The Role of Retinoic Acid in the Formation and Modulation of Invertebrate Electrical Synapses

Joel Wingrove, Hons. B.Sc

A thesis submitted to the Department of Biological Sciences
in partial fulfillment of the requirements for the
degree of Master of Science

Faculty of Mathematics and Sciences

Brock University

St. Catharines, ON

© Joel Wingrove, 2023

Abstract

Communication between cells in the nervous system is dependent upon structures known as synapses. Synapses are broadly characterized as either chemical or electrical in nature, owing to the type of signals that are transmitted across them. Factors that can affect chemical synapses have been extensively studied. However, the factors that can influence the formation and modulation of electrical synapses are poorly understood. Retinoic acid, a vitamin A metabolite, is a known regulator of chemical synapses, yet its capacity to regulate electrical synapses is not as well established. Preliminary evidence from the central neurons of both invertebrates and vertebrates suggests that it is also capable of regulating the strength of electrical synapses. In this study, I provide further insights into how retinoic acid can act as a neuromodulator of electrical synapses. My findings suggest that retinoic acid is capable of rapidly altering the strength of electrical synapses in a dose- and isomer-dependent manner. Further, I provide evidence that this acute effect might be independent of either the retinoid receptors or a protein kinase. In addition, I provide novel findings to suggest retinoic acid is also capable of regulating the formation of electrical synapses. Long term exposure to two isomers of retinoic acid, all-*trans*-retinoic acid and 9-*cis*-retinoic acid, reduces both the proportion of cell pairs, and the average synaptic strength between cells that form electrical synapses. In summary, these investigations provide novel insights into the role that retinoids play in the both the formation and modulation of electrical synapses in the CNS.

Acknowledgments

I would like to take this moment to show my appreciation to all those who have helped me reach this point, including all my family, friends, mentors and colleagues.

Sunlight kissed upon the morning; a new day is dawning.

Table of Contents

Abstract .... *ii*
Acknowledgments .... *iii*
Table of Contents .... *iv*
List of Figures .... *v*
List of abbreviations .... *vi*

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW 1

GENERAL INTRODUCTION 2
RETINOIC ACID: METABOLISM, MACHINERY AND SIGNALING 3
A COMPARISON OF RETINOID SIGNALING IN VERTEBRATES AND INVERTEBRATES 7
NONGENOMIC EFFECTS OF RETINOID SIGNALING 9
A BRIEF HISTORY OF ELECTRICAL SYNAPSES 13
THE STRUCTURE OF ELECTRICAL SYNAPSES 14
ROLE OF ELECTRICAL SYNAPSES IN THE CNS 18
PROPERTIES OF ELECTRICAL SYNAPSES 19
MODULATION OF ELECTRICAL SYNAPSES 24

CHAPTER 2: METHODOLOGY 26

ANIMALS 27
CELL CULTURE 27
CHEMICALS 28
ELECTROPHYSIOLOGY 28
ACUTE AND LONG-TERM EXPERIMENTS 29

CHAPTER 3: RESULTS 33

ALL-*TRANS* RETINOIC ACID REDUCES ELECTRICAL COUPLING BETWEEN PEA NEURONS 34
ISOMER-SELECTIVE EFFECT OF RETINOIC ACID ON ELECTRICAL COUPLING 37
CONCENTRATION-DEPENDENT EFFECTS OF ALL-*TRANS* RA ON ELECTRICAL COUPLING 42
ALL-*TRANS* RETINOIC ACID'S UNCOUPLING EFFECT IS INDEPENDENT OF PROTEIN SYNTHESIS 45
THE UNCOUPLING EFFECT OF ALL-*TRANS* RA IS INDEPENDENT OF PKC AND RETINOID RECEPTORS 48
THE SYNTHETIC RETINOID EC23 MIMICS ALL-*TRANS* RA-INDUCED UNCOUPLING 51
RETINOIDS INHIBIT THE FORMATION OF ELECTRICAL SYNAPSES 54
LONG-TERM RETINOID EXPOSURE REDUCES SYNAPTIC STRENGTH OF ELECTRICAL SYNAPSES 57

CHAPTER 4: DISCUSSION 62

ACUTE EFFECTS OF RETINOIDS ON ELECTRICAL SYNAPSES 64
ISOMER-SELECTIVE EFFECTS OF RETINOID SIGNALING 73
CELL-SPECIFIC EFFECTS OF RETINOIDS 75
PHYSIOLOGICAL ROLE OF RETINOID-MEDIATED UNCOUPLING? 76
CONCLUSION 79

REFERENCES 80

List of Figures

Figure 1. Biochemical synthesis of retinoids 4

Figure 2. Retinoid signaling by nuclear receptors in vertebrates 6

Figure 3. General structure of gap junctions 17

Figure 4. Detecting and measuring electrical synapses 23

Figure 5. atRA's effect on electrical coupling is independent of culture duration 37

Figure 6. atRA but not 9-*cis* RA reduces electrical coupling between PeA neurons in the high coupling direction 39

Figure 7. atRA, but not 9-cis RA reduces electrical coupling in the low coupling direction 41

Figure 8. atRA's effect on electrical coupling also occurs at lower concentrations 44

Figure 9. atRA-mediated reduction in electrical coupling involves a mechanism that is independent of protein synthesis 47

Figure 10. atRA-mediated uncoupling is independent of PKC or retinoid receptors 50

Figure 11. atRA-induced uncoupling is mimicked by an RAR pan-agonist 53

Figure 12. Long-term (48 h) exposure to atRA inhibits PeA-PeA electrical synapse formation 56

Figure 13. Long-term exposure to RA reduces synaptic strength of PeA-PeA pairs in the high-coupling direction 58

Figure 14. Long-term exposure to RA reduces synaptic strength of PeA-PeA pairs in the low coupling direction 59

Figure 15. Long-term exposure (48 h) to RA reduces synaptic strength of PeA-PeA pairs (pooled directions) 61

List of abbreviations

ADH Alcohol dehydrogenase

CNS Central nervous system

CREB cAMP response element binding protein

ELH Egg laying hormone

LBD Ligand binding domain

LTP Long-term potentiation

LTD Long-term depression

*Lym*RAR *Lymnaea* retinoic acid receptor

*Lym*RXR Lymnaea retinoid X receptor

PeA Pedal A

PKC Protein kinase C

CRABP-1(2) Cellular retinoic acid binding protein

RA Retinoic acid

atRA all-*trans* retinoic acid

RALDH Retinaldehyde dehydrogenases

RARE Retinoic acid response element

RAR Retinoic acid receptor

RXR Retinoid X receptor

SDR Short-chain reductases

Chapter 1: Introduction and Literature Review

General Introduction

Retinoic acid (RA), a vitamin A metabolite, is a well-established signaling molecule involved in several neurological processes, spanning across early development into adulthood. During vertebrate neural development, retinoid signaling plays a critical role in differentiation (Schuldiner et al., 2001), patterning (Begemann & Meyer, 2001) and neurite outgrowth (Clagett-Dame et al., 2006). In the adult CNS of vertebrates, it is important for neuroplasticity (Lenz et al., 2021), neurogenesis (Jacobs et al., 2006) and vision (Travis et al., 2007). Over the past half-century, extensive research efforts have established RA as a critical bioactive molecule for normal physiology.

Canonically, RA exerts its biological effects through its interaction with retinoid receptors; the retinoic acid receptor (RAR) and retinoid X receptor (RXR), which are part of the nuclear receptor superfamily and function as ligand-activated transcription factors to regulate gene expression (Tanoury et al., 2013). In contrast to this genomic pathway, over the past two decades RA has also been shown to exert its effects in a nongenomic manner. In these cases, RA signaling can involve activation of extra-nuclear retinoid receptors (Poon & Chen, 2008) or can include RA binding directly to other signaling molecules, such as protein kinases (Radominska-Pandya et al., 2000).

Investigations into the role of RA signaling in the invertebrate CNS are limited. The effects of RA in the CNS of the pond snail, *Lymnaea stagnalis,* appear to be well conserved with those in the vertebrate CNS. Our research group has provided evidence for its involvement in embryonic development (Johnson et al., 2019), regeneration (Dmetrichuk et al., 2006), neuroplasticity (Rothwell et al., 2017; Wingrove et al., 2023) and memory formation in molluscs (Rothwell & Spencer, 2014; Wingrove et al., 2023).

Furthermore, RA (Dmetrichuk et al., 2008), as well as the retinoid receptors (Carter et al., 2010, 2015) have been detected in the *Lymnaea* CNS. However, the extent to which RA mediates its biological effects in *Lymnaea via* either genomic or nongenomic pathways is not yet clear. For instance, RA (Vesprini & Spencer, 2014), as well as both RAR and RXR agonists (de Hoog et al., 2018) and antagonists (de Hoog et al., 2019) rapidly induce changes in the biophysical properties of ion channels in a diverse set of neurons. There is also evidence that RA plays a nongenomic chemotropic role in the guidance of intact and isolated (transected from soma) growth cones (Farrar et al., 2009). The rapid time course of these effects suggests they are mediated by a nongenomic mechanism. As such, it is my goal to further our understanding of a potential nongenomic role for RA signaling in the formation and modulation of electrical synapses in cultured *Lymnaea* neurons.

Retinoic Acid: Metabolism, Machinery and Signaling

Animals are incapable of *de novo* synthesis of vitamin A (retinol), and must acquire it through dietary means (Blomhoff & Blomhoff, 2006; Theodosiou et al., 2010); however, they can metabolize it into its bioactive form: retinaldehyde, a visual chromophore, and RA, a 'regulator of gene expression' (Blomhoff & Blomhoff, 2006). Dietary vitamin A is acquired by way of carotenoids (from plants) or retinyl esters (from animal tissues; (Theodosiou et al., 2010)), and it is then synthesized into endogenous RA via a two-step enzymatic process (Fig. 1). This enzyme-mediated process involves oxidation of vitamin A into retinaldehyde by alcohol dehydrogenases, followed by a second, irreversible oxidation of retinaldehyde into RA by retinaldehyde dehydrogenases (RALDH).

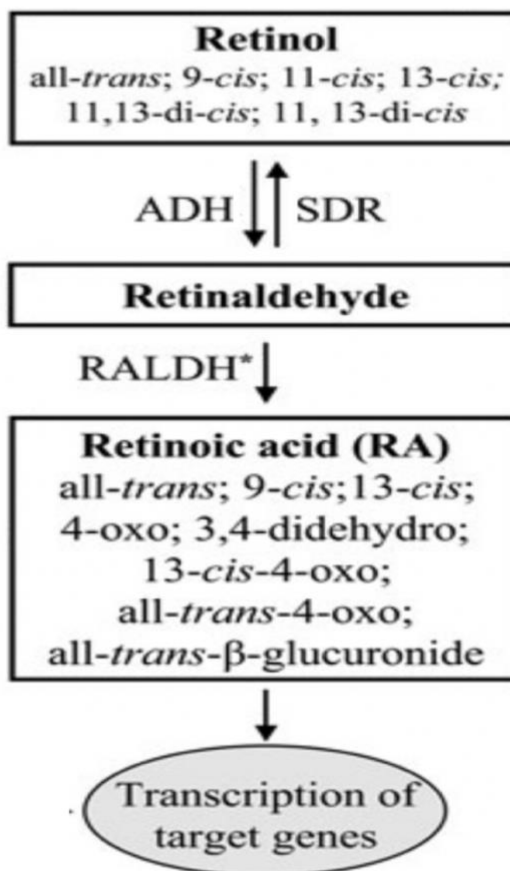

Figure 1. Biochemical synthesis of retinoids. Alcohol dehydrogenase (ADH) catalyzes the oxidation of vitamin A (retinol) isoforms into retinaldehyde. Short-chain reductases (SDR) catalyze the reverse reaction, reducing retinaldehyde back into retinol. Retinaldehyde dehydrogenase (RALDH*) catalyzes the irreversible reduction of retinaldehyde into RA of which there are multiple stereoisomers. Modified from Theodosiou et al. (2010).

In the cytoplasm, RA is associated with the cellular retinoic acid binding proteins (CRABP-1 and CRABP-2), which promote its translocation into the nucleus (Napoli, 2017). Upon nuclear entry, RA then binds to nuclear receptors, which function as ligand-activated transcription factors (Blomhoff & Blomhoff, 2006). Two families of these nuclear receptors exist: the retinoic acid receptor (RAR) and retinoid X receptor (RXR) (Blomhoff & Blomhoff, 2006). Three RARs (RARα, RARβ, RARγ) and three RXRs (RXRα, RXRβ, RXRγ) are encoded by the vertebrate genome. For biological activity,

heterodimerization of the RAR and RXR receptors is thought to be necessary (Blomhoff & Blomhoff, 2006), but RXRs can also either homodimerize or heterodimerize with a number of other non-retinoid nuclear receptors (Lefebvre et al., 2010), which allows for activation of many divergent signaling pathways. Thus, the composition of the receptor complex is thought to vary as it can consist of multiple retinoid receptor isoforms and/or other nuclear receptors. Upon binding of RA to the ligand binding domain (LBD) on each retinoid receptor, dimerization occurs, which activates the receptor complex allowing it to bind to its target DNA sequence, the retinoic acid response element (RARE) (Maden, 2007). In vertebrates, RARs are responsive to all-*trans* RA (atRA) and 9-*cis* RA, whereas RXRs are only thought to be responsive to 9-*cis* RA (Fig. 2; (Simões-Costa et al., 2008)).

Retinoid receptors, in particular the RXRs, are found in most invertebrate phyla including *Placazoa* (Reitzel et al., 2018), *Mollusca* (Bouton et al., 2005; Castro et al., 2007; Urushitani et al., 2011, 2018) and *Arthropoda* (Eichner et al., 2015; Hayward et al., 1999), though the presence or function of RARs in invertebrate species is far less clear (Albalat & Cañestro, 2009; Campo-Paysaa et al., 2008; Gutierrez-Mazariegos et al., 2014; Urushitani et al., 2013). In *Lymnaea*, both a *Lym*RXR (Genbank Accession No. AY846875) and *Lym*RAR (Genbank Accession No. GU932671) have been successfully cloned from its CNS, but unlike vertebrates, they only express one isoform for each receptor subtype. The *Lym*RALDH (Genbank Accession No. FJ539101) has also been cloned. Furthermore, RA has been shown to be present in *Lymnaea's* CNS, while RALDH activity has been demonstrated in *Lymnaea* CNS extracts (Dmetrichuk et al., 2008). Unlike in vertebrates, the binding affinities of RA isomers to RARs and RXRs are less known in invertebrates. AtRA and 9-*cis* RA seem to have similar binding affinities to the locust RXR

(Nowickyj et al., 2008), which suggests an evolutionary divergence in the retinoid signaling machinery in some protostomes.

