_{1\text{-}2}$ between conditions 1 and 2) is then calculated as the conductance difference between the average of all measurements during the last 3 min before the end of condition 1 (first green circle) and the average of all measurements during the 3 min period bracketing the minimum peak in conductance during condition 2 (first red circle). The conductance change between condition 3 and condition 1 is calculated as $\Delta g_{1\text{-}2} + \Delta g_{2\text{-}3}$.

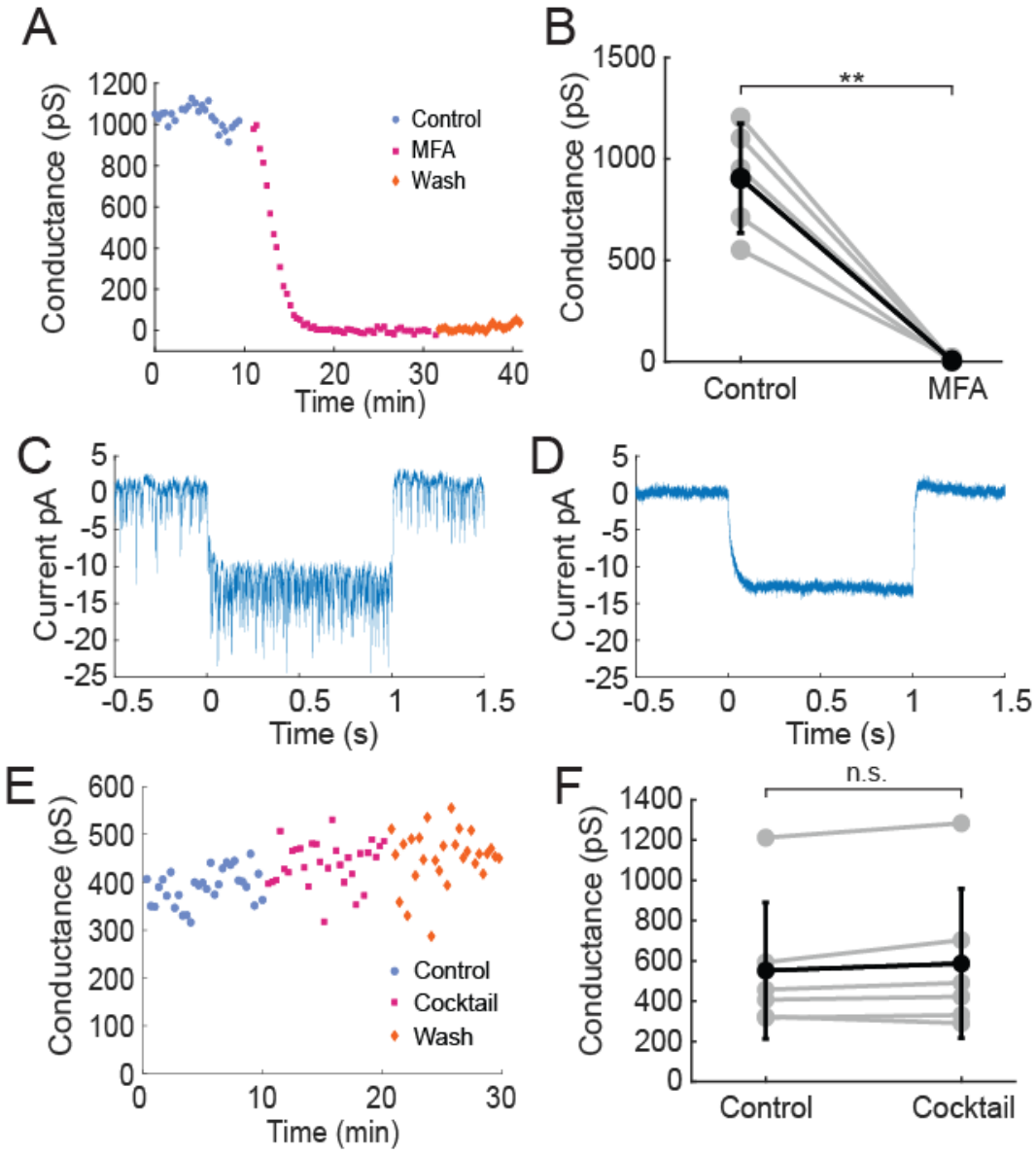

Figure 2.3 Validation of AII amacrine cell junctional conductance measurements. **A & B**, AII amacrine cell junctional conductance is eliminated by addition of the gap junction blocker MFA. **A**, Example experiment and **B**, summary data showing that MFA reduces the junctional conductance between AII cells (n=5). **C-F,** Electrical coupling between AII cells is preserved following block of chemical synaptic transmission. Example traces of currents recorded from Cell 2, responding to a 100 mV depolarizing voltage step in Cell

1, in the absence (**C)** and in the presence (**D**) of chemical synapse antagonists and channel blocker CNQX, gabazine, strychnine, and TTX, blocking AMPA, $GABA_A$, and glycine receptors and voltage-gated sodium channels, respectively. The antagonist cocktail silenced the robust chemical synaptic inputs to AII amacrine cells. **E**, Example experiment and **F**, summary data showing that blocking chemical synaptic transmission does not alter electrical coupling between AII cells (n=6). **B** & **F,** Individual experiments (gray lines) and mean ± SD (black lines). Student's paired t-test. **, $p < 0.01$; n.s., not significant.

Adenosine receptor activation does not affect AII electrical synapse conductance

With our approach for assessing AII electrical synapse plasticity validated, we tested whether adenosine modulates junctional conductance. Tracer coupling experiments in photoreceptors (Li et al., 2013, 2014) demonstrate that adenosine modulates electrical synapses containing Cx36, the gap junction protein that also comprises AII electrical synapses (Feigenspan et al., 2001). In addition, background illumination alters tracer coupling in the coupled AII cell network (Bloomfield et al., 1997; Bloomfield and Völgyi, 2004), as well as adenosine levels in the retina (Ribelayga and Mangel, 2005). With these previous studies in mind, we hypothesized that adenosine receptor activity would modulate the conductance of AII electrical synapses. Addition of 500 µM adenosine, however, did not alter the junctional conductance between AII cells, which equaled 564 ± 169 pS in control solution and 517 ± 171 pS in adenosine (n=5, p = 0.08; Fig. 4A, B). However, addition of 500 µM aminophylline, a non-selective AR antagonist, decreased conductance to 43.5% of control, which equaled 457 ± 96 pS in control solution and 199 ± 96 pS in aminophylline (n=7, $p = 5.5x10^{-7}$; Figure 5A, B). In order to pin down which receptor type mediates this plasticity, we investigated the effect of selectively inhibiting A1 and A2a receptors, which are the most commonly expressed AR subtypes in the retina. The presence of neither A1R antagonist DPCPX (50 -100 nM; Fig. 4C, D) nor A2aR antagonist ZM 241385 (1 µM; Fig. 4 E, F) significantly altered conductance, which equaled 486 ± 225 pS in control solution, 486 ± 215 pS in DPCPX, and 461 ± 128 pS in control solution, 420 ± 121 pS in ZM 241385 (p = 0.95 and 0.06, respectively). Because selective antagonism of receptors yielded negative results, we

concluded that adenosine receptors most likely do not modulate the junctional conductance of AII - AII electrical synapses.

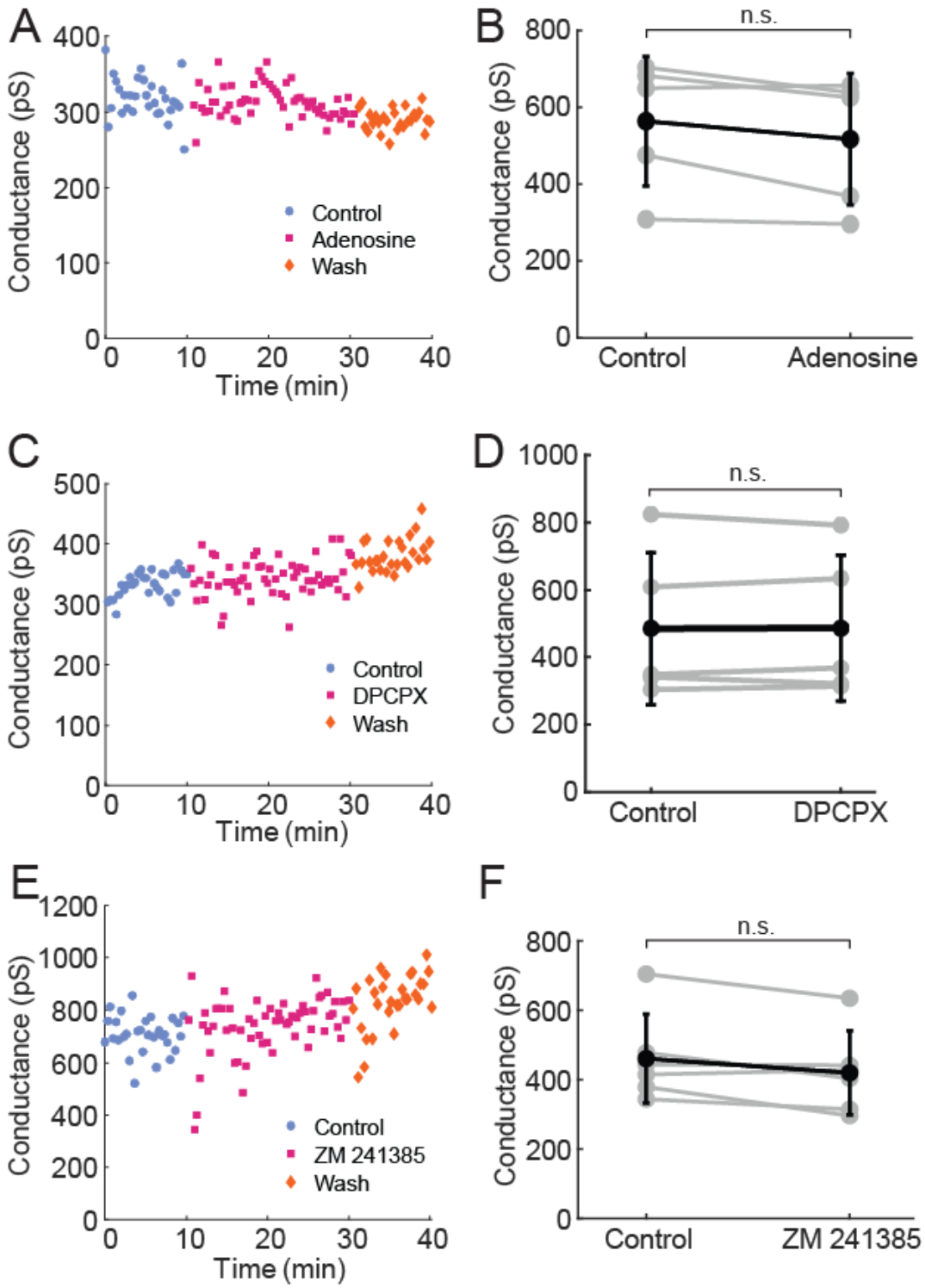

Figure 2.4 AII amacrine cell junctional conductance is not modulated by adenosine. **A,** Example experiment and **B**, summary data showing that adenosine does not alter

junctional conductance between AII cells (n=5). **C-F,** Junctional conductance between AII cells is unaffected by adenosine receptor antagonism. **C,** Example experiment and **D,** summary data showing that block of A1 receptors with the selective antagonist DPCPX does not alter junctional conductance between AII cells (n=5). **E,** Example experiment and **F,** summary data showing that block of A2a receptors with the selective antagonist ZM 241385 does not alter junctional conductance (n=6). **B**, **D**, **F,** Individual experiments (gray lines) and mean ± SD (black lines). Student's paired t-test. n.s., not significant.

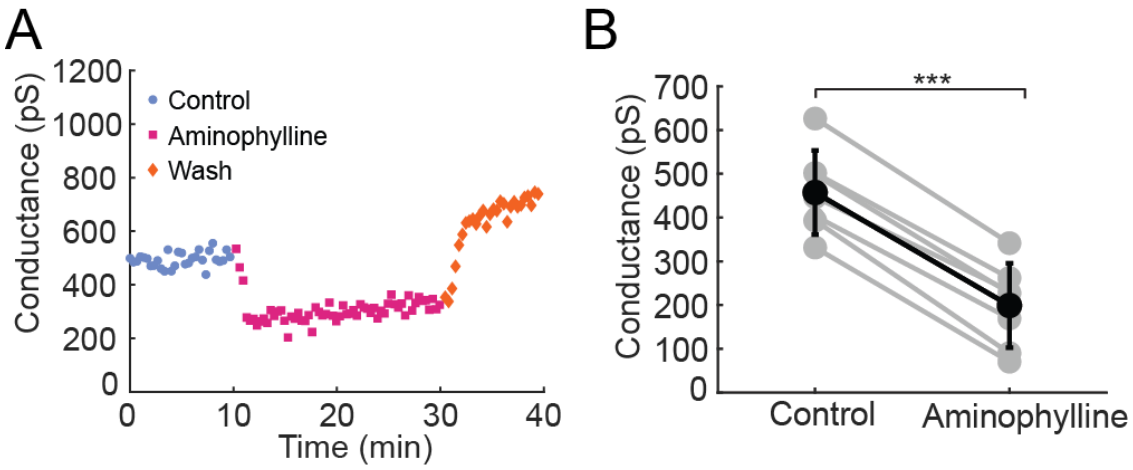

Figure 2.5 AII amacrine cell junctional conductance is modulated by aminophylline. **A,** Example experiment and **B**, summary data showing that aminophylline decreases junctional conductance between AII cells (n=7). Individual experiments (gray lines) and mean ± SD (black lines). Student's paired t-test. ***, $p < 0.001$

NMDA receptor activation decreases AII electrical synapse conductance

NMDARs are commonly studied in the context of chemical synapse plasticity. Tracer coupling experiments in AII amacrine cells have implicated NMDARs as a potential modulator of electrical synapses as well (Kothmann et al., 2012). We hypothesized that activation of NMDARs would modulate AII electrical synapse junctional conductance.