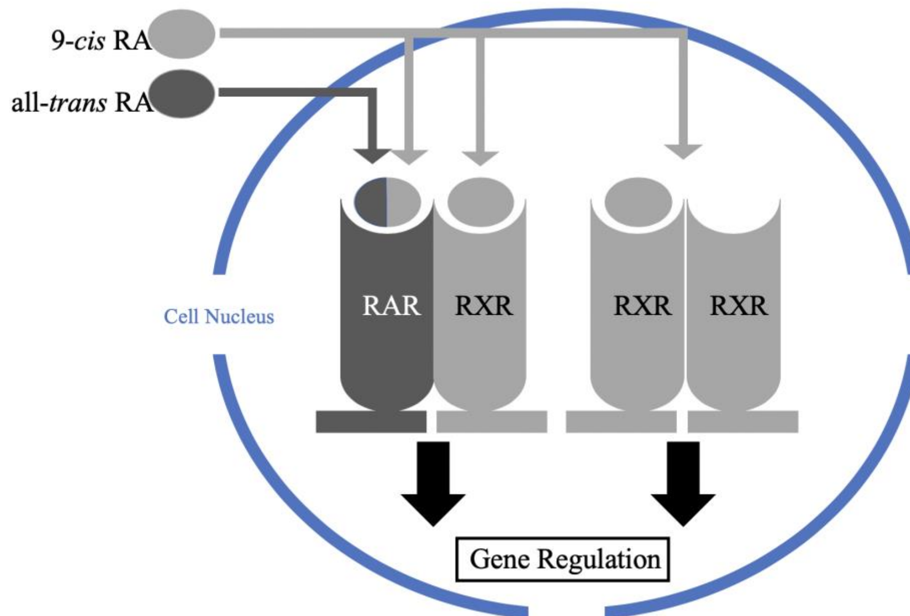

Figure 2. Retinoid signaling by nuclear receptors in vertebrates. In vertebrates, the nuclear retinoid receptors are bound by different RA isomers. The retinoic acid receptors (RARs) can be activated by either atRA or 9-*cis* RA, while retinoid X receptors (RXRs) are primarily bound and activated by 9-*cis* RA. Ligand-bound nuclear receptors either heterodimerize (RAR/RXR) or homodimerize (RXR/RXR). Once dimerization occurs the transcription complex binds to the RARE to regulate gene expression. Modified from Simões-Costa et al. (2008)

To date, RAR orthologs cloned from molluscs, including *Nucella lapillus* (Gutierrez-Mazariegos et al., 2014) and *Tillandsia clavigera* (Urushitani et al., 2013), have not been shown to activate transcription in the presence of atRA. Although recent evidence suggests that all-*trans* and 9-*cis* RA are both capable of binding to and activating transcription via the *Lym*RXR (de Hoog et al., 2022) and other molluscan RXRs (Bouton et al., 2005; Castro et al., 2007; Urushitani et al., 2018), as of yet, both the *Lym*RAR (Carter et al., 2015) and

*Lym*RXR (Carter et al., 2010) have only been localized to non-nuclear compartments of *Lymnaea* neurons.

A Comparison of Retinoid Signaling in Vertebrates and Invertebrates

Although initially thought to be a vertebrate innovation, it is now clear that many of the effects of RA signaling are evolutionarily conserved in some invertebrate lineages. Akin to vertebrates, retinoid signaling in *Lymnaea* plays an important role in neurite outgrowth, axonal pathfinding and neuroplasticity. In vertebrates, the role that RA plays in neurite outgrowth and axonal pathfinding is most commonly studied in primary neuronal cell cultures. AtRA increases the survival rate and density of dissociated rat embryonic spinal cord neurons (Wuarin et al., 1990) and mouse spinal cord explants display increased neurite outgrowth (but not number of neurites) following exposure to atRA (Quinn & De Boni, 1991). Further, dissociated cultures of chick neural stem tubes also exhibit increased neurite length and number in the presence of RA (Corcoran et al., 2000). In *Lymnaea*, all-*trans* and 9-*cis* RA increase the number and length of neurites extended from cultured CNS neurons (Dmetrichuk et al., 2006, 2008).

There is also evidence that RA exerts chemotropic effects and acts as a guidance molecule. Neurite extensions from chick neural tubes (Maden et al., 1998), spinal cord explants from the newt (Dmetrichuk et al., 2005) and cultured embryonic spinal cord neurons from *Xenopus* (Rand et al., 2017), display preferential growth towards gradients of atRA. This chemotropic effect is well conserved in *Lymnaea*, as again, both all-*trans* and 9-*cis* RA elicit chemoattractive responses in regenerating CNS neurons (Dmetrichuk et al., 2006, 2008). Nevertheless, retinoid-mediated growth cone turning has only been conclusively demonstrated in invertebrate species that also express RARs. For instance,

although the locust RXR has been identified, this species lacks an RAR and preliminary evidence indicates that locust growth cones are not responsive to RA (Sukiban et al., 2014). In *Lymnaea*, both RAR (Johnson et al., 2019) and RXR (Carter et al., 2010) agonists can induce chemoattractive responses in *Lymnaea* growth cones, responses that are counteracted by complementary RAR/RXR antagonists (Carter et al., 2010, 2015). However, the specificity of retinoid receptor agonists in *Lymnaea* is not clear. For instance, there is new evidence to suggest that the synthetic retinoid, EC23, used by Johnson et al. (2019) to induce growth cone turning in *Lymnaea* neurons and previously thought to be a selective RAR agonist (based on vertebrate studies), is capable of binding to and activating transcription via the *Lym*RXR (de Hoog et al., 2022). Hence, it is uncertain whether the effects of RA on neurite outgrowth and axonal pathfinding are mediated by *Lym*RARs and/or *Lym*RXRs, or an alternative (though less likely) signaling pathway (discussed below).

RA also plays an important role in memory and synaptic plasticity in vertebrates. In rodents, for example, decreased RA bioavailability is associated with deficits in hippocampal-dependent memory (Bonnet et al., 2008; Cocco et al., 2002), whereas disruptions to retinoid signaling machinery can lead to interruptions in the underlying cellular phenomena of long-term potentiation (LTP) (Jiang et al., 2012; Misner et al., 2001) and long-term depression (LTD) (Misner et al., 2001). Abnormal signaling via the various isoforms of the retinoid receptors influences behavioral and cellular deficits. For example, knockout of RARβ eliminates LTP and LTD (and also impairs performance in learning and spatial memory tasks), whereas knockout of RXRγ eliminates only LTD, but also leads to deficits in spatial learning and memory (Chiang et al., 1998). RA and its receptors are also

important for long-term memory formation following operant conditioning of *Lymnaea's* aerial respiratory behaviour (Rothwell & Spencer, 2014) and classical conditioning of its feeding behaviour (unpublished observations). Incubation in either an RALDH inhibitor (Rothwell & Spencer, 2014; Wingrove et al., 2023) or a retinoid receptor antagonist (Rothwell & Spencer, 2014) prevents long-term memory formation, whereas retinoid receptor agonists (Carpenter et al., 2016) promote or enhance long-term memory formation.

Although it is clear that RA signaling contributes to a subset of analogous functions in the nervous systems of distinct evolutionary lineages, whether these functions are the result of similar mechanisms is less clear. In terms of producing translatable research on retinoid signaling, *Lymnaea* offers a suitable target of investigation to explore related mechanisms, the results of which might yield important insights into the fundamental principles involved in this evolutionarily ancient signaling pathway.

Nongenomic Effects of Retinoid Signaling

The basic mechanisms that govern retinoid signaling, specifically with respect to the role of RA as a transcriptional regulator, have been called into question in recent years. Demonstrations from both vertebrate and invertebrate species have provided evidence for a number of nongenomic effects of RA. It is suggested that these pathways might involve RA binding directly to other signaling molecules, such as protein kinases (Khatib et al., 2019; Ochoa et al., 2003; Radominska-Pandya et al., 2000) or cAMP response element binding protein (CREB) (Aggarwal et al., 2006), or by binding directly to retinoid receptors that mediate signaling outside of the nucleus (N. Chen & Napoli, 2008; Piskunov et al., 2014). It is also feasible that RA signaling, at times, depends on the involvement of both

genomic and nongenomic components. In human neuroblastoma cells (SH-SY5Y), both transcriptional activation and non-genomic ERK-1/2 phosphorylation is required to induce maximal neurite outgrowth following application of various retinoids (Khatib et al., 2019).

In an invertebrate nervous system, RA is capable of exerting its effects on growth cone guidance rapidly and independently of gene transcription. For example, RA can induce growth cone turning only minutes after its local application to growth cones, a response too fast to require gene transcription (Dmetrichuk et al., 2006). Furthermore, RA is not only capable of eliciting chemoattractive responses in intact neurites of cultured, regenerating *Lymnaea* neurons (Dmetrichuk et al., 2006, 2008), but also in isolated neurites, transected from the cell body (thus with no nucleus) (Farrar et al., 2009). Crucially, application of protein synthesis inhibitors abolishes the chemoattractive effects of RA on isolated neurites/growth cones. This evidence suggests that a local, nongenomic mechanism acting rapidly within the neurite/growth cone, mediates the effects of RA. One possibility is the local translation of protein(s) involved in RA-induced chemoattraction. Considering that growth cones must sometimes travel long distances from their cell body to reach synaptic targets, local translation provides a mechanism for single growth cones to act independently from their cell body. Although local translation plays a demonstrable role in regulating axonal and dendritic processes within the adult brain (Sasaki, 2020), in regenerating neurons there is less direct evidence for local protein synthesis in neurites/growth cones. However, in the isolated neurites of regenerating *Lymnaea* neurons, it was determined that local synthesis of actin binding proteins regulated neurite outgrowth (Van Kesteren et al., 2006), and there is also evidence that the conopressin receptor (Spencer et al., 2000) and egg laying hormone (Van Minnen et al., 1997) can be locally

synthesized. Injection of mRNA encoding for the conopressin receptor and egg laying hormone (proteins which are not endogenously expressed in some identified *Lymnaea* neurons) into the isolated axons of such neurons, was shown (via immunocytochemistry) to lead to expression of both proteins. The presence of miRNAs, which are well established translational repressors (Wilczynska & Bushell, 2015), within the regenerating neurites and growth cones of *Lymnaea* motorneurons (Walker et al., 2018), also implies a mechanism for the control of local protein synthesis during the regenerative process. In terms of RA-induced chemoattraction, the retinoid receptors are candidates for the nongenomic pathway as: 1. the *Lym*RXR and *Lym*RAR display extra-nuclear localization patterns in the growth cones and neurites of regenerating *Lymnaea* neurons (Carter et al., 2010, 2015); 2. receptor agonists are capable of mimicking the chemoattractive effects of RA (Johnson et al., 2019; Nasser, 2017); 3. receptor antagonists inhibit the chemoattractive effects of retinoids (Carter et al., 2010, 2015).

Perhaps one of the most well studied nongenomic retinoid signaling pathways in vertebrates is that underlying homeostatic plasticity. RA has been identified as a key signaling molecule coordinating homeostatic mechanisms at both excitatory (Aoto et al., 2008; Sarti et al., 2012; Wang et al., 2011) and inhibitory synapses (Sarti et al., 2013) stabilizing the excitatory / inhibitory balance of circuit activity via a process known as synaptic scaling. The mechanisms underlying this effect are dependent on the RAR acting as an RNA-binding protein, which represses translation of mGluR1 transcripts (Poon & Chen, 2008).

In *Lymnaea*, RA is also capable of altering the biophysical properties of identified neurons; acute application of RA modulates the firing properties (such as spike broadening,

complex spiking and cell silencing) of peptidergic and dopaminergic neurons in a dose- and isomer-dependent manner (Vesprini & Spencer, 2014), effects that were, in part, attributed to Ca^{2+} signaling (Vesprini et al., 2015). Although the exact molecular mechanisms by which RA exerts its effects are not yet clear, de Hoog et al. (2018) found that atRA and the retinoid agonist, EC23, inhibit voltage-gated Ca^{2+} channels (of the Cav2 subtype) in *Lymnaea* neurons. Further, a follow-up study by de Hoog & Spencer (2022) found that RA-induced spike broadening and complex spiking is likely mediated by enhanced inactivation of delayed rectifier voltage-gated K^{+} channels. Complex spiking and spike broadening extend the depolarization phase of the action potential increasing Ca^{2+} influx through voltage-gated Ca^{2+} channels. However, as RA has inhibitory effects on Cav2 but not Cav1 channels, the authors suggest that RA-induced spike broadening and complex spiking "fine tunes" calcium influx through specific Ca^{2+} channel subtypes. These findings suggest that retinoid signaling might be important for modulating specific Ca^{2+} signaling pathways underlying synaptic plasticity.

RA also influences both the formation and modulation of central synapses of *Lymnaea* neurons (Rothwell et al., 2017) and such effects have been shown at both chemical and electrical synapses. Incubation in atRA increases the proportion and strength of excitatory chemical synapses formed between *Lymnaea* neurons cultured in a soma-soma configuration. At electrical synapses, atRA reduces the synaptic strength of coupling in a cell-specific manner over the course of one hour. The rapid time course of these effects provides evidence that atRA is acting in a nongenomic manner to modulate certain electrical synapses within *Lymnaea's* CNS.

A Brief History of Electrical Synapses

Deep in the annals of neuroscience exists the historical record of a fervent debate over the nature of interneuronal communication. Early 20th century contributions from Santiago Ramón y Cajal and Charles Scott Sherrington, established that nervous systems were made of discrete elements, neurons, which exchanged information across specialized structures, synapses. However, the mechanism by which neurons communicated – via chemical or electrical signals – was a point of heated contention, known as *The War of Soups and Sparks* (Valenstein, 2005), and persisted throughout the first half of the 20th century. Those who argued in favor of electrical transmission posited that action potentials in the presynaptic cell induce passive currents in the postsynaptic cell. Others suggested that action potentials in the presynaptic cell cause the liberation of chemicals (neurotransmitters) from the presynaptic cell which interact with the postsynaptic cell to propagate the signal.

Early investigations providing exquisite evidence that chemicals were capable of driving physiological responses in neurons laid the groundwork for the prevailing view that chemical transmission was the sole means of interneuronal communication. This view persisted until direct demonstrations of electrical transmission from crayfish giant motor neurons. That is, it was shown that the time course of a presynaptic signal mirrored that of a postsynaptic response, was bidirectional and voltage-dependent (Furshpan & Potter, 1959). In subsequent years, evidence for electrical synaptic transmission was confirmed in vertebrates, including fish (Bennett et al., 1959), birds (Martin & Pilar, 1963), and mammals (Baker & Llinás, 1971).

It is now established that both means of synaptic transmission co-exist, providing distinct and, at times, overlapping contributions to nervous system functioning. Electrical connections are perhaps still under-investigated and under-appreciated, and publication records reflect heavy attentional bias towards chemical transmission. This is likely due, in part, to the fact that electrical synapses (relative to chemical synapses) account for a distinct minority of synapses in mature neural circuits. Nonetheless, the ubiquity of electrical synapses in metazoan nervous systems underscores the importance of future research aiming to decipher the role of these seemingly "simple" structures in fundamental neural processes.

The Structure of Electrical Synapses

Early anatomical investigations using electron microscopy identified peculiar structural connections between apposed cells, which are now known as gap junctions. Through electrophysiological and electron microscopic analysis, it became apparent that gap junctions provided the structural basis for electrical transmission in vertebrates and invertebrates (Hanna et al., 1978; Peracchia, 1973). Morphologically, gap junctions can be readily identified. The spacing between adjacent cells connected by a gap junction (3-4 nm) is considerably narrower than other membrane interfaces (Hormuzdi et al., 2004). The size of the gap across a chemical synaptic cleft is ~20-40 nm, while the gap between apposed neurons in non-synaptic regions is closer to ~20 nm.