Activation of NMDARs requires release of the Mg^{2+} block inherent to NMDARs. Bath application of Mg^{2+}-free aCSF relieves the Mg^{2+} block and allows for activation of NMDARs (Nowak et al., 1984). We observed a decrease in junctional conductance between AII cells when switching from Mg^{2+}-containing to Mg-free solution. Junctional conductance was reduced to 57.7% of control from 442 ± 97.0 pS in Mg^{2+}-containing solution, to 255 ± 151 pS in Mg^{2+}-free solution (n=5, p = 0.01; Fig. 6A, B).

A second approach to relieving the Mg^{2+} block of NMDARs is to depolarize the cell. When the holding potential under voltage clamp was raised from -60 mV to 0 mV, in both cells of the recorded pairs, the junctional conductance between AII cells decreased to 41.4% of control, from 526 ± 178 pS at -60 mV to 218 ± 118 pS at 0 mV (n=5, p = 0.02; Figs 6C, D). Taken together, these results support the hypothesis that NMDARs contribute to the regulation of AII electrical synapses. Note that both the Mg^{2+}-free and depolarization experiments were performed without the addition of NMDA or glutamate, suggesting that under our experimental conditions there was sufficient endogenous glutamate in the retinal extracellular space to activate NMDARs when the Mg^{2+} block

was removed. From this point forward, all experiments investigating the role of NMDARs in modulating AII electrical synapses were performed in Mg^{2+}-free aCSF.

We directly tested whether activation of NMDARs modulates AII amacrine cell electrical coupling by addition of NMDA, as well as the NMDAR coagonist D-serine; a coagonist, either D-serine or glycine, is necessary for activation of NMDARs (Johnson and Ascher, 1987; Mothet et al., 2000). Addition of 100 µM NMDA and 200 µM D-serine led to a decrease in junctional conductance to 46.9% of control, with conductance decreasing from 508 ± 181 pS to 238 ± 127 pS (n=6, $p = 3.9x10^{-4}$; Fig. 6E, F). Experiments were also conducted in the presence of the non-selective NMDAR antagonist APV to test whether NMDA was acting specifically on NMDARs. We found that 50 µM APV blocked the NMDA + D-serine-induced decrease in junctional conductance, validating our findings. In the presence of APV, junctional conductance did not change, equaling 734 ± 417 pS in control solution and 769 ± 501 pS after addition of NMDA + D-serine (n=6, one-way repeated measures ANOVA, p = 0.45; Fig. 6G, H).

Two NMDAR subtypes are commonly implicated in chemical synapse plasticity: GluN2A and GluN2B subunit-containing NMDARs. GluN2B-containing receptors have been shown to colocalize with Cx36 gap junctional proteins in AII amacrine cell arboreal dendrites (Veruki et al., 2019). We tested whether NMDA-N2B receptors mediate electrical synapse modulation by selectively blocking the receptors with the N2B-selective antagonist Ro 25-6981. We found, however, that addition of Ro 25-6981 did not significantly block the NMDA+D-serine-mediated decrease in conductance. In the presence of 3 µM Ro 25-6981, NMDA+D-serine reduced the junctional conductance to 44.3% of control, from 469 ± 126 pS to 208 ± 136 pS (n=5, p = 0.04; Fig. 6I, J),

suggesting that N2B-containing NMDARs do not mediate the observed NMDAR-evoked decrease in junctional conductance.

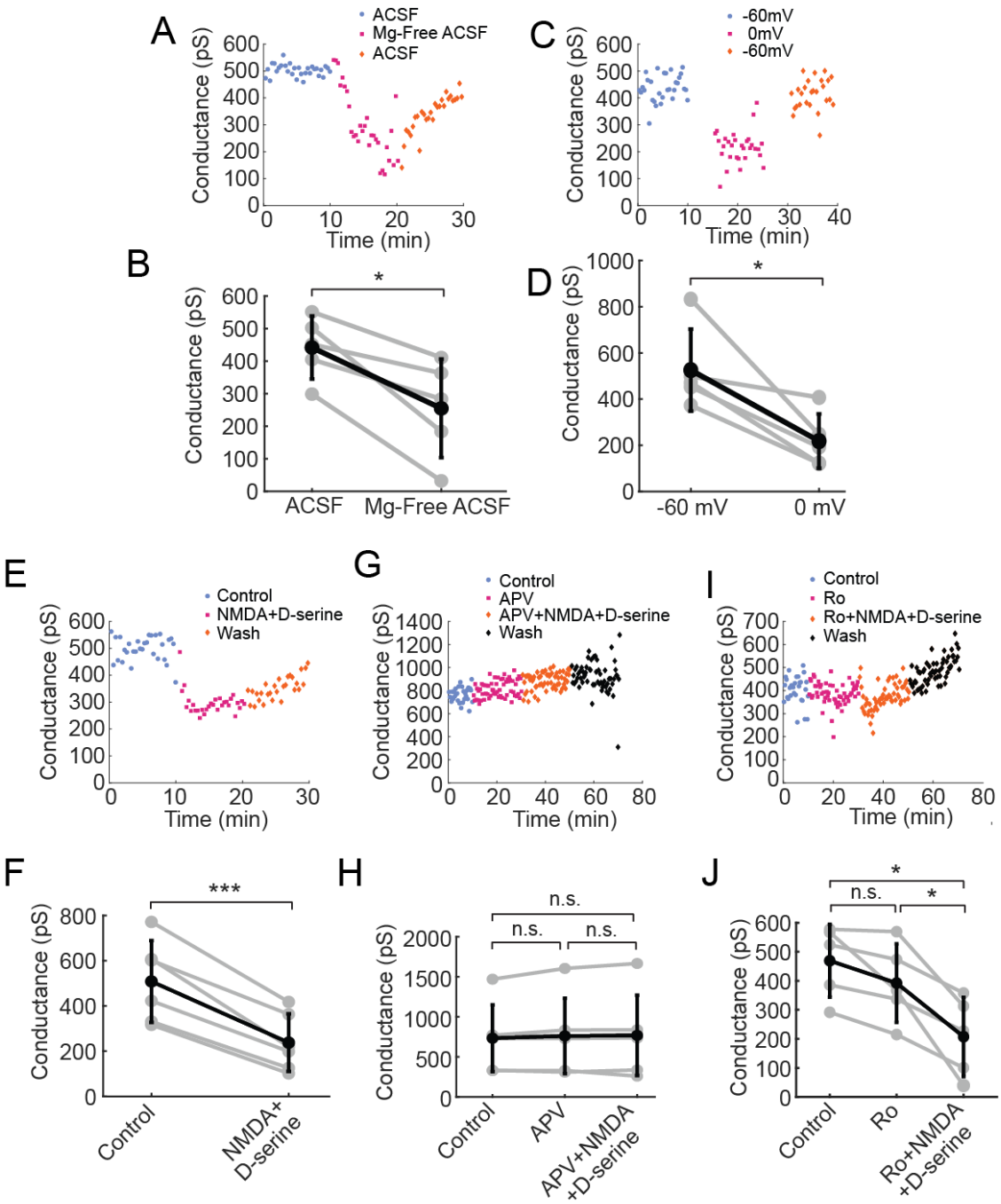

Figure 2.6 NMDA receptor activation decreases AII cell junctional conductance.

A-D, AII cell junctional conductance is decreased by relief of the NMDAR Mg^{2+} block. **A,** Example experiment and **B**, summary data showing that removal of Mg^{2+} from the aCSF results in a decrease in the conductance between AII cells (n=5). **C,** Example

experiment and **D,** summary data showing that membrane potential depolarization decreases the conductance between AII cells (n=5). **E,** Example experiment and **F,** summary data showing that NMDAR activation by addition of NMDA and the coagonist D-serine decreases conductance between AII cells (n=6). **G,** Example experiment and **H,** summary data demonstrating that block of NMDARs with D-APV prevents NMDAR-mediated decreases in junctional conductance between AII cells (n=6). **I,** Example experiment and **J,** summary data demonstrating that block of GluN2B-NMDARs with the selective antagonist Ro 26-6981 (Ro) does not prevent NMDAR-mediated decreases in the junctional conductance between AII cells (n=5). **B**, **D**, **F, H, J,** Individual experiments (gray lines) and mean ± SD (black lines). **B, D, F**, Student's paired t-test. **H, J,** One-way repeated measures ANOVA followed by Tukey-Kramer post hoc multiple comparisons. *, $p < 0.05$; ***, $p < 0.001$; n.s., not significant.

NMDAR coagonist D-serine potentiates, but is not necessary, for NMDAR-mediated decreases in AII cell junctional conductance

We further probed the role of the NMDAR coagonist D-serine in AII electrical synapse plasticity by evaluating the effect of application of D-serine alone. Addition of D-serine without NMDA yielded a small but significant decrease in junctional conductance. Addition of 200 µM D-serine reduced the conductance to 85.6% of control, from 646 ± 268 pS to 533 ± 299 pS (n=5, $p = 4.7x10^{-3}$; Fig. 7A, B)**.** Next, we determined the effect of D-serine on NMDA-mediated changes in conductance. We first applied 100 µM NMDA alone for 20 minutes, followed by 100 µM NMDA in conjunction with 200 µM D-serine for another 20 minutes. NMDA alone decreased conductance to 63.2% of control, from 517 ± 197 pS to 327 ± 179 pS (n=10, $p = 2.3x10^{-5}$; Fig. 7C, D). The addition of D-serine along with NMDA treatment further decreased the conductance, from 327 ± 179 pS in NMDA to 192 ± 123 pS in NMDA+D-serine (n=10, $p = 2.1x10^{-4}$; Fig. 7C, D).

It is well known that agonist binding alone to NMDARs is insufficient for channel opening, and that the presence of a coagonist is necessary (Johnson and Ascher, 1987). This led us to conclude that for NMDA application to decrease conductance, D-serine or some other coagonist must already be present in the tissue. We employed inositol 1,4,5-trisphosphate receptor type 2 (IP3R2) and serine racemase (SR) knockout genetic mouse models, which are believed to have reduced D-serine levels in the retina, to explore this possibility.

D-serine is a known gliotransmitter and its release is triggered by astrocytic Ca^{2+} signaling (Mothet et al., 2005). Müller cells, the primary glial cells of the retina (Newman and Reichenbach, 1996), display decreased glial Ca^{2+} signaling in IP3R2 knockout mice

(IP3R2KO) (Biesecker et al., 2016), and therefore, should have decreased D-serine release. We hypothesized that, compared to WT retinas, IP3R2KO retinas treated with NMDA alone would result in a smaller junctional conductance decrease, and that only with the addition of exogenous D-serine would NMDA cause the same magnitude of decrease as seen in the WT retina. Contrary to this expectation, the NMDA-evoked decrease in conductance in IP3R2KO retinas did not differ from the decrease seen in WT retinas. Junctional conductance decreased to 54.9% of control, from 494 ± 152 pS to 271 ± 95.1 pS (n=7, $p = 3.02 \times 10^{-3}$; Fig. 7E), indicating that either D-serine levels in IP3R2KO tissue are the similar to those in WT, or that glycine, the other known NMDAR coagonist, served as the coagonist.

We then repeated the previous experiments in serine racemase knockout (SRKO) mice to determine if D-serine is necessary to mediate the decrease in NMDA-evoked conductance. Serine racemase is an enzyme that converts L-serine to D-serine and mice deficient in the enzyme lack D-serine in their retinas (Wolosker et al., 1999b; Sullivan et al., 2011). We hypothesized that, compared to WT retinas, the NMDA-evoked conductance decrease in SRKO retinas would be smaller, and that only with the addition of exogenous D-serine would the NMDA decrease in conductance be as large as in WT mice. Again, as observed in the IP3R2KO mice, the NMDA-evoked conductance decrease in SRKO retinas did not differ from the decrease seen in WT retinas, decreasing to 64.2% of control, from 544 ± 221 pS to 349 ± 202 pS (n=10, $p = 3.6 \times 10^{-6}$; Fig. 7F).

It was possible that we observed negative results in the genetic models due to compensatory mechanisms in the global knockouts leading to increased D-serine levels. To confirm that D-serine is not necessary for NMDA-mediated decreases in junctional

conductance, we used another approach in WT mice. We first treated retinas with D-amino acid oxidase (DAAO), an enzyme which breaks down D-serine in tissue. Following a 20 min incubation in 300 µg/ml DAAO, NMDA + DAAO was added, followed by NMDA + D-serine in the absence of DAAO. DAAO treatment did not prevent the NMDA-mediated decrease in conductance. Conductance equaled 710 ± 238 pS in control and 689 ± 247 pS in DAAO and decreased to 62.1% of control in NMDA + DAAO (441 ± 180 pS, n=8, $p = 4.4x10^{-3}$ compared to control), and to 36.8% of control in NMDA + D-serine (261 ± 161 pS) (Fig. 7G, H). These results indicate that D-serine can act as a coagonist for NMDAR-mediated decreases in conductance but is not necessary for NMDAR-mediated plasticity to occur.