Gap junctions represent an aggregated cluster of either hundreds or thousands of intercellular channels (Skerrett & Williams, 2017). Individual gap junction channels consist of a multimeric protein ensemble composed of two separate hemichannels, one present in each cell. Alignment of two hemichannels produces a pore directly connecting

the cytoplasm of the two cells. The individual protein subunits that make up hemichannels, connexins (Cxs) in vertebrates and innexins (Inxs) in invertebrates, are derived from evolutionarily distinct protein families (Beyer & Berthoud, 2018; Welzel & Schuster, 2022). Although innexins and connexins bear little resemblance in terms of their primary amino acid sequences, they are topologically similar, and carry out similar functions across both lineages (Beyer & Berthoud, 2018). Connexin and innexin proteins contain four transmembrane domains, with cytosolic facing C- and N- termini (Fig. 3A) (Sánchez et al., 2019). The quaternary structure of connexins and innexins are also strikingly similar to one another. In vertebrates, 6 connexin subunits combine to form a *connexon*-based hemichannel, while in invertebrates 6-8 innexin subunits are thought to combine and constitute *innexon*-based hemichannels (Fig. 3B). Connexon- and innexon-based hemichannels can be derived from either a homomeric (same) or heteromeric (variable) assembly of connexin/innexin subunits respectively (Fig. 3B). Further, cells can combine two identical hemichannels (each with the same subunit composition) to form a *homotypic* channel or combine two different hemichannels (each with a different subunit composition) to form a *heterotypic* channel (Figure 3B). The capacity to "mix and match" at these different levels provides a diverse set of gap junction channel isoforms to mediate a myriad of functional capacities. A third protein family, known as pannexins, shares considerable sequence homology to innexins, and is also found in vertebrates. Although overexpression of pannexins can lead to the formation of intercellular channels (Vanden Abeele et al., 2006), under normal conditions it is assumed that pannexin-based channels only form hemichannels (due to glycosylation of their extracellular domains) (Boassa et al., 2007; Penuela et al., 2007; Sosinsky et al., 2011).

Innexin- and connexin-encoding genes have been identified in a large number of phyla, including *Arthropoda* (Calkins et al., 2015; Ganfornina et al., 1999; Stebbings et al., 2002), *Annelida* (Kandarian et al., 2012; Potenza et al., 2003), *Nematoda* (Starich et al., 2001), *Mollusca* (Mersman et al., 2020; Sadamoto et al., 2021) and *Chordata* (White et al., 2004). The number of connexin and innexin-encoding genes varies across distinct lineages. The human genome encodes for 21 connexin genes and the mouse for 20 (Söhl & Willecke, 2004). In contrast, the genomes of the fruit fly, *Drosophila melanogaster* (*Arthropoda*) (Stebbings et al., 2002), the medicinal leech, *Hirudo verdana (Annelida)* (Kandarian et al., 2012), *Caenorhabditis elegans (Nematoda)* (Starich et al., 2001), and *Limax valentianus* (*Mollusca*) (Sadamoto et al., 2021) encode for 8, 21, 25, and 12 distinct innexin-encoding genes, respectively. In *Lymnaea*, at least 10 innexin-encoding genes have been identified (Mersman et al., 2020; Sadamoto et al., 2021).

Both connexins and innexins display tissue- and cell-dependent expression patterns. For instance, in the human brain Cx36 is widely expressed in neurons, whereas expression of other connexin isoforms is upregulated in glial cells, such as astrocytes (Cx26, Cx30, and Cx43) and oligodendrocytes (Cx29, Cx32, Cx47) (Connors & Long, 2004). In *Lymnaea,* 8 innexin paralogs display heterogenic expression across various tissues (buccal mass, foot, albumin gland, penis and CNS). The most thoroughly characterized innexin isoform, *Lymnaea* innexin 1 (*Lst Inx-1)*, is most highly expressed in the CNS (Mersman et al., 2020). Transcriptome data from *Lymnaea's* CNS also suggests the presence of 2 other innexin paralogs, though their presence and relative expression in other tissues is currently unknown (Sadamoto et al., 2021).

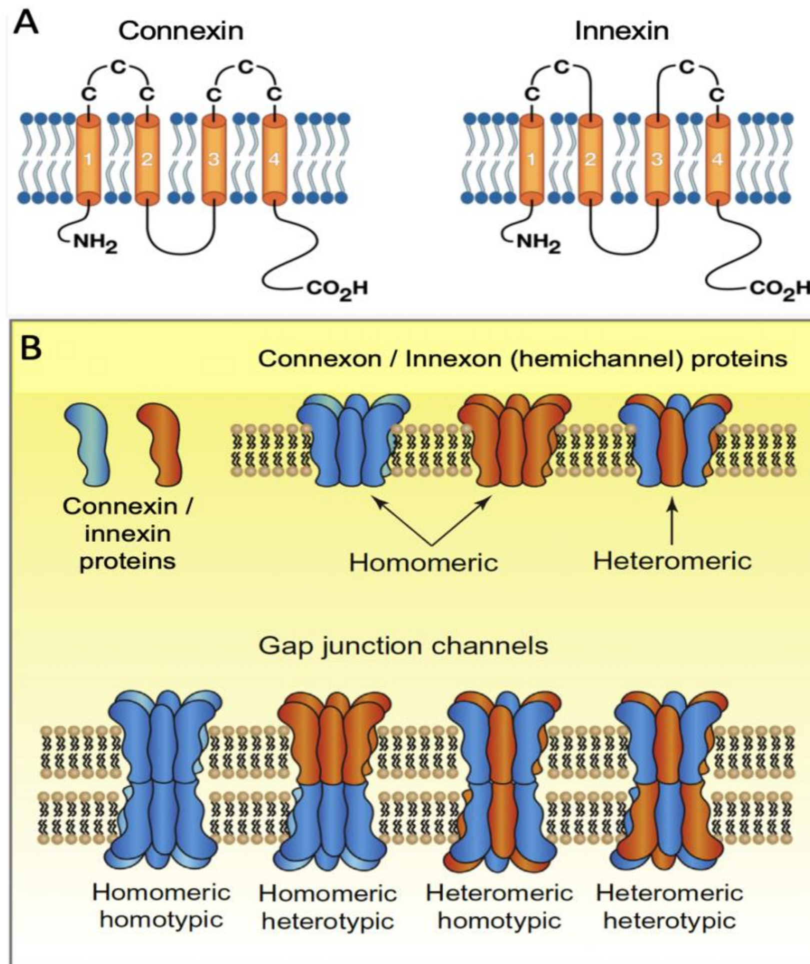

Figure 3. General structure of gap junctions. A) Transmembrane topology of connexin and innexin proteins. Connexins and innexins both contain four transmembrane regions with cytosolic facing C- and N-termini. Connexins and innexins contain three and two cysteine (C) residues (which participate in tethering connected hemichannels) in their extracellular loops, respectively. Panel A modified from Sánchez et al., (2019). **B)** Formation of connexon / innexon hemichannels can be constituted by the same (homomeric) or different (heteromeric) protein subunits (top). Vertebrate connexon hemichannels are composed of six connexins and invertebrate innexon hemichannels can be derived from 6-8 innexin subunits. Combination of two hemichannels (one from each cell) leads to the formation of a functional gap junction channel (bottom). Arrangement of homomeric or heteromeric hemichannels from each cell can produce variable gap junction channel types with different functional properties. Panel B modified from Meriney & Fanselow (2019).

Role of Electrical Synapses in the CNS

The presence of electrical synapses in neural circuitry plays a critical role in network functions such as lateral excitation (DeVries et al., 2002), oscillatory activity (Hormuzdi et al., 2001), coincidence detection (Galarreta & Hestrin, 2001) and synchronicity (Bennett & Zukin, 2004; Perez Velazquez & Carlen, 2000). In a historical sense, electrical synapses in certain neural populations have been most commonly linked to synchronizing network activity (Bennett, 1966; Llinas et al., 1974; Watanabe, 1958). That is, either the currents underlying synaptic potentials and/or action potentials can pass into electrically coupled neighboring cells, thereby influencing their activity in a concomitant fashion. The ability for suprathreshold (Galarreta & Hestrin, 2002; Gibson et al., 1999; Landisman et al., 2002) and subthreshold voltage changes (Beierlein et al., 2000; Benardo & Foster, 1986; Christie et al., 1989) to promote synchronicity has been detailed across various vertebrate and invertebrate nervous systems. Synchronicity is particularly important for producing the patterned oscillatory output of central pattern generating (CPG) networks, which control rhythmic motor behaviors such as respiration (Rekling et al., 2000), feeding (Susswein et al., 2002) and locomotion (Sakurai & Katz, 2016). In general, such CPG-based networks rely on the presence of gap junctions to synchronize the activity of interneurons (and even motorneurons) necessary to produce these rhythmic behaviors. In crustaceans, gap junctions underlie the synchronized activity in stomatogastric ganglion neurons and cardiac motorneurons critical for maintaining the rhythmic contractile output of gastric and heart muscles, respectively (See review: (Otopalik et al., 2019)). Similarly, electrical coupling is thought to be key for producing the synchronicity observed in the locomotor circuitry of vertebrates (Hinckley & Ziskind-

Conhaim, 2006) and invertebrates (Kristan & Calabrese, 1976). Locomotion in *Lymnaea* is partly controlled by the activity of a large cluster of electrically coupled Pedal A (PeA) motorneurons, which innervate the cilia of the foot (Syed & Winlow, 1989). However, whether these PeA neurons may be altered by intrinsic and/or extrinsic factors, and the functional outcome of such changes, has yet to be determined.

Properties of Electrical Synapses

A key physical feature of gap junction channels is the size of the pore, as it is the limiting factor determining the type of molecules that can be transmitted through a channel. The two main ways of characterizing the type of molecules which can pass through gap junctions are the atomic mass of the molecule and the size of the pore itself. It is estimated that molecules < 1000 Da can generally be transmitted through a channel pore, whereas the actual size of a channel pore is estimated to be 10-20 Å in diameter (Hormuzdi et al., 2004). The variant protein subunits that constitute gap junctions influence the size of the channel pore, and thus the type of molecules that can pass through a particular gap junction (Beblo & Veenstra, 1997; Gong & Nicholson, 2001; Veenstra, 2001; H. Z. Wang & Veenstra, 1997). Inorganic molecules, such as Na^+, K^+, Ca^{2+} and Cl^- ions are estimated to be 22-40 Da, and even when hydrated the estimated diameters of these ions are only 3-5 Å. Thus, the size of channel pores does not limit the flow of these inorganic ions (carrying current) across gap junctions. In addition to the inorganic molecules, the size of a channel pore also permits the flow of many important signaling molecules, including cAMP (Lawrence et al., 1978), ATP (Lohman & Isakson, 2014), and inositol 1,4,5-trisphosphate (IP3) (Sáez et al., 1989) to name a few. The type of molecules able to flow across gap junctions carry

important functional consequences, insofar as cells connected via a gap junction can transmit chemical (second messengers) and electrical signals (ions).

The ability for ions to freely flow across gap junctions represents a low resistance pathway for the direct flow of electrical current between cells. In contrast to chemical synapses, which require the probabilistic release of an intermediate messenger (neurotransmitters) to mediate intercellular communication, the mechanism for transmission across electrical synapses is both rapid and analog (i.e. continuously variable). In other words, in cells connected via an electrical synapse, changes in the membrane potential of a pre-synaptic cell directly influence the membrane potential of the postsynaptic cell without the need for an intermediate messenger (as is this case in chemical synapses). Gap junctions are analogous to simple ohmic resistors, where current (I) flow is derived from the membrane voltage (V) between coupled cells and the resistance (R) of the gap junction. As stated by Ohm's Law:

$$V = \frac{I}{R}$$

Due to this property, current transmission across gap junctions is often bidirectional; yet, current flow across the junction is not always equal in magnitude in each direction. Gap junctions are classified as non-rectifying (passing ionic current equally well in both directions) or rectifying (passing current preferentially or exclusively in one direction). Non-rectifying channels are generally homotypic, whereas rectifying channels are heterotypic (Marder, 2009). Nonetheless, the occurrence of action potentials or changes

in membrane potential in any cell within a coupled pair, translates into a complementary potential in the coupled cell (albeit reduced in size).

In an experimental setting, the strength of an electrical synapse between two neurons is often investigated by simultaneously recording from two electrically coupled cells (Fig. 4A). In this paradigm, a voltage change is induced by delivering a hyperpolarizing current step in the pre-synaptic cell, which in turn induces the potential (or coupling potential) in the post-synaptic cell. Quantification of the strength of electrical coupling between two neurons is commonly defined as the coupling coefficient (CC), which is measured by taking the ratio of the voltage change in the post-synaptic cell (generally smaller in size) to that of the voltage change in the pre-synaptic cell:

$$CC = \frac{V_{Post}}{V_{Pre}}$$

The coupling coefficient varies between 0 and 1, where increased values correspond to an increased strength of an electrical synapse. Quantification of a voltage change in two electrically coupled cells is taken at the point where the voltage change in both cells reaches a steady state (Fig. 4B). Two cells connected via an electrical synapse can be represented by an equivalent electrical circuit (Fig. 4C), wherein R_j represents the junctional resistance, and R_1 (pre-synaptic cell) and R_2 (post-synaptic cell) the membrane resistance of the coupled cells. In this instance, current injected into one cell can travel along two parallel pathways towards ground, either through R_1 or R_j and R_2, consequently, inducing a voltage change in both the presynaptic and postsynaptic cell. Yet, as R_j and R_2 are connected in series they represent a voltage divider: a passive linear circuit that produces an output

voltage that is a fraction of the input voltage. The input voltage is divided across two components proportionally depending on their respective resistance. The relationship between an input voltage and output voltage in a voltage divider is represented by the following equation:

$$V_{Post} = V_{Pre} * \frac{R_2}{R_2 + R_j}$$

From this equation:

$$\frac{V_{Post}}{V_{Pre}} = \frac{R_2}{R_2 + R_j}, \qquad if \;\; CC = \frac{V_{Post}}{V_{Pre}}, \qquad then\; CC = \frac{R_2}{R_2 + R_j}$$

From this relationship it is evident that the coupling coefficient is dependent not only on the junctional resistance (R_j) but also the membrane resistance (R_2) of the postsynaptic cell. As such, factors which control either junctional conductance or membrane resistance can dynamically alter the strength of electrical synapses.