A summary of the genetic model and DAAO results is given in Fig 7I and shows that there are no significant differences in junctional conductance decreases in IP3R2KO and SRKO mice and in DAAO experiments compared to WT.

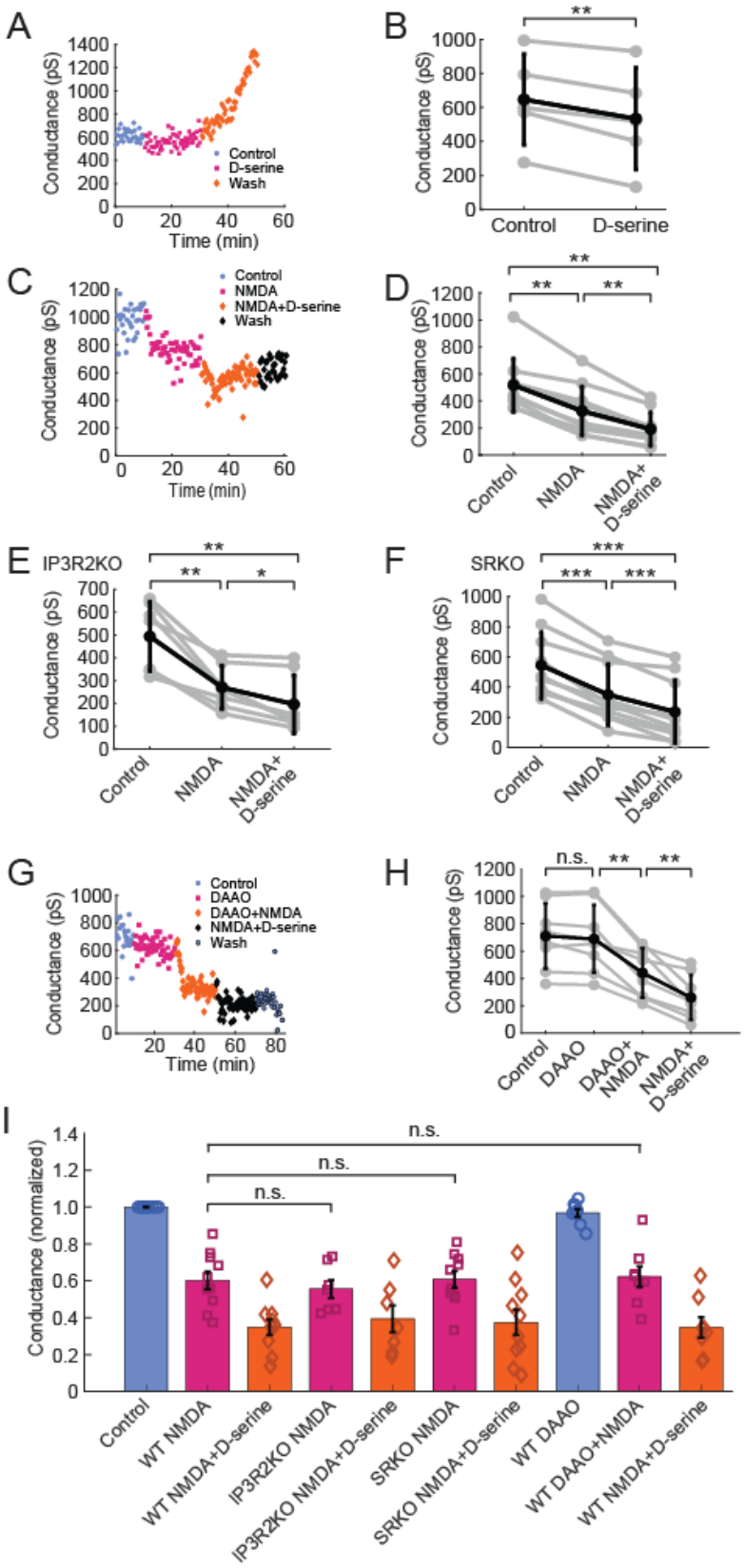
A
Conductance (pS)
Time (min)
Control
D-serine
Wash
B
Control
D-serine
C
Control
NMDA
NMDA+D-serine
Wash
D
Control
NMDA
NMDA+ D-serine
E
IP3R2KO
F
SRKO
G
Control
DAAO
DAAO+NMDA
NMDA+D-serine
Wash
H
Control
DAAO
DAAO+ NMDA
NMDA+ D-serine
I
Conductance (normalized)
n.s.
Control
WT NMDA
WT NMDA+D-serine
IP3R2KO NMDA
IP3R2KO NMDA+D-serine
SRKO NMDA
SRKO NMDA+D-serine
WT DAAO
WT DAAO+NMDA
WT NMDA+D-serine

Figure 2.7 The NMDAR coagonist D-serine potentiates but is not necessary for NMDAR-mediated decreases in AII cell junctional conductance. **A,** Example experiment and **B,** summary data showing that D-serine alone decreases junctional conductance between AII cells (n=5). **C,** Example experiment and **D,** summary data showing that NMDA alone decreases junctional conductance between AII cells and that addition of D-serine along with NMDA further decreases the conductance (n=10). **E-F**, NMDA-mediated decreases in conductance are not prevented in genetic models meant to decrease D-serine levels. **E,** Experiments on retinas from IP3R2KO mice, which have reduced Ca^{2+} signaling in Müller glial cells. Summary data showing that the NMDA-mediated conductance decrease between AII cells is maintained in these mice (n=7). **F,** Experiments on retinas from SRKO mice, where production of D-serine is blocked. Summary data showing that the NMDA-mediated conductance decrease between AII cells is also maintained in these mice (n=10). **G,** Example experiment and **H,** summary data demonstrating that the presence of DAAO, which degrades D-serine, does not prevent the NMDA-mediated decrease in conductance (n=10). **I,** Summary bar graph showing that the NMDA-mediated decreases in AII junctional conductances are maintained in IP3R2KO and SRKO mice as well as in WT mice following DAAO addition. NMDA-mediated conductance decreases (purple bars) under these conditions are all similar to the conductance decrease in WT mice. All data are normalized to the conductance in control aCSF for each condition and show individual experimental data and the mean ± SD. **B**, **D-F, H,** Individual experiments (gray lines) and mean ± SD (black lines). **B,** Student's paired t-test. **D-F, H** One-way repeated measures ANOVA followed by Tukey-Kramer post hoc multiple comparisons. **I**, two-way repeated measures ANOVA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., not significant.