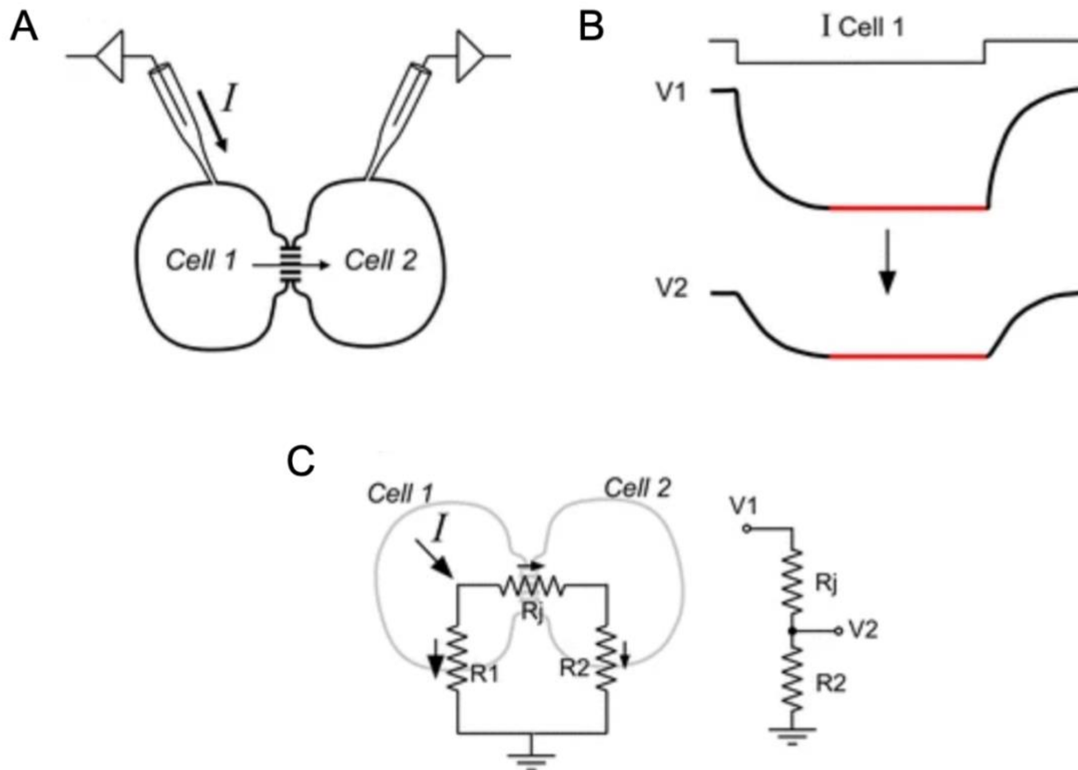

Figure 4. Detecting and measuring electrical synapses. **A)** Dual channel intracellular recordings from a soma-soma electrical synapse. Injection of hyperpolarizing current (*I*) in cell 1 is transferred to cell 2 through gap junctions. **B)** Voltage changes in the membrane potential of cell 1 (V_1) induced by a hyperpolarizing current step, produces a sign-conserving, attenuated voltage change in cell 2. The coupling coefficient is a measure of the ratio of voltage change in cell 2 to cell 1 and is typically calculated when the voltage change reaches a steady state (as indicated by the red line in the diagram). This coefficient reflects the strength of the electrical coupling between the cells, with a higher coefficient indicating stronger coupling. **C, left)** The diagram shows the equivalent circuit for a pair of coupled cells during current injection (*I*) into cell 1 (small arrows denote direction of current flow). The circuit includes the membrane resistance (cell 1, R_1 and cell 2, R_2) and the junctional resistance (R_j) of the gap junctions connecting cell 1 to cell 2 (or vice versa). **C, right)** The circuit also represents a voltage divider, with the junctional resistance (R_j) connected in series to the membrane resistance of the postsynaptic cell (R_2). The input voltage is the membrane voltage change in the presynaptic cell (cell 1, V_1), and the output voltage of the divider is the membrane voltage change in the postsynaptic cell (cell 2, V_2). Figure taken and modified from Curti & O'Brien (2016)

Modulation of Electrical Synapses

The perceived structural simplicity of electrical synapses (relative to chemical synapses) was another reason that led many researchers to posit a limited potential for plasticity at electrical synapses (Pereda, 2014). However, it is now clear that protein dense matrices made up of scaffolding and modulatory proteins associated with the gap junctions resemble, in terms of complexity, the pre- and post-synaptic densities that populate chemical synapses (Martin et al., 2020). Nonetheless, this misconception likely served as another factor biasing research efforts away from electrical transmission and towards chemical transmission. It is now well documented, however, that synaptic efficacy at electrical synapses is highly dynamic and sensitive to both preceding activity and chemical action. Moreover, plasticity at gap junctions is intricately linked to their interactions with multimeric protein assembles affecting trafficking, docking, cell adhesion, scaffolding, and post-translational modifications (Hervé et al., 2004; Lynn et al., 2012; Miller & Pereda, 2017). Changes in gap junctions can vary over a wide range of timescales, milliseconds to days, involving different mechanisms at different time points. Although changes in the single channel conductance or channel gating (open versus closed states) enable rapid modulation, factors influencing the number or type of channels being expressed (via biochemical signaling pathways which affect protein synthesis, assembly, trafficking, post-translational modifications and/or proteolysis) provide a slower means of regulation. The mechanisms responsible for changes across different timeframes are not mutually exclusive. For instance, phosphorylation can influence both single channel conductance as well as protein trafficking and proteolysis (Goodenough & Paul, 2009). One such phosphorylating enzyme, protein kinase C (PKC) is thought to regulate electrical synapse

communication in vertebrates (Pogoda et al., 2016) and invertebrates (Beekharry et al., 2018).

An increasing number of neuromodulators such as dopamine (Zhang & McMahon, 2000), serotonin (Rörig & Sutor, 1996) and glutamate (Wang et al., 2012) have been identified as neuromodulators of electrical synapses. Of particular interest to my investigation is RA, which has now been shown to affect electrical coupling in Bass (fish) retinal horizontal cells (Zhang & McMahon, 2000), and more recently, identified *Lymnaea* central neurons (Rothwell et al., 2017). The latter study was the first instance showing the capacity of RA to modulate electrical coupling in central neurons of an invertebrate. Yet, this previous report did not seek to examine the mechanisms underlying this modulation. As such the mechanisms by which RA exerts its effects on electrical coupling are not yet known. Thus, it is my overall goal to further characterize the modulatory effects of RA on electrical synapses by combining pharmacological agents and intracellular recording techniques. As investigations on the extrinsic and intrinsic factors that influence electrical synapses (especially in invertebrate species) are limited, a more comprehensive description of RA's action on electrical synapses is warranted. These investigations will also be useful in further characterizing the nongenomic roles of RA signaling.

Chapter 2: Methodology

Animals

All animals were bred and raised in a temperature-controlled (21°C) laboratory environment and kept within well aerated housing tanks filled with dechlorinated water. A 12:12 h dark:light schedule was maintained to mimic their natural habitat. *Lymnaea*'s diet consisted of romaine lettuce, NutraFin Max Spirulina fish food and carrots. Only animals that were 16-22 mm in length (>10 weeks of age) were used for these studies.

Cell Culture

Dissection of *Lymnaea's* CNS was performed under sterile conditions. *Lymnaea* were anesthetized by submersion (1 min) in *Lymnaea* saline containing 25% Listerine (containing menthol; 0.042% w/v). Anesthetized animals were then placed into a dissection dish filled with antibiotic saline (ABS) [normal *Lymnaea* saline containing 225 ug/mL gentamicin (Sigma-Aldrich, St. Louis, MO)] and their CNS dissected and placed into a sterile plastic culture dish. Three five-minute washes in ABS were performed to remove residual contaminants from the dissection procedure. CNS were then exposed to a trypsin treatment (2mg/mL) in defined medium (DM; 50% Leibovitz's L-15 Medium; ThermoFisher Scientific, Waltham, MA) for 18-20 minutes at room temperature. One 10-minute incubation in a trypsin inhibitor (2 mg/mL in DM; Sigma-Aldrich) was then used to inactivate trypsin. Subsequently, in a sterile dissection dish containing high-osmolarity DM, CNS were pinned out to remove the outer and inner sheath encapsulating the PeA neurons. Individual PeA neurons were isolated from the pedal ganglia using fire polished pipettes (coated in Sigma-cote [Sigma-Aldrich] to reduce cell adhesion), by gentle application of negative pressure via a Gilmont microsyringe. Cells were cultured in collagen-coated 35 mm glass bottom dishes (MatTex Corp.) filled with 3 mL of DM.

Individual PeA neurons were isolated and plated in pairs in a soma-soma configuration (making direct physical contact) to promote electrical synaptogenesis. Following plating, cells were kept in the dark at room temperature for at least 40 hours (unless otherwise stated).

Chemicals

All-*trans* and 9-*cis* RA (Sigma-Aldrich) were dissolved in 100% EtOH. The RAR agonist EC23 (Tocris Bioscience), and RXR agonists PA024 (provided by Dr. H. Kagechika [University of Tokyo, Japan]) and SR11237 (Tocris Bioscience) were dissolved in 100% DMSO. The RAR pan-antagonist LE540 (Tocris Bioscience), RXR pan-antagonist HX531 (Tocris Bioscience) were dissolved in 100% DMSO. The protein kinase C inhibitors, Gö6976 (Sigma-Aldrich) and H7 (Sigma-Aldrich), were also dissolved in 100% DMSO (Lacchini et al., 2006; Van Soest et al., 2000). Anisomycin (Sigma-Aldrich), a well-established translational inhibitor, was prepared in sterile distilled water (Feng et al., 1997). All chemicals were prepared to a final stock concentration of 100 mM. All further dilutions were carried out in DM (50 % Leibovitz‟s L-15 medium, Gibco) to the desired bath concentration ranging from 1 to 50 µM (noted in each experiment). All stock solutions of atRA were made fresh (daily) prior to each experiment.

Electrophysiology

Dual intracellular sharp electrode recordings were made from PeA-PeA pairs to detect the presence or absence of electrical synapses. Upon cell entry, hyperpolarizing current steps were delivered to test for electrical coupling between PeA-PeA pairs. At least 5-10 minutes between cell entry and the onset of any experiment enabled cells to recover

from cell injury and to minimize fluctuations in the RMP or input resistance. Prior to experiments, hyperpolarizing current steps sufficient to produce ~50 mV deflections were used to standardize recording parameters across all conditions. Reasons for omitting PeA-PeA pairs from analysis included: (1) 'weak' baseline coupling coefficients (< 0.15) in the acute modulation experiments; (2) RMPs (< -30mV); (3) loss of seal; (4) blebbing of cell membrane. All intracellular recording were made using glass electrodes (40-80 MΩ) pulled by a Sutter Instrument electrode puller (Model P-1000; Sutter Instrument, CA, USA) and backfilled with saturated K_2SO_4. Signal amplifications were made with a dual channel intracellular recording amplifier (NeuroData IR283A; Cygnus 113 Technology Inc.). A Powerlab 4sp digitizer (AD Instruments) synched with Chart v4.2.4 software (AD Instruments) was used for acquisition and subsequent storage/analysis of all electrophysiological data.

Acute and Long-Term Experiments

The experiments performed in this study were designed to test the effects of retinoids on PeA electrical synapses over two differing times scales. The first series of experiments aimed to characterize acute retinoid-mediated *modulation* of electrical synapses over 1-2 h recording durations. The second series of experiments aimed to characterize the long-term (48 h) effects of retinoid signaling on the *formation* of electrical synapses. For all acute modulation experiments, bi-directional coupling coefficients were separately calculated three times and then averaged for each cell pair at any given time point (i.e. at the time of [T0] and 60-minutes following [T60] the addition of any drug treatment, unless otherwise stated). Electrical coupling was also monitored at 10-minute intervals across all recordings. Notably, as electrical coupling between PeA-PeA pairs

often exhibited rectification, the baseline bi-directional coupling coefficients calculated for each cell in a pair were categorized as either high or low coupling (discussed in detail within results section). This step was taken to standardize all comparisons between experimental and control conditions and to assess whether any of the drug treatments mediated an effect on electrical coupling that was a function of the basal (or initial) synaptic strength. All statistical analyses were performed using GraphPad Prism. All data are represented as mean ± SEM.

To test the acute effects of retinoids, PeA-PeA pairs were incubated in DM for a minimum of 40 h prior to recordings (unless otherwise stated). After this time, and following the start of electrophysiological recordings retinoids were applied to the bathing media to produce the desired bath concentration: atRA (10 or 5 µM), 9-*cis* RA (10 µM). Similar pharmacological concentrations of RA have been previously used to study the effects of retinoid signaling in both *Lymnaea* (de Hoog et al., 2018; Rothwell et al., 2017; Vesprini & Spencer, 2014) and vertebrates (Zhang & McMahon, 2000). The final bath concentration of the vehicle control (EtOH) was 0.1 or 0.05%. Electrical coupling was monitored for 60 minutes (unless otherwise stated). Mixed factor ANOVAs (with repeated measures), with drug treatment as the fixed factor and time as the repeated measures factor, were used to compare and analyze electrical coupling over time (unless otherwise stated). A Sidak *post hoc* test was performed when a significant interaction or main effect at a significance level of $P < 0.05$ occurred.

The acute modulation experiments also utilized various pharmacological agonists and antagonists to identify potential intermediate messengers involved in the RA-mediated reduction in electrical coupling. PeA-PeA pairs were exposed to either 0.1% DMSO

(vehicle control), an RXR agonist (either SR11237 or PA024) or the RAR agonist (EC23) to determine whether these retinoid receptor agonists could mimic the effects of RA. Retinoid receptor antagonists (RXR: HX531; RAR: LE540) or protein kinase C inhibitors (Gö6976 and H7) were utilized to determine whether these antagonists were capable of counteracting retinoid-mediated effects on electrical coupling. Prior to the addition of atRA, PeA-PeA pairs were pre-incubated in antagonists for 1 h. For both agonist and antagonist experiments, data were analyzed as a % change in average coupling coefficient between T0 and T60. A one-way ANOVA was used to compare % change in average coupling coefficient across conditions and a Sidak *post hoc* test utilized if the significance level reached $P < 0.05$.

In the long-term experiments to examine the effects of retinoids on synapse formation, in cases where electrical synapses formed (discussed in detail below), bi-directional coupling coefficients were again measured three times and averaged for each pair *once* at the completion of a 10-minute recording. For this series of experiments, PeA-PeA pairs were cultured in DM for 40 h prior to recording and retinoids were added at the time of cell plating. Retinoids used, included atRA (either 10 or 5 μM) or 9-*cis* RA (10 μM), whereas EtOH (0.1% or 0.05%) was added as the vehicle control. Only PeA-PeA pairs making direct soma-soma physical contacts were recorded and included in analysis. Photographs of each PeA-PeA pair included in the analysis were captured with a Retiga EXi Fast 1394 CCD digital camera (Quantitative Imaging Corp.).

To determine whether electrical synapse formation differed following each drug treatment, the proportion of PeA-PeA pairs which exhibited electrical coupling was calculated and analyzed using a Fishers Exact test. Comparison of the average coupling

coefficients between retinoid treatments and controls were conducted using unpaired t-tests. Due to drug availability, all-*trans* and 9-*cis* RA experiments were carried out many months apart, and thus were analyzed separately with their own control data, using unpaired t-tests.

Chapter 3: Results

All-*trans* Retinoic Acid Reduces Electrical Coupling Between PeA Neurons

The first goal of this study was to characterize the effects of RA on soma-soma electrical synapses formed between PeA motorneurons. There is preliminary evidence that RA rapidly (over 1 h) reduces electrical coupling between *in vitro* cultured PeA-PeA pairs (Rothwell et al., 2017). Yet, this previous report only provided an analysis of this effect using one isomer (atRA), one concentration (10 µM) and one culture duration (24 h). Thus, the first series of experiments aimed to expand the analysis of RA's acute uncoupling effect on PeA neurons.

Dual intracellular sharp electrode recordings were used to establish that co-cultured PeA-PeA pairs exhibited bidirectional electrical coupling (Fig. 5A). Subsequently, atRA (5 or 10 µM), 9-*cis*-RA (10 µM), or EtOH as the vehicle control (0.1% or 0.05%) was added to the bath. Measurements of bidirectional coupling coefficients were then made at 10-minute intervals over a 1 h recording period (unless otherwise stated). PeA-PeA pairs often exhibited rectification, wherein the ratio of the voltage change (i.e. coupling coefficient) produced by hyperpolarizing current injection into either cell within a PeA-PeA pair, was not equal in magnitude in both directions. Thus, the two cells within a PeA-PeA pair were classified as either "PeA-1" or "PeA-2" following the measurement of bidirectional coupling coefficients. The "pre-synaptic" cell within a PeA-PeA pair that exhibited the higher baseline coupling coefficient (before drug treatment) was defined as "PeA-1", conversely, the cell that exhibited the lower baseline coupling coefficient was defined as "PeA-2". In this manner, the labelling of cells within a PeA-PeA pair as PeA-1 or PeA-2, corresponds to a *post hoc* classification used in this report and does not represent the identity of any specific cell-type within the PeA cluster proper (Fig. 5B). This

classification was conducted to assess whether retinoid treatments differentially altered the two coupling coefficients within a cell pair, based on their initial strength of coupling. As such, separate mixed factor ANOVAs were used to analyze the strength of coupling in a PeA-PeA pair in the high and low coupling directions.

As the previous investigation from Rothwell et al. (2017) only characterized atRA's effect on electrical coupling in PeA-PeA pairs following a 24 h incubation, it was first determined whether RA's effect on electrical coupling was dependent on culture duration. Electrical synapse formation is a multistep process that requires the coordinated recruitment of not only the innexin/connexin proteins that constitute the channels themselves, but other adapter, scaffolding and modulatory proteins (Martin et al., 2020). Yet, when and how these proteins are recruited into a developing electrical synapse is not well understood. Thus, RA's modulatory capacity may be dependent on the presence of, and action on (either directly or indirectly), specific protein effectors which are integrated at specific temporal stages during synapse formation.

For this initial investigation, PeA-PeA pairs were cultured for either 24 h or 48 h prior to recording and 10 µM atRA application. A repeated-measures design (mixed factor ANOVA) was used to determine whether any atRA-induced change in coupling resulted from an interaction between culture duration (i.e. whether PeA-PeA cells were cultured for 24 h or 48 h; between-subjects factor) and time exposed to RA (i.e. T0 = at the time of atRA application and T60 = 1 h after atRA application; within-subjects factor).

Separate mixed factor ANOVAs revealed that the atRA-induced change in electrical coupling did not result from an interaction between culture duration and exposure time to RA in either the high ($F_{(1, 16)} = 0.018$, $P = 0.894$) or low ($F_{(1, 16)} = 0.540$, $P = 0.473$)

coupling directions. For the high coupling direction, exposure to RA ($F_{(1, 16)} = 35.93$, $P < 0.001$) but not culture duration ($F_{(1, 16)} = 0.103$, $P = 0.752$) had a significant effect on PeA coupling. *Post hoc* analysis indicated that application of atRA significantly reduced electrical coupling (from T0 to T60) between PeA-PeA pairs cultured for both 24 h (n = 8; $P = 0.002$) and 48 h (n = 10; $P < 0.001$; Fig 5C). Similarly, in the low coupling direction, only exposure to RA ($F_{(1, 16)} = 20.36$, $P < 0.001$) and not culture duration ($F_{(1, 16)} = 0.022$, $P = 0.884$) had a significant effect on PeA coupling. *Post hoc* analysis confirmed that atRA significantly reduced electrical coupling (from T0 to T60) for PeA-PeA pairs cultured for both 24 h (n = 8; $P = 0.006$) and 48 h (n = 10; $P = 0.024$; Fig 5D). Thus, the acute effect of atRA on electrical coupling between PeA-PeA cell pairs was independent of culture duration.

With the caveat that only synapses with a minimum of 15% coupling coefficient were used for these studies, the initial strength of coupling of these cell pairs did not vary as a function of culture duration in either the high or low coupling direction. However, as preliminary data suggested that more PeA-PeA pairs tended to form electrical synapses if cultured for 48 h rather than 24 h, a 48 h culture duration was used as the standard culture time for all subsequent studies.

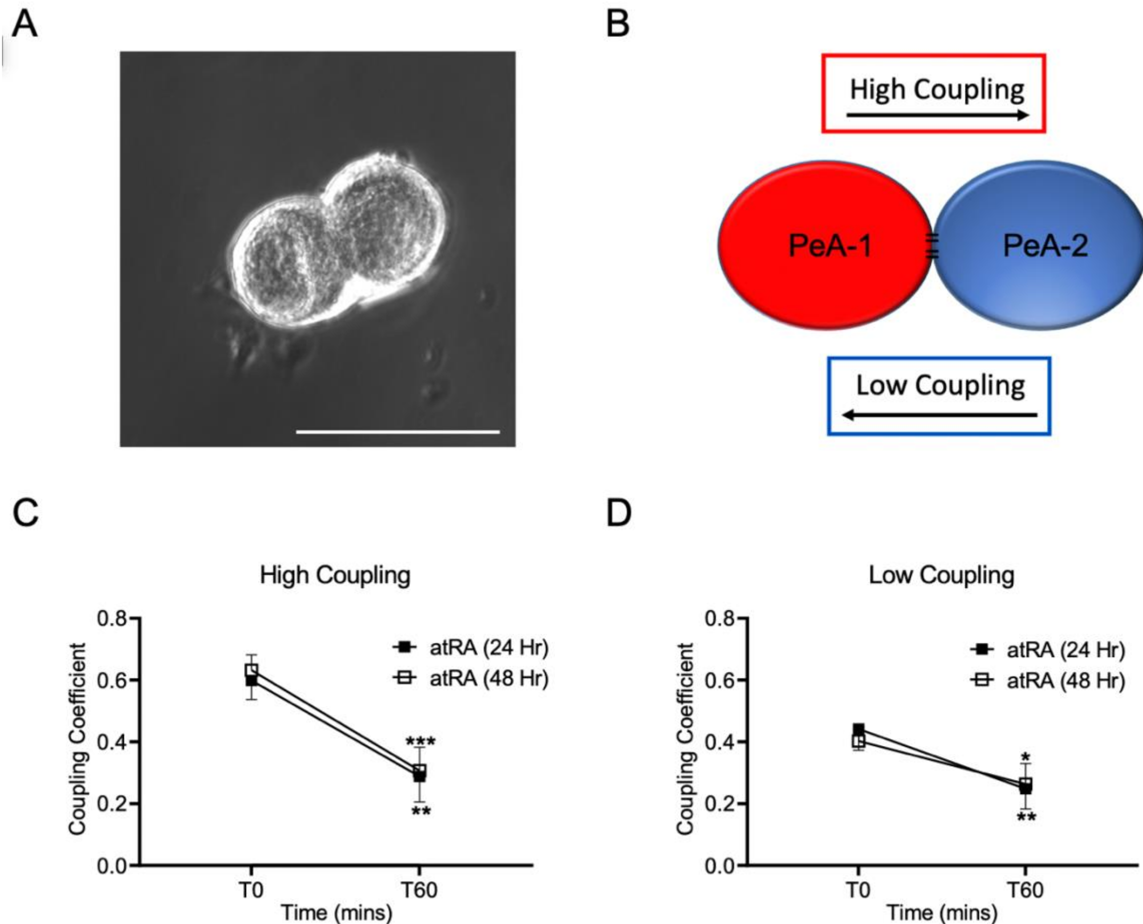

Figure 5. atRA's effect on electrical coupling is independent of culture duration. (**A**) Representative photo of *in vitro* cultured PeA neurons in a soma-soma configuration (Scale bar = 50 µm). (**B**) Schematic illustrating rectification of bidirectional electrical coupling between a PeA-PeA pair. In PeA-PeA cell pairs, the cell exhibiting a high coupling coefficient is defined as PeA-1 (red), while the cell exhibiting a low coupling coefficient is defined as PeA-2 (blue). (**C,D**) Acute application of atRA (10 µM) reduces the average coupling coefficient of PeA-PeA pairs cultured for 24 h and 48 h in high (**C**) and low (**D**) coupling directions. Measurements of coupling coefficient were taken at the time of drug application (T0) and 1 h later (T60). Significance indicates *post hoc* analysis (T60 vs. T0; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). All data are shown as mean and SEM.

<u>**Isomer-Selective Effect of Retinoic Acid on Electrical Coupling**</u>

I next investigated the acute effects of two isomers, atRA and 9-*cis* RA, on PeA coupling, as prior evidence suggests that different isomers of RA can exert divergent effects on retinoid signaling (Hellemans et al., 1999; Lovat et al., 1999; Redfern et al., 1995).

Along with atRA, 9-*cis*-RA has been localized to the *Lymnaea* CNS (Dmetrichuk et al., 2008), and both isomers can exert rapid, but divergent effects on the electrical properties of *Lymnaea* neurons (de Hoog et al., 2018; Vesprini & Spencer, 2014). Thus, it was the goal here to determine whether the two RA isomers produced similar or different effects on PeA coupling. To ensure that the uncoupling effect of RA between PeA-PeA pairs was not caused by the vehicle (EtOH), its effect on coupling was also examined. A repeated-measures design (mixed factor ANOVA) was employed to assess whether acute exposure (over 1 h) to 0.1% EtOH, 10 µM atRA, or 10 µM 9-*cis* RA (between-subjects factor denoted as "drug treatment") affected the average coupling coefficient measured at T0 (time of drug application) and T60 (1 h post drug application; within-subjects factor denoted as "time"). Similar to the previous analysis, bidirectional coupling coefficients were categorized into high and low coupling groups and subjected to separate analysis.

In the high coupling direction, a main effect of time was obviated by a significant interaction of time and drug treatment ($F_{(2,27)} = 7.686$, $P = 0.002$; Fig. 6A,B). PeA-PeA pairs exposed to the vehicle 0.1% EtOH (n = 10) exhibited no change in coupling from T0 (0.61 ± 0.05) to T60 (0.56 ± 0.04; $P = 0.722$; Fig. 6B,C). In contrast, bath application of 10 µM atRA (n = 10) led to a reduction in electrical coupling from T0 (0.63 ± 0.05) to T60 (0.30 ± 0.07; $P < 0.001$; Fig. 6B,D). Acute exposure to 10 µM 9-*cis* RA (n = 10) did not however alter electrical coupling from T0 (0.64 ± 0.06) to T60 (0.56 ± 0.06; $P = 0.447$; Fig. 6B,E).

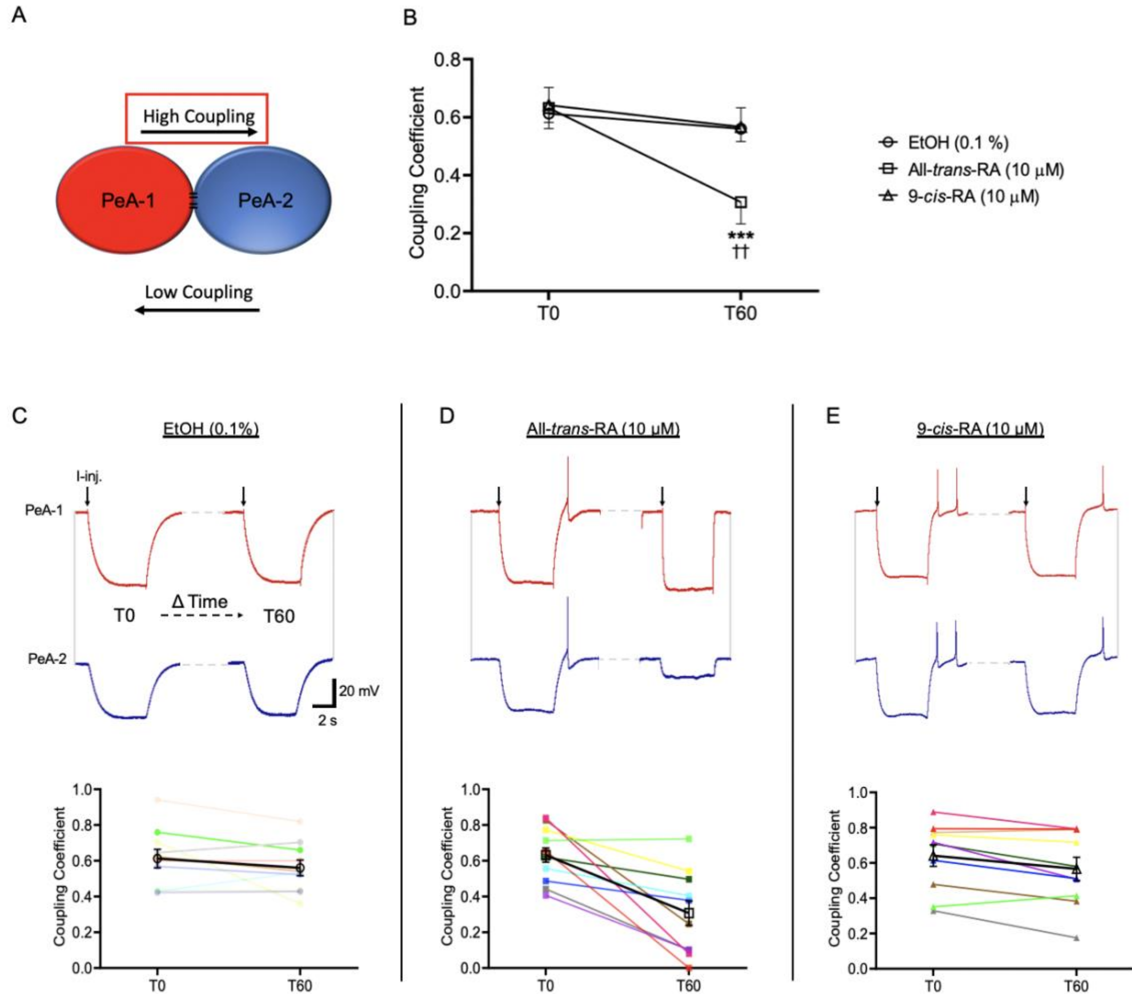

Figure 6. atRA but not 9-*cis* RA reduces electrical coupling between PeA neurons in the high coupling direction. Electrical coupling of PeA motorneurons was measured following acute exposure (1 h) to EtOH, atRA, or 9-*cis* RA. (**A**) Schematic of bidirectional coupling between PeA-PeA cell pair. Only data from the high coupling direction grouping is shown. (**B**) Summary graph of average coupling coefficients from PeA-PeA pairs at T0 and 1 h (T60) after drug treatment with either 0.1% EtOH, 10 µM atRA, or 10 µM 9-*cis*-RA. Only bath application of atRA significantly ($P < 0.05$) reduces electrical coupling from T0 to T60. (**C,D,E**; Top) Raw electrophysiological recordings of PeA-1 (red) and PeA-2 (blue); downward arrow indicates injection of hyperpolarizing current (I inj.) into PeA-1. (Bottom). Average change in coupling coefficient is shown in black, with individual cells (n = 10 for each condition) depicted by different colored lines. *Post hoc* significance level (T0 vs. T60, ***$P < 0.001$; T60 [EtOH] vs. T60 [atRA], ††$P < 0.01$). All data represent mean ± SEM.