Glycine modulates NMDAR-mediated decreases in conductance at AII electrical synapses

Glycine is the other known NMDAR coagonist besides D-serine (Johnson and Ascher, 1987; Mothet et al., 2000). Because none of our manipulations to lower D-serine in the retina reduced NMDA-mediated decreases in AII electrical coupling, we hypothesized that endogenous glycine could serve as the coagonist for NMDA-mediated changes in coupling. To test this, we first added 400 □M glycine alone to the tissue. Similar to the results when D-serine was added alone, glycine yielded a small, but significant, decrease in conductance, with conductance decreasing to 86.3% of control following glycine addition. Conductance decreased from 570 ± 146 pS to 492 ± 99.1 pS (n=6, p = 0.02; Fig. 8A, B). In a following experiment, NMDA and then NMDA + glycine was added sequentially. Addition of NMDA alone decreased the conductance to 48.1% of control, from 801 ± 620 pS to 385 ± 357 pS (n=6, p = 0.03; Fig 8C, D). Addition of NMDA + glycine then led to a further decrease in conductance to 28.7% of control from 385 ± 357 pS in NMDA to 230 ± 182 pS in NMDA + glycine (n=6, p = 0.04; Fig. 8C, D). These results mirror the effects that were observed with D-serine and indicate that both glycine and D-serine may play a role as coagonists in NMDAR-mediated AII electrical synapse plasticity.

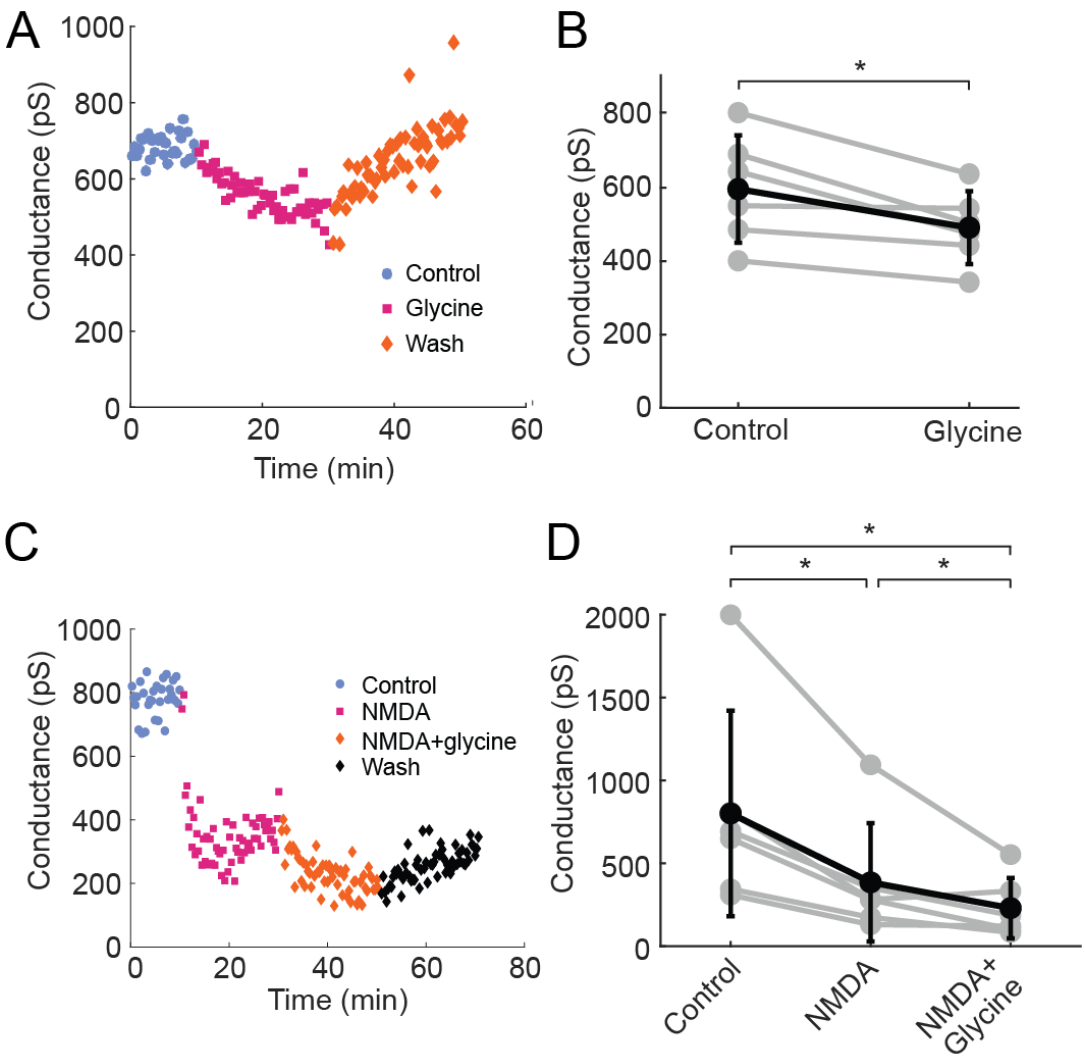

Figure 2.8 The NMDAR coagonist glycine potentiates the NMDAR-mediated decrease in AII cell junctional conductance. **A,** Example experiment and **B,** summary data showing that glycine addition alone decreases junctional conductance between AII cells (n=6). **C,** Example experiment and **D,** summary data showing that NMDA alone decreases junctional conductance between AII cells, and that addition of glycine along with NMDA further decreases the conductance (n=6). **B**, **D,** Individual experiments (gray lines) and mean ± SD (black lines). **B,** Student's paired t-test. **D,** One-way repeated measures ANOVA followed by Tukey-Kramer post hoc multiple comparisons. *, $p < 0.05$.

Discussion

A variety of methods have been used in previous studies to analyze electrical synapse strength, including tracer coupling, electrical synchronization, noise analysis, and coupling coefficients (Connors, 2017; Nagy et al., 2018). Here, we have used dual whole-cell patch-clamp electrophysiology to assess the strength of electrical coupling in AII amacrine cells, a method that directly measures electrical synapse strength. We found that NMDAR activation substantially reduces junctional conductance and that the addition of NMDAR coagonists D-serine or glycine further reduces conductance. NMDA modulation of coupling was not prevented by blocking NMDARs containing the N2B subunit, indicating that these receptors are not responsible for electrical synapse plasticity. These results represent the first demonstration of neurotransmitter-mediated plasticity at AII amacrine cell electrical synapses as measured directly by an electrophysiological approach.