Rectification at electrical synapses is thought to result from the presence of heterotypic gap junction channels (Marder, 2009), and so the rectification between PeA-PeA pairs is likely due to the expression of heterotypic channels. Hence, the RA-mediated reduction in electrical coupling might be dependent on RA's action on specific innexin isoforms which constitute gap junction channels between PeA neurons. A separate analysis of RA's effect on electrical coupling was thus performed in the low coupling direction.

I found that RA's effect on electrical coupling in the low coupling direction was similar to that of its effects in the high coupling direction. A mixed factor ANOVA, again, revealed that the main effect of time was obviated by a significant interaction of time and drug treatment ($F_{(2,27)} = 4.896$, $P = 0.015$) in the low coupling direction (Fig. 7A,B). No reduction in electrical coupling was observed in the PeA-PeA pairs exposed to 0.1% EtOH from T0 (0.44 ± 0.06) to T60 (0.47 ± 0.07; $P = 0.819$; Fig. 7B,C). However, a reduction in electrical coupling was observed in 10 µM atRA-incubated cells from T0 (0.40 ± 0.04) to T60 (0.26 ± 0.06; $P = 0.004$; Fig. 7B,D). Again, acute exposure to 10 µM 9-*cis*-RA failed to induce a change in electrical coupling from T0 (0.43 ± 0.06) to T60 (0.40 ± 0.06; $P = 0.924$; Fig. 7B,E).

In summary, these findings suggest that (1) the two RA isomers exhibit differential effects on electrical coupling (atRA but not 9-*cis* RA rapidly reduces coupling) in PeA neurons and (2) atRA's effect on electrical coupling is bidirectional, occurring in both high and low coupling directions.

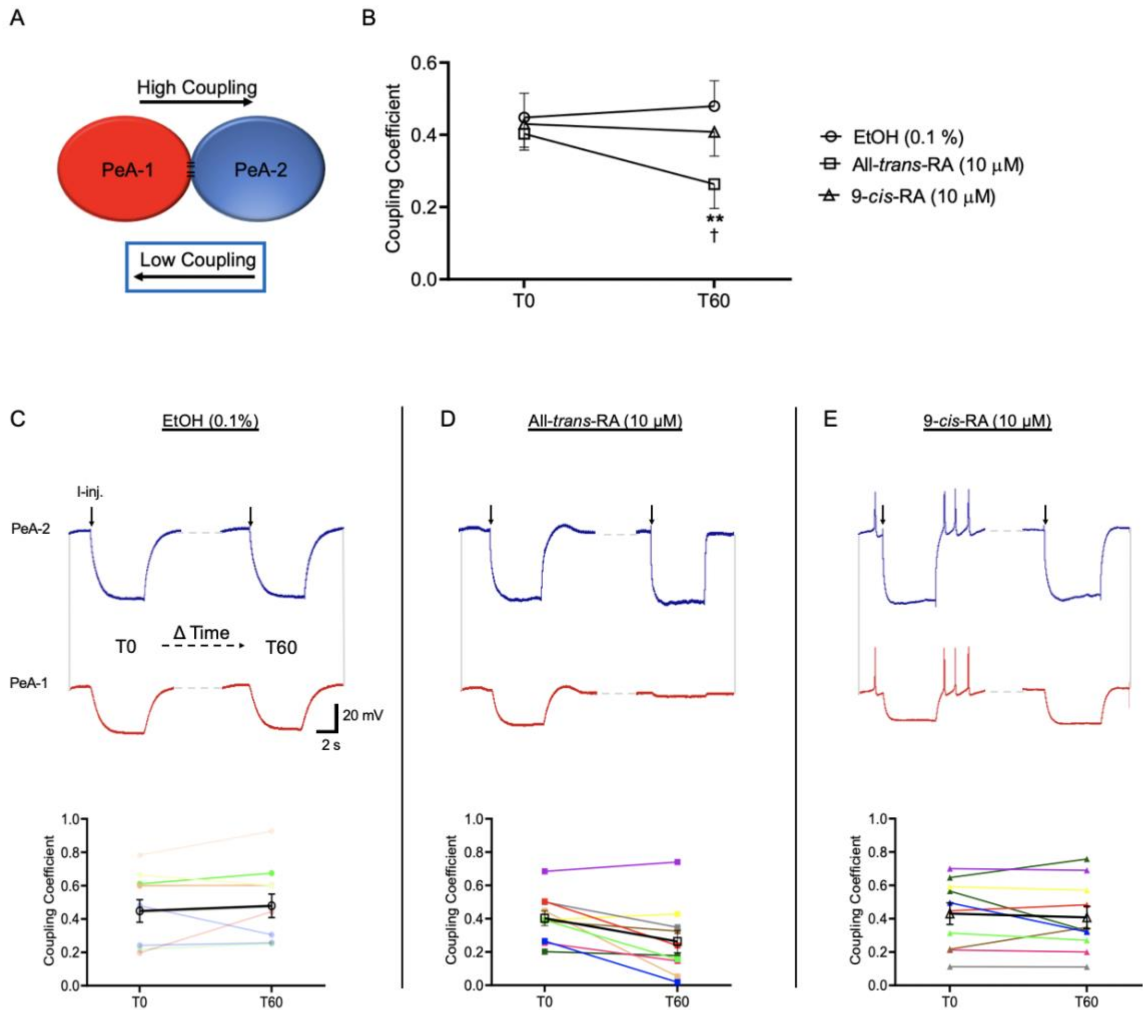

Figure 7. atRA, but not 9-cis RA reduces electrical coupling in the low coupling direction. Electrical coupling between PeA-PeA pairs after acute exposure (1 h) to either 0.1% EtOH, 10 µM atRA, or 10 µM 9-*cis*-RA. (**A**) Cartoon diagram of PeA-PeA pairs indicating that data shown represents coupling coefficients measured in the low coupling direction. (**B**) Summary graph of average coupling coefficients from PeA-PeA pairs across each condition both at the time of (T0) and 1 h (T60) after drug treatment. Only acute exposure to atRA significantly reduces electrical coupling from T0 to T60. (**C,D,E;** Top) Representative traces of electrical coupling depicting PeA-1 (red) and PeA-2 (blue); downward arrow indicates hyperpolarizing current (I inj.) injected into PeA-2. (Below) Average change in coupling coefficient (black lines) and individual cells (colored lines). *Post hoc* significance level (T0 vs. T60, $^{**}P < 0.01$; and T60 [EtOH] vs. T60 [atRA], $^{\dagger}P < 0.05$). All data represent mean ± SEM.

Concentration-Dependent Effects of all-*trans* RA on Electrical Coupling

Next, I sought to determine whether lower concentrations of atRA could also rapidly modulate electrical coupling between PeA-PeA pairs. Given that acute application of 9-*cis*-RA did not produce a significant reduction in electrical coupling at higher concentrations (10 µM) only lower concentrations (5 µM) of atRA were tested. Initial testing suggested that treatment with 5 µM atRA did not appear to alter coupling over a 1 h recording period (data not shown), and it was hypothesized that a lower concentration of atRA might take longer to affect electrical coupling. Therefore, the recording time was increased from 1 h to 2 h for this experiment.

A mixed factor ANOVA revealed a significant main effect of time ($F_{(2,32)} = 7.506$, $P = 0.002$) but not drug treatment ($F_{(1,16)} = 1.080$, $P = 0.314$) in the high coupling direction. *Post hoc* analysis revealed that 5 µM atRA (n = 9) reduced electrical coupling relative to baseline (T0; 0.62 ± 0.04) at both the 1 h (0.50 ± 0.03, $P = 0.024$) and 2 h timepoints (0.47 ± 0.05, $P = 0.004$; Fig. 8Ai,iii). In contrast, vehicle-treated (0.05% EtOH) PeA-PeA pairs (n = 9) exhibited no significant reduction in electrical coupling relative to baseline (T0: 0.50 ± 0.06) either at the 1 h (T60: 0.43 ± 0.07, $P = 0.288$) or 2 h timepoint (T120: 0.42 ± 0.07, $P = 0.157$; Fig. 8Aii,iii).

A mixed factor ANOVA also revealed a significant main effect of time ($F_{(2,32)} = 9.408$, $P < 0.001$), but not drug treatment ($F_{(1,16)} = 0.027$, $P = 0.869$) in the low coupling direction. Exposure to 5 µM atRA again reduced coupling in PeA-PeA pairs relative to baseline (T0: 0.50 ± 0.05) at both the 1 h (T60: 0.38 ± 0.05, $P = 0.007$) and 2 h timepoints (T120: 0.33 ± 0.05, $P < 0.001$; Fig. 8Bi,iii). In contrast, no difference in coupling was observed in the vehicle-treated (0.05% EtOH) PeA-PeA pairs relative to baseline (T0: 0.42

± 0.07) after either 1 h (T60: 0.39 ± 0.07, $P = 0.680$) or 2 h (T120: 0.36 ± 0.08, $P = 0.210$; Fig 8Bii,iii).

Taken together, these data suggest that RA's effect on electrical coupling is concentration-dependent, as the reduction in electrical coupling was most prominent at the higher concentration of atRA tested.

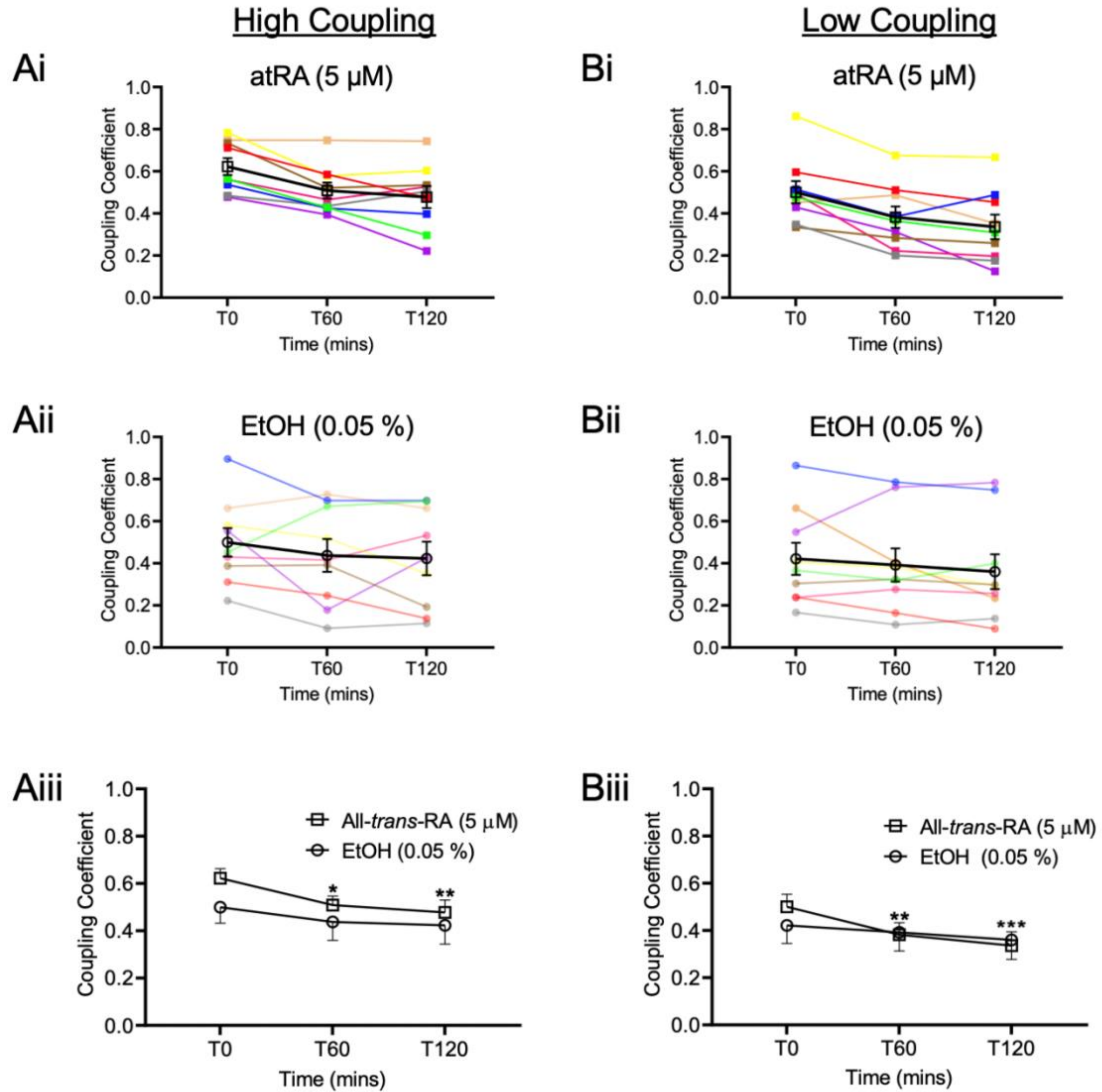

Figure 8. atRA's effect on electrical coupling also occurs at lower concentrations. Individual (colored lines) and average (black lines) coupling coefficients from PeA-PeA pairs. Coupling measurements at three timepoints at the time of (T0), 1 h (T60) and 2 h (T120) after addition of 5 μM atRA (**Ai, Bi**) or 0.05 % EtOH (**Aii, Bii**) in the high (left) and low (right) coupling directions. Summary graphs depicting a direct comparison of coupling for atRA and EtOH treatment conditions in the high **(Aiii)** and low (**Biii**) coupling directions. *Post hoc* significance level (T0 vs. T60 or T120; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). All data represent mean ± SEM.

All-*trans* Retinoic Acid's Uncoupling Effect is Independent of Protein Synthesis

It is estimated that the half-life of innexon/connexon hemichannel proteins within gap junction plaques is ~1-6 h (Curtin et al., 2002; Falk et al., 2014). The constant turnover of individual gap junction proteins within a gap junction plaque is also thought to be important for maintaining homeostasis and provides a mechanism to alter gap junction function. Insertion of new gap junction proteins (connexins/innexins) or gap junction-associated proteins (such as modulatory and scaffolding proteins), into a gap junction plaque could dynamically alter the strength of an electrical synapse. The rapid time course of RA's uncoupling effect suggests that it is controlled via a nongenomic mechanism, and prior evidence shows that some nongenomic effects of RA in *Lymnaea* neurons require protein synthesis (Farrar et al., 2009). Thus, it is important to determine whether RA's uncoupling effect is dependent on a signaling pathway involving (1) the synthesis of new proteins and/or (2) extant proteins.

To test whether the RA-mediated reduction in electrical coupling between PeA neurons involves protein synthesis, PeA-PeA pairs (n = 9) were pre-incubated in the translational inhibitor, anisomycin (50 µM), for 1 h and then exposed to 10 µM atRA for 1 h. To ensure that anisomycin itself did not induce uncoupling, these experiments involved a 2 h recording period where coupling coefficients were calculated at the time of application of anisomycin (-T60), 1 h after the addition of anisomycin, but at the time of addition of atRA (T0), and 1 h after the addition of atRA (T60).

A one-way repeated-measures ANOVA design was employed to compare the average coupling coefficient measured across the three timepoints. There was a difference in the coupling coefficients measured over the 2 h recording period in both the high ($F_{(2,16)}$

= 29.57, $P < 0.0001$) and low ($F_{(2,16)} = 16.80$, $P = 0.0001$) coupling directions. *Post hoc* analysis indicated that in both the high and low coupling directions, coupling was only reduced following the addition of 10 µM atRA. In the high coupling direction, the average coupling coefficient was not significantly changed ($P = 0.88$) from -T60 (0.56 ± 0.04) to T0 (0.54 ± 0.06) indicating that anisomycin alone had no effect on coupling, but was reduced ($P < 0.0001$) from T0 to T60 (0.32 ± 0.06; Fig. 9A) suggesting that atRA still reduced coupling in the presence of anisomycin. Similarly, in the low coupling direction the average coupling coefficient did not differ ($P = 0.30$) from -T60 (0.43 ± 0.05) to T0 (0.37 ± 0.05) in the presence of anisomycin alone, but again, was reduced ($P = 0.002$) from T0 to T60 (0.20 ± 0.05; Fig. 9B) in the presence of atRA.

Together these data suggest that anisomycin does not, by itself, reduce electrical coupling and that the RA-mediated reduction in electrical coupling is not dependent on protein synthesis. This implies that if the retinoid signaling pathway mediating a reduction in electrical coupling involves downstream protein effectors, it likely does so via interaction(s) with the existing pool of translated proteins.

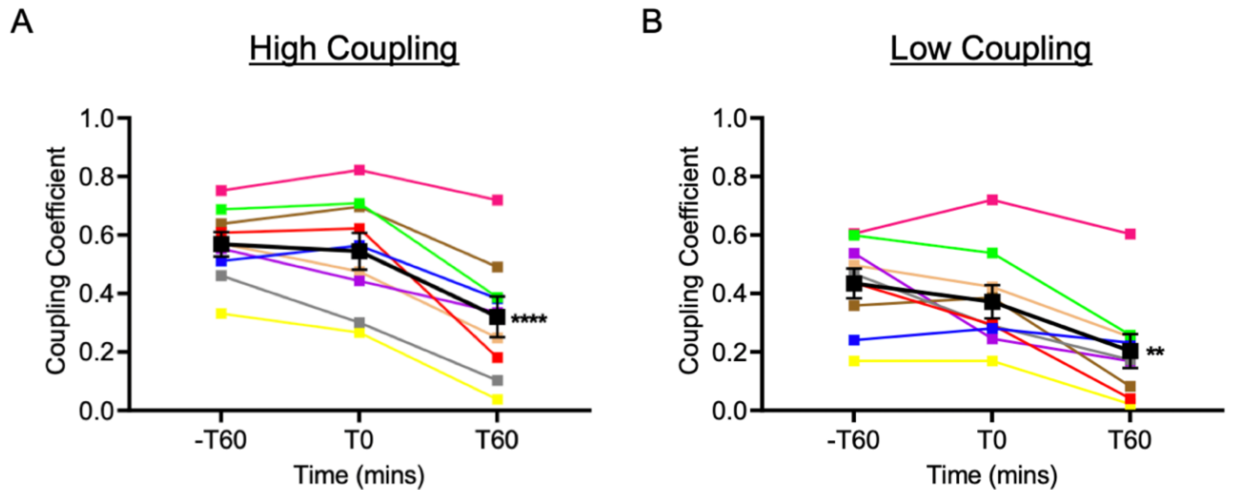

Figure 9. atRA-mediated reduction in electrical coupling involves a mechanism that is independent of protein synthesis. The data show electrical coupling between PeA-PeA pairs over a 2 h recording period. Average coupling coefficient of PeA-PeA pairs in the presence of 50 µM anisomycin alone (-T60 to T0) and co-incubation with 10 µM atRA (T0 to T60). A significant reduction in electrical coupling occurs only after the addition of atRA in both the high (**A**) and low (**B**) coupling directions. Changes in the coupling coefficients for individual cell pairs (colored lines) with combined averages (black lines) superimposed on the same graph. Error bars denote SEM. *Post hoc* significance level (T0 vs. T60; $**P < 0.01$, $****P < 0.0001$)

The Uncoupling Effect of All-*trans* RA is Independent of PKC and Retinoid Receptors

Given the results demonstrating that atRA's uncoupling effect is likely independent of protein synthesis, the next step was to examine candidate proteins that have either (1) shown to be involved in the modulation of electrical synapses and/or (2) shown to be downstream effectors of RA. If activation of certain target proteins were required for RA's uncoupling effect, then pre-incubation in antagonists of these target proteins 1 h prior to recording should prevent or reduce RA-mediated effects. To directly compare the changes in coupling produced by atRA in the presence or absence of various inhibitors, all subsequent data (for acute modulation experiments) are presented as the proportional (%) change in average coupling coefficient from T0 to T60 and compared to the effects of atRA alone. As previous data showed no differences in RA-mediated effects based on initial coupling strength, only coupling in the high coupling direction is shown in subsequent sections examining the acute effects of retinoids.

Two classes of proteins were targeted with antagonists: PKC and the retinoid receptors, RAR and RXR. PKC is a known modulator of electrical coupling in both vertebrates (Pogoda et al., 2016) and invertebrates (Beekharry et al., 2018), and has also been implicated in the nongenomic effects of RA, and contains a binding site for RA (Ochoa et al., 2003; Radominska-Pandya et al., 2000). Two separate membrane-permeable PKC inhibitors, H7 (10 µM) and Gö6976 (10 µM), were used to inhibit PKC (based on previous studies demonstrating the effective use of these compounds in *Lymnaea* neurons (Lacchini et al., 2006; Van Soest et al., 2000)). The second targets for these antagonist experiments were the retinoid receptors, *Lym*RAR and *Lym*RXR. RA can activate retinoid receptors which mediate nongenomic signaling outside the nucleus (N. Chen & Napoli,

2008; Piskunov et al., 2014), suggesting that the signaling pathway leading to the reduction in coupling might be mediated by activation of the receptors themselves. The RAR pan antagonist, LE540 (1 µM), and the RXR pan-antagonist, HX531 (1 µM), were chosen, based on their ability to block the effects of retinoids in *Lymnaea* (Carter et al., 2010, 2015).

A one-way ANOVA revealed that the proportional change in coupling (relative to baseline [T0]) produced by 10 µM atRA did not differ across treatment groups ($F_{(4,45)}$ = 2.041, P = 0.10). AtRA still induced a change in coupling in the presence of Gö6976 (-38.57 ± 10.66 %; n =10) and H7 (-36.94 ± 7.12 %; n = 10) that did not differ from atRA alone (-46.07 ± 9.50 %; n = 11; Fig 10A,B). Thus, acute exposure to atRA continued to significantly reduce coupling between PeA neurons in the presence of the PKC inhibitors. Additionally, the proportional change in coupling following bath application of atRA was not different from that produced by atRA in the presence of either the RAR pan-antagonist, LE540 (-68.34 ± 6.49 %; n = 9; Fig. 10 A,B), or the RXR pan-antagonist, HX531 (-35.79 ± 10.45 %; n = 10; Fig 10A,B). These observations suggest that atRA's acute effect on electrical coupling in PeA-PeA pairs might involve a mechanism that is independent of the retinoid receptors.

At 10 µM, the retinoid receptor antagonists are toxic and this was the primary rationale for the application of a lower concentration of (1 µM). Thus, as equimolar concentrations of atRA and retinoid receptor antagonists were not employed in this analysis, it could be argued that insufficient receptor blockade obfuscated any potential nongenomic role of the receptors. Nonetheless, these antagonist experiments suggest that signaling via either of the retinoid receptors or PKC is not likely involved in the RA-mediated reduction in coupling between PeA neurons.

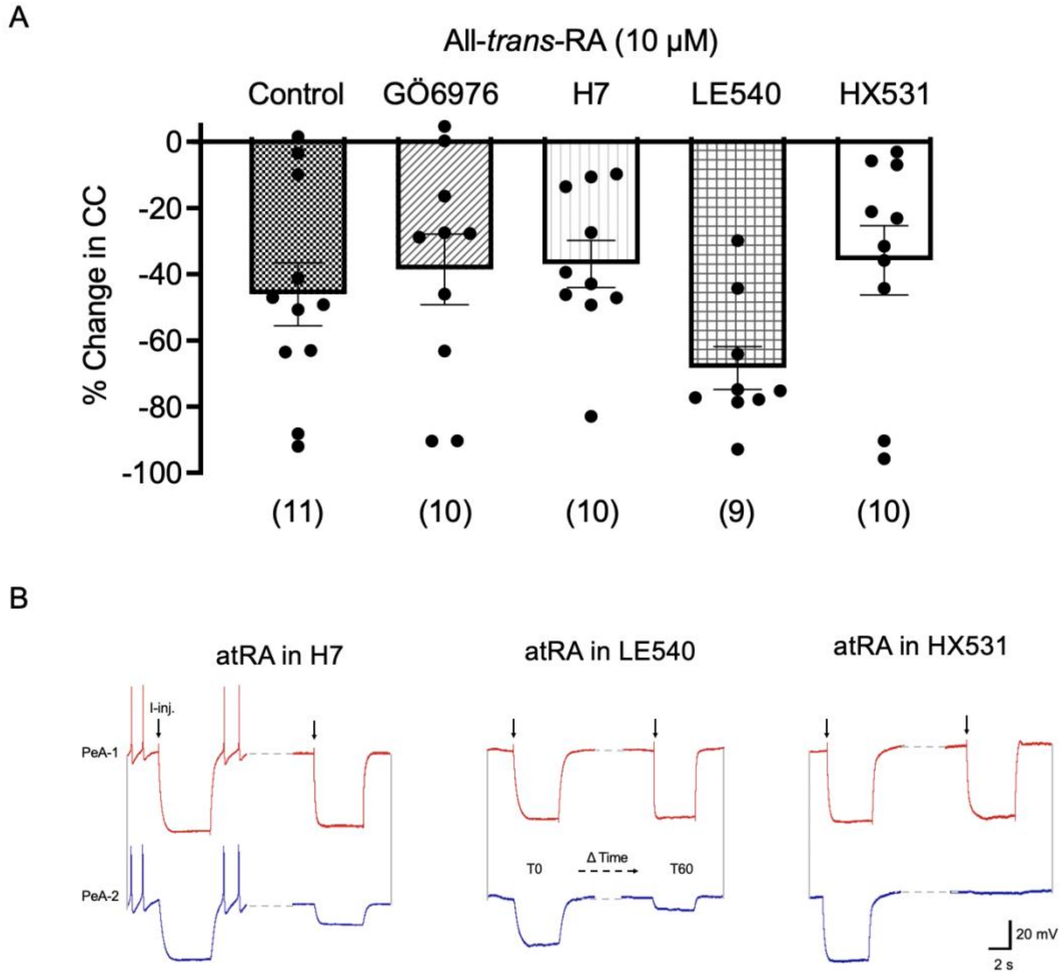

Figure 10. atRA-mediated uncoupling is independent of PKC or retinoid receptors. Measurements of electrical coupling between PeA-PeA pairs pre-incubated in antagonists 1 h prior to addition of 10 µM atRA. The data show a proportional (%) change in the average coupling coefficient from T0 to T60 for each treatment condition. (**A**) A one-way ANOVA found no significant difference ($P = 0.10$) in the proportional change in average coupling coefficient across treatment groups (left to right): 10 µM atRA alone (control: in the presence of DMSO), or 10 µM atRA in the presence of PKC inhibitors Gö6976 (10 µM) and H7 (10 µM), the RXR antagonist HX531 (1 µM) or the RAR antagonist LE540 (1 µM). (**B**) Raw electrophysiological traces showing electrical coupling from 10 µM atRA-treated PeA-PeA pairs in the presence of H7, LE540 and HX531 (left to right) indicating a failure to block the effects of atRA over 1 h. Error bars show SEM. Numbers in brackets indicate N values.

The Synthetic Retinoid EC23 Mimics all-*trans* RA-Induced Uncoupling

To address the possibility that the concentration of the antagonists used in the previous experiments were insufficient to counteract the effects of 10 µM atRA, it was next determined whether pharmacological activation of the retinoid receptors could mimic the effects of atRA. Cells were exposed to various RXR or RAR agonists (previously shown to mimic the physiological effects of RA (Carter et al., 2010; Johnson et al., 2019; Nasser, 2017). The proportional (%) change in the coupling coefficients over 1 h, following application of the retinoid receptor agonists were compared to those produced by application of the vehicle 0.1% DMSO (n = 10) and 10 µM atRA alone (n = 11).

A one-way ANOVA found that the proportional change in coupling differed among drug treatments ($F_{(5,55)} = 5.840$, $P = 0.0002$). *Post hoc* analysis revealed a significant difference in the proportional change in coupling for PeA-PeA pairs incubated in 10 µM atRA (-46.07 ± 9.5 %, $P = 0.003$) compared with 0.1% DMSO (-3.35 ± 3.53 %), indicating that application of the vehicle did not significantly alter coupling over 1 h (Fig. 11A,B). The addition of two RXR agonists, PA024 (1 µM) and SR11237 (10 µM), had no effect on electrical coupling, as the coupling coefficient was not significantly reduced after application of either RXR agonist. The change in coupling produced by atRA alone was greater than that produced by the RXR agonists, PA024 (-13.56 ± 4.62 %, $P = 0.04$) or SR11237 (-12.43 ± 6.68 %, $P = 0.03$; Fig. 11A,B), which did not differ from that produced by the vehicle ($P > 0.05$) alone.

The RAR pan-agonist EC23 (1 µM) mimicked the uncoupling effect of atRA (10 µM) on PeA cell pairs. The proportional change in electrical coupling induced by EC23 (-46.42 ± 10.23 %) was not different from that produced by atRA ($P > 0.99$) but was

significantly greater than that produced by DMSO alone ($P = 0.004$). These data indicate that EC23 reduced electrical coupling between PeA neurons (Fig. 11A,B). In contrast to the previous results with the RAR antagonist, these results suggest a putative role for the *Lym*RAR receptor in mediating the RA-induced reduction of coupling between PeA cell pairs. To address this inconsistency, PeA-PeA pairs were pre-incubated (for 1 h) in an RXR/RAR antagonist cocktail containing both retinoid receptor antagonists, HX531 (1 μM) and LE540 (1 μM), and their coupling coefficients were then measured over 1 h following bath application of EC23 (1 μM). Both retinoid receptor antagonists were utilized in this experiment, because although EC23 is thought to be a selective RAR agonist in vertebrates there is evidence that it also binds to the *Lym*RXR (de Hoog et al., 2022). Yet, in the presence of this retinoid receptor antagonist cocktail, EC23 still induced a reduction in electrical coupling between PeA neurons. The proportional change produced by EC23 in cells pre-incubated in the HX531/LE540 antagonist cocktail (-37.64 ± 9.59 %) did not differ from that produced by EC23 alone ($P = 0.96$) or atRA ($P = 0.97$), but was different from that produced by DMSO ($P = 0.03$; Fig. 11A).