Changes in background illumination have been shown to alter tracer coupling between AII amacrine cells, as well as adenosine levels (Bloomfield et al., 1997; Bloomfield and Völgyi, 2004; Ribelayga and Mangel, 2005). Additionally, tracer coupling experiments have implicated adenosine as a modulator of photoreceptor electrical synapses composed of connexin36 (Cx36) gap junction proteins (Li et al., 2013; Cao et al., 2021), the same gap junction proteins that comprise AII amacrine cell electrical synapses (Feigenspan et al., 2001). While these previous studies point to adenosine as a potential modulator of AII electrical coupling, our results indicate that adenosine does not directly affect plasticity at these electrical synapses. Neither AR agonists nor selective antagonists changed AII electrical synapse strength. While the non-selective

AR antagonist aminophylline did in fact decrease AII electrical synapse strength, we concluded that this effect was not likely mediated through ARs, but through phosphodiesterase inhibition. Further investigation into the role of phosphodiesterase activity in AII electrical synapse plasticity was out of scope for this study but will be instructive for future experiments addressing second messenger signaling mechanisms behind electrical synapse plasticity.

While we found that activation of NMDARs results in a decrease in electrical coupling between AII amacrine cells, a previous study found the opposite result. Using tracer spread to evaluate coupling in the AII cell network in rabbit, Kothmann et. al reported that NMDAR activation increased coupling between cells (Kothmann et al., 2012). It is not clear what accounts for this difference in results, although differences between species may contribute. Differences in the techniques used to evaluate electrical synapse strength may also be important. For instance, using tracer coupling, several groups have published robust evidence showing that dopamine receptor activation decreases coupling within the AII amacrine cell network (Hampson et al., 1992; Urschel et al., 2006; Kothmann et al., 2009); yet, other studies measuring junctional conductance directly with paired recordings do not support a role for dopamine in modulating electrical synapses between AII cells (Demb and Singer, 2012; Hartveit and Veruki, 2012). Contradictory results concerning the effects of NMDA on electrical coupling in the inferior olive have also been reported (Mathy et al., 2014; Turecek et al., 2014). These conflicting findings indicate that modulation of electrical coupling may depend on multiple processes that could vary depending on the preparations and techniques used to assess coupling.

We have shown that both glycine and D-serine can serve as coagonists in NMDA-mediated modulation of coupling between AII amacrine cells, further reducing AII cell junctional conductance. Glycine is released from small-field amacrine cells (Menger et al., 1998), a subset of which are activated by the ON pathway and release glycine onto AII arboreal dendrites with illumination (Marc et al., 2014).

The source of D-serine is less clear and there is an ongoing debate whether the coagonist is released from glial cells or neurons (Wolosker et al., 1999a; Miya et al., 2008). We tested whether the D-serine which potentiates NMDA-mediated electrical coupling plasticity originated from glial cells. D-serine is thought to be released from glia in a Ca^{2+}-dependent manner (Mothet et al., 2005). We found that the NMDA-mediated junctional conductance decrease was not reduced in IP3R2KO mice, which show reduced Ca^{2+}-signaling in Müller cells (Biesecker et al., 2016), the glial cells present in the inner plexiform layer (Newman and Reichenbach, 1996). This result suggests that the D-serine that contributes to AII amacrine cell plasticity does not originate from retinal glial cells. However, D-serine release from Müller cells may not be solely dependent on IP3R2 receptor Ca^{2+} increases. Sherwood et. al have shown that other glial IP3Rs besides IP3R2 mediate D-serine release (Sherwood et al., 2017), and Shigetomi et. al have shown that astrocytic TRPA1 Ca^{2+} activity can mediate D-serine release and subsequent long-term potentiation at hippocampal synapses (Shigetomi et al., 2013). In addition, the presence of glycine may compensate when there is decreased D-serine release in IP3R2KO mice.

Interpretation of our results is complicated by potential off target effects of our pharmacological manipulations and the location of the NMDARs that modulate

conductance cannot be definitively localized to AII amacrine cells, even though they express these receptors (Veruki et al., 2019). Although our approach directly measures the conductance of electrical synapses between AII cells, it is possible that the effects of the pharmacological agents are due to activation of NMDARs on upstream neurons. AII amacrine cells receive input from as many as 28 distinct classes of neurons (Marc et al., 2014). We controlled for this by suppressing general chemical synapse communication through a cocktail of antagonists. However, this did not block all chemical synapse communication, nor the abundant electrical synapse communication; AII amacrine cells are electrically coupled to ON cone bipolar cells (Strettoi et al., 1992). It is possible that the NMDAR modulation of electrical coupling in AII cells we observed was due to activation of NMDARs on other cell types. Further investigation using interventions that specifically target AII amacrine cell receptors would address this issue.

Our finding that NMDAR activation decreases AII electrical synapse strength suggests a possible mechanism by which AII amacrine cells contribute differentially to the processing of both rod and cone signals in the retina. Tracer coupling and receptive field sizes of AII cells are maximal at scotopic light levels (Bloomfield et al., 1997; Bloomfield and Völgyi, 2004). Extensive electrical coupling within the AII network under these conditions is proposed to enhance the sensitivity of rod-mediated vision by reducing noise within the rod bipolar cell pathway (Smith and Vardi, 1995; Dunn et al., 2006). As background light levels increase towards the photopic range, tracer coupling and receptive field sizes of AII cells decrease (Bloomfield and Völgyi, 2004). A reduction in AII-AII coupling at higher background light levels is thought to increase the spatial

resolution of cone-mediated vision (Demb and Singer, 2012). Our results demonstrating NMDAR modulation of AII cell electrical synapses links NMDARs to the mechanism mediating this light-dependent uncoupling of the AII cell network, which allows the AII amacrine cell to serve different visual functions under different lighting conditions.

NMDA receptors are commonly associated with plasticity at chemical synapses in the CNS. Here, we add to the accumulating evidence (Pereda and Faber, 1996; Mentis et al., 2002; Arumugam et al., 2005; Kothmann et al., 2012; Mathy et al., 2014; Turecek et al., 2014; Kourosh-Arami et al., 2023) that NMDARs also contribute to the plasticity of a key electrical synapse in the retina, the electrical synapse coupling AII amacrine cells together. Electrical synapses are an understudied subject in the field of neuroscience. Our study adds to mounting evidence that NMDARs mediate plasticity at electrical as well as chemical synapses.

Chapter 3: Conclusion

Conclusions

Our findings indicate that NMDAR, but not AR, activity decreases communication between AII homologous electrical synapses as determined by dual whole-cell patch-clamp electrophysiology. We also observed that the NMDAR coagonist D-serine potentiates NMDAR-mediated plasticity. Due to previous publications indicating that D-serine is the primary NMDAR coagonist in the retina and necessary for NMDAR-mediated currents in ganglion cells (Gustafson et al., 2007), we hypothesized that D-serine is also necessary for NMDAR-mediated AII electrical synapse plasticity. This hypothesis was thoroughly negated through two genetic and one pharmacological intervention. These negative results are likely explained by the observation that the NMDAR co-agonist glycine also potentiated NMDAR-mediated decreases in junctional conductance. These results are not the first to provide electrophysiological evidence of NMDAR-mediated electrical synapse plasticity, but they are the first to do so in the retina. These results are also the first to investigate the role of coagonists D-serine and glycine within NMDAR-mediated electrical synapse plasticity.

Future Directions

Our findings support a study published by Kothmann et al. indicating that NMDARs mediate electrical synapse plasticity between AII amacrine cells. However, that study reports that NMDAR activation increases coupling between AIIs, while our findings claim the opposite. Either way, these studies have only just begun to scratch the surface of what underlying mechanisms might mediate this plasticity, and the function it

serves within rod and cone signaling pathways mediated by AIIs. With that in mind, the following questions are raised for future investigation:

Can we replicate the results observed with dual whole-cell recordings using other approaches, such as tracer coupling? As mentioned above, compared to our findings, Kothmann et al. found opposite results regarding NMDAR-mediated AII electrical synapse plasticity. However, that study employed tracer coupling and immunohistochemical techniques in rabbit, and ours used an electrophysiological approach in mouse. This dissonance is not surprising. Multiple labs have published robust evidence that dopamine receptor activation decreases electrical synapse strength between AII amacrine cells in tracer coupling experiments (Hampson et al., 1992; Urschel et al., 2006; Kothmann et al., 2009). Yet, two groups have indicated they could not replicate this finding using dual patch-clamp electrophysiology (Demb and Singer, 2012; Hartveit and Veruki, 2012). This lack of reproducibility may be due to species differences, or perhaps, these techniques are more disparate than previously thought, and reveal differential properties and nuances of electrical synapse communication. It would be instructive to repeat our experiments using a tracer coupling approach to find out if we could replicate the results we found here.

Is junctional conductance determined by dual patch clamp modulated by different background light levels? It is widely believed that the distinct role of AII electrical synapse plasticity is to facilitate transmission of either the rod or cone pathways based on adaptation to background light levels. However, light modulation of electrical synapses has only been proposed based on data from tracer coupling and single cell receptive field size measurements (Bloomfield et al., 1997; Bloomfield and Völgyi, 2004),

rather than dual whole-cell recordings. In order to address this question, we could measure AII junctional conductance in response to various background light intensities.

What role do NMDARs play in light modulation of AII electrical synapse plasticity? We found that NMDAR activation decreases AII junctional conductance, which may mirror decreased AII tracer coupling in complete darkness and bright light (Bloomfield et al., 1997; Bloomfield and Völgyi, 2004). If background light intensities do indeed modulate junctional conductance, we could determine if NMDARs play a role in this modulation. We could design a viral vector knocking down NMDARs that target cells expressing the AII cell marker Prox1 (Pérez de Sevilla Müller et al., 2017), with a GFP label. Sparse viral expression in the retina would allow us to record from pairs with and without NMDARs. We could compare light modulation of junctional conductance between those two groups in order to determine if NMDARs influence this modulation.

What NMDAR subtypes mediate this decrease in AII electrical synapse strength? While Veruki et al. demonstrated that NMDARs localized near AII Cx36 proteins contain GluN2B subunits, we found that blocking GluN2B-NMDARs with selective antagonist Ro 25-6981 did not prevent NMDAR-mediated decreases in junctional conductance (Veruki et al., 2019). GluN2A-NMDARs would be the next likely candidate to mediate the effect we observed; however, no selective antagonists for GluN2A-NMDARs are commercially available. In order to investigate this, we would use various pharmacological approaches that partially antagonize GluN2A-NMDARs, such as tricine or PEAQX (Veruki et al., 2019), in order to accumulate evidence testing whether these receptor subtypes mediate AII electrical synapse plasticity.

Do D-serine and glycine differentially contribute to NMDAR-mediated plasticity? We observed that both NMDAR coagonists D-serine and glycine further potentiate NMDAR-mediated plasticity. Through genetic and pharmacological approaches, we also determined that D-serine is not necessary for NMDAR-mediated plasticity; however, we did not evaluate if this is also true for glycine. We would do so by treating the slice with glycine oxidase, which enzymatically breaks down glycine in the extracellular space. Determining which coagonist preferentially binds to the NMDARs mediating the plasticity might also reveal the circuitry and signaling mechanisms that carry out this effect.

What second messenger signaling cascades affect AII electrical synapse plasticity? We did not investigate the cellular mechanisms behind NMDAR-mediated plasticity, which could be facilitated through changes in gap junction protein phosphorylation state or expression. We could determine this expression by employing immunohistochemical or other imaging techniques. Additionally, we could identify the second messenger signaling cascades that lead to these changes. Several studies have demonstrated that NMDAR-mediated electrical synapse plasticity requires CamKII activity (Pereda et al., 1998; Kothmann et al., 2012; Turecek et al., 2014). In order to address this possibility, we would repeat our experiments in the presence of a CamKII antagonist.

In addition, we found that aminophylline, a non-selective, general adenosine receptor antagonist and phosphodiesterase inhibitor, reduces junctional conductance at electrical synapses; however, no specific AR antagonists reproduced this effect. We concluded that phosphodiesterase inhibition likely yielded the reduction in junctional conductance. Repeating experiments in the presence of selective phosphodiesterase

inhibitors would reveal more about the mechanisms at play behind AII electrical synapse plasticity.

Do glia play a role in electrical synapse plasticity? Our results proposing a role for the gliotransmitter D-serine in AII electrical synapse plasticity prompted us to hypothesize that reduction of glial IP3R2 calcium signaling would decrease D-serine release into the extracellular space, and thus prevent NMDAR-mediated decreases in junctional conductance. Although we found that NMDAR-mediated plasticity in the IP3R2KO mouse was not significantly different to that in WT, this does not rule out glial-derived D-serine as a modulator of AII electrical synapse communication. Other publications have claimed that D-serine release is mediated by other glial IP3Rs as well as TRPA1 (Shigetomi et al., 2013; Sherwood et al., 2017). We could repeat the experiments performed in IP3R2KO in mouse models targeting these receptor types in order to determine if glial D-serine mediates the effects we observed. Additionally, optogenetic or chemogenetic techniques that enable direct and specific stimulation or inhibition of glial cells may be combined with electrophysiological measurements in order to answer this question.

While many questions remain, our findings provide electrophysiological evidence for NMDAR modulation of electrical synapses in the retina. Our results and future experiments will further define the role of electrical synapses in health and disease of the retina, as well as the rest of the nervous system.

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