In summary, the RAR agonist, EC23, was capable of mimicking the uncoupling effect of atRA, though the retinoid receptor antagonists were ineffective at preventing the effects of either atRA or EC23.

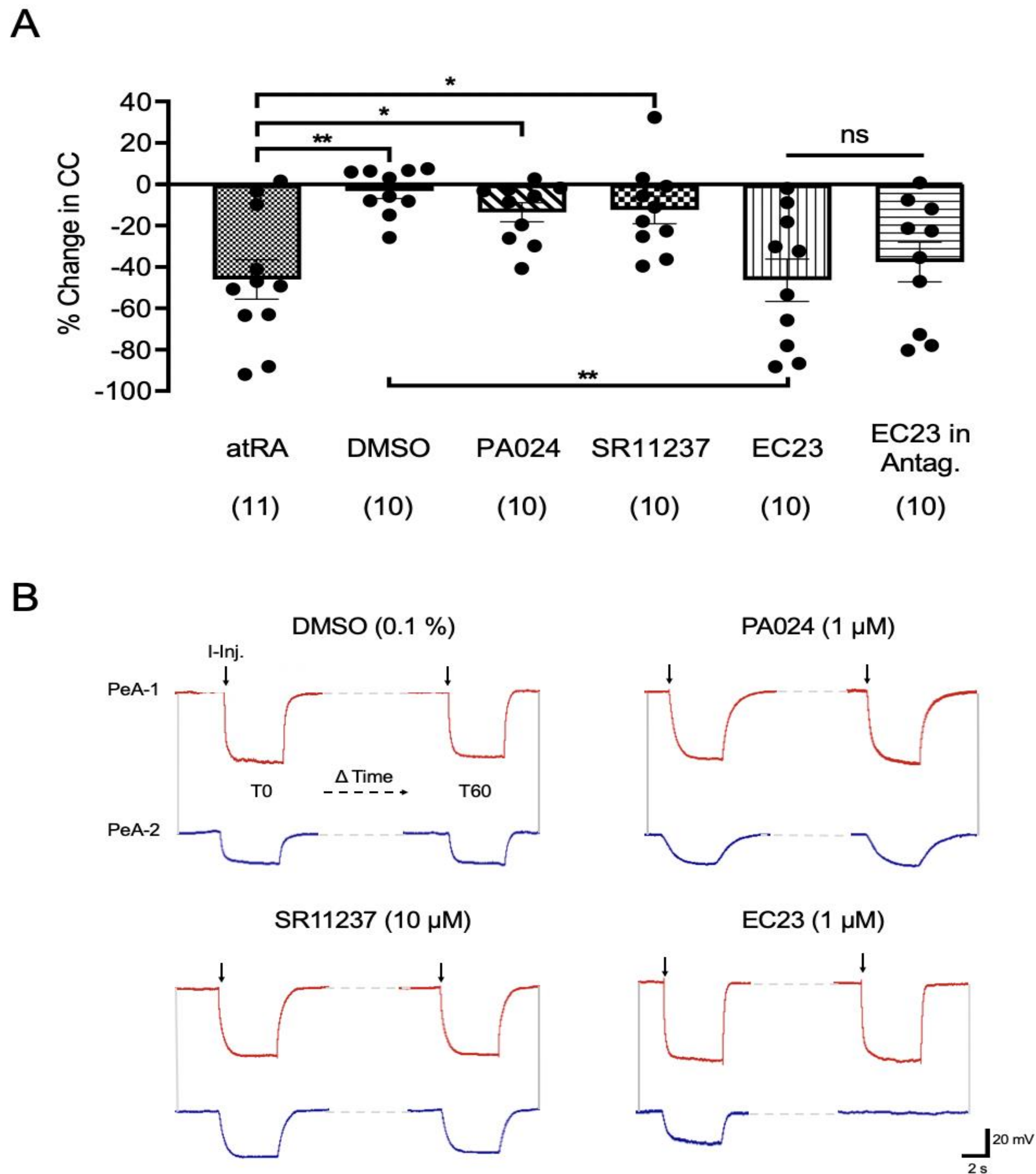

Figure 11. atRA-induced uncoupling is mimicked by an RAR pan-agonist. (**A**) Data show the proportional (%) change in average coupling coefficient between PeA-PeA pairs following acute exposure to atRA (10 µM), vehicle control (DMSO 0.1%), RAR agonist (1 µM EC23) and RXR agonists (1 µM PA024 and 10 µM SR11237). The RAR agonist, EC23, but not the RXR agonists, PA024 and SR11237, mimic the uncoupling effect of atRA. A retinoid receptor antagonist cocktail, containing 1 µM HX531 and 1 µM LE540, failed to block the uncoupling effect of 1 µM EC23. Error bars indicate SEM. Significance level of Sidak *post hoc* comparisons (*$P < 0.05$; ** $P < 0.01$). (**B**) Raw electrophysiological traces of PeA-PeA electrical coupling following acute exposure (T0 to T60) to vehicle (DMSO), RXR agonists (PA024 and SR11237) or RAR agonist (EC23). Downward arrows represent injection of hyperpolarizing current (I-inj) into PeA-1.

Retinoids Inhibit the Formation of Electrical Synapses

Having shown the acute effects of atRA on electrical synapses it was next determined whether retinoids could affect the formation of electrical synapses. AtRA and 9-*cis* RA were shown to affect the formation of gap junctions in nonneuronal tissues such as human prostate cancer cells (Kelsey et al., 2012), though there are no reports demonstrating this capacity in neurons (either in invertebrates or vertebrates). Immediately after cell plating, retinoids were applied. Specifically, PeA-PeA pairs were incubated in atRA (10 μM or 5 μM), 9-*cis* RA (10 μM) or vehicle (EtOH: 0.1% or 0.05%). The proportion of cells that formed electrical synapses was assessed after 48 h.

AtRA, in a dose-dependent manner, reduced the number of cells which formed electrical synapses (Fig 12A). Although fewer cell pairs exhibited electrical coupling following incubation in 5 μM atRA (82.7 %; 24 out of 29) relative to those incubated in the vehicle 0.05% EtOH (93.1 %; 27 out of 29) this difference was not significant (P = 0.42; Fig. 12Bi). In contrast, following 10 μM atRA incubation, significantly fewer (P = 0.008) PeA-PeA pairs (68%; 17 out of 25) exhibited electrical coupling compared to PeA-PeA pairs incubated in 0.1% EtOH (96.4%; 27 out of 28; Fig. 12Bii).

Despite having no acute effect on coupling, 9-*cis* RA affected the formation of electrical synapses. After a 48 h incubation in 9-*cis* RA, the proportion of PeA-PeA pairs that formed electrical synapses was reduced, compared to vehicle controls ($P < 0.05$). After long-term exposure to 10 μM 9-*cis* RA, there was a reduction in the number of PeA-PeA pairs that formed electrical synapses (76.4 %; 26 out of 34), compared to pairs incubated in the vehicle (0.1% EtOH; 96.4 %; 27 out of 28) (Fig. 12Biii).

RA is known to affect the electrical properties of *Lymnaea* neurons, so to rule out the possibility that cell pairs incubated for 48 h might be electrically compromised their resting membrane potentials (RMPs) were also examined. Separate comparison of the RMPs from cell pairs incubated in either RA or their respective vehicle controls, revealed no significant differences across conditions. The average RMP from cells incubated in 5 µM atRA (n = 58; -57.04 ± 1.56 mV) was not different from those incubated in 0.05% EtOH (n = 54; -54.93 ± 1.39 mV; *P* = 0.31; Fig 12Ci). No significant differences between the average RMP of cells incubated in the vehicle 0.1% EtOH (n = 50; -55.87 ± 1.87 mV) or 10 µM atRA (n = 50; -58.23 ± 2.07 mV; *P* = 0.89) were observed (Fig. 12Cii). Finally, the average RMP of cells incubated in 10 µM 9-*cis* RA (n = 68; -51.21 ± 1.49 mV) was also not different from the cells incubated in 0.1% EtOH (n = 58; -50.51 ± 1.62 mV; *P* = 0.74; Fig. 12Ciii). Together, these data suggest that the reduced electrical synaptogenesis following long-term retinoid exposure was not attributable to the electrical integrity of the cultured cells.

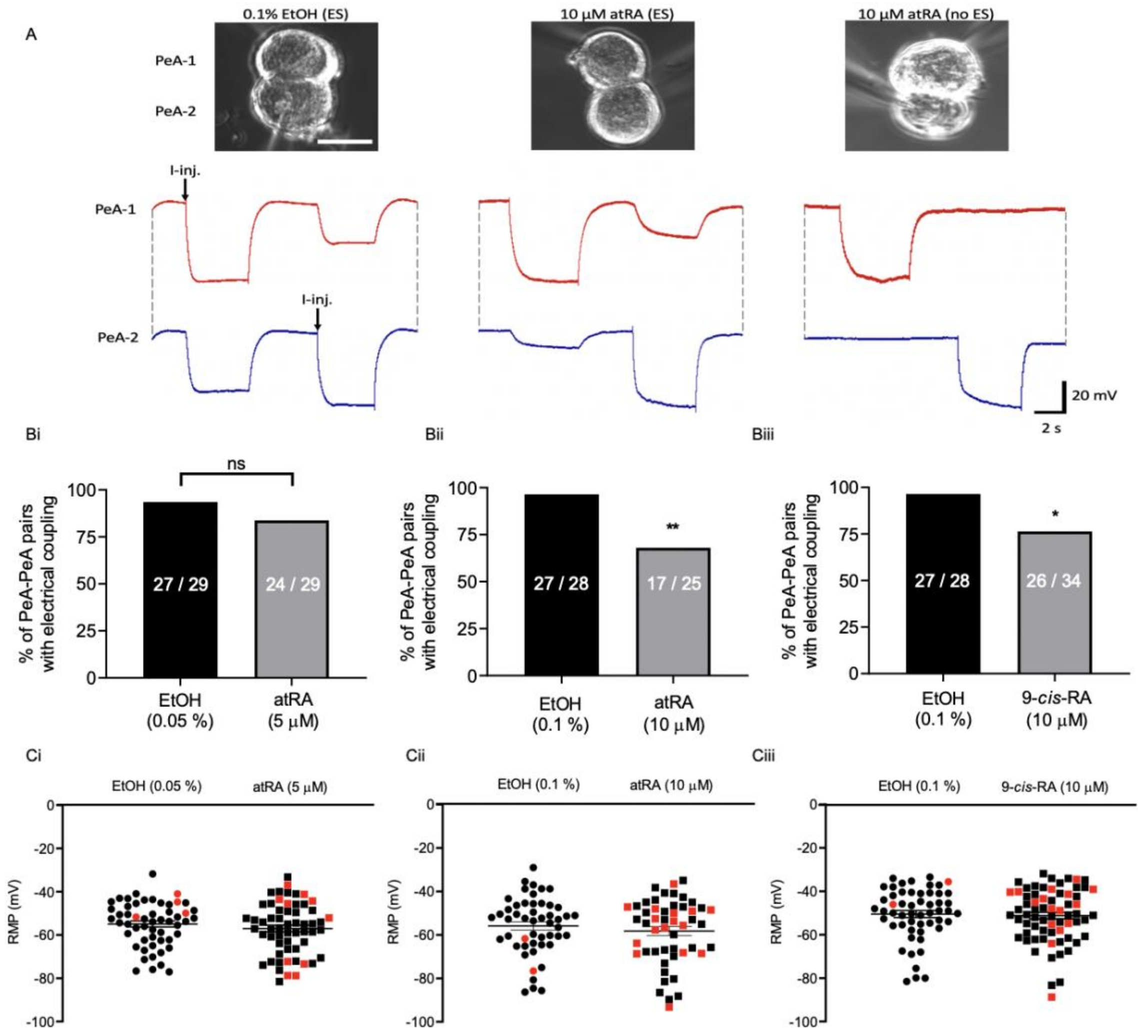

Figure 12. Long-term (48 h) exposure to atRA inhibits PeA-PeA electrical synapse formation. (**A**) Representative images and electrophysiological traces of PeA-PeA pairs after long-term exposure to 0.1% EtOH and 10 µM atRA. Representative examples show presence of electrical synapses (ES) formed following incubation in EtOH (left) and atRA (center), as well as the absence of an electrical synapse following incubation in atRA (right). (**B**) The proportion of PeA-PeA pairs in which electrical synapses formed in the presence of retinoids (either 5 µM atRA, 10 µM atRA or 10 µM 9-cis RA) or their complementary concentration of vehicle (0.05% or 0.1% EtOH). Long-term exposure to 10 µM atRA ($P < 0.01$) and 10 µM 9-*cis* RA ($P < 0.05$) significantly reduces the proportion of cells which form electrical synapses. (**C**) Long-term retinoid exposure does not affect the baseline RMP of PeA neurons (mean ± SEM). The data include pooled RMPs from PeA-PeA pairs both with (black) and without (red) electrical synapses. *$P < 0.05$, ** $P < 0.01$.

Long-Term Retinoid Exposure Reduces Synaptic Strength of Electrical Synapses

As the type of innexins/connexins that constitute electrical synapses influence junctional conductance, factors which control the expression of different innexins/connexins can affect coupling strength (Oyamada et al., 2005). Thus, if RA signaling alters the expression of innexin(s) proteins constituting electrical synapses, changes in the coupling strength of PeA-PeA pairs might reflect RA's action on certain innexin isoforms.

The influence of long-term retinoid exposure on the relative strength of synapses formed between PeA-PeA pairs was next assessed. Long-term exposure (48 h) to retinoids affected the average synaptic strength of electrically coupled PeA-PeA pairs. Akin to the last series of experiments examining the acute effects of retinoids, bidirectional coupling coefficients from each PeA-PeA pair were classified into high or low coupling groupings based on their initial strength. Despite 5 µM atRA having no significant effect on the proportion of cells exhibiting electrical coupling it did, however, reduce baseline synaptic strength in those cells which formed electrical synapses. The average coupling coefficient measured in the high coupling direction for cells incubated in 5 µM atRA (n = 24; 0.40 ± 0.04) was reduced ($P = 0.02$) relative to cells incubated in the vehicle, 0.05% EtOH (n = 27; 0.56 ± 0.05; Fig. 13). The average coupling coefficient of cell pairs incubated in the higher concentration of 10 µM atRA (n =17; 0.34 ± 0.04) was also reduced ($P = 0.006$) compared to PeA-PeA pairs incubated in 0.1 % EtOH (n = 27; 0.55 ± 0.05) in the high coupling direction (Fig. 13). Although 9-*cis*-RA had no acute effect on electrical coupling between PeA-PeA pairs, long-term exposure to this isomer did affect baseline synaptic strength in the high coupling direction. The average coupling coefficient of PeA-PeA pairs

was reduced ($P = 0.004$) following long-term incubation in 10 µM 9-*cis*-RA (n = 26; 0.46 ± 0.04) relative to pairs in 0.1% EtOH (n = 27; 0.66 ± 0.04; Fig. 13).

Taken together, these results suggest that long-term exposure to RA affects the coupling strength of cultured PeA neurons in a dose-dependent and isomer-independent manner.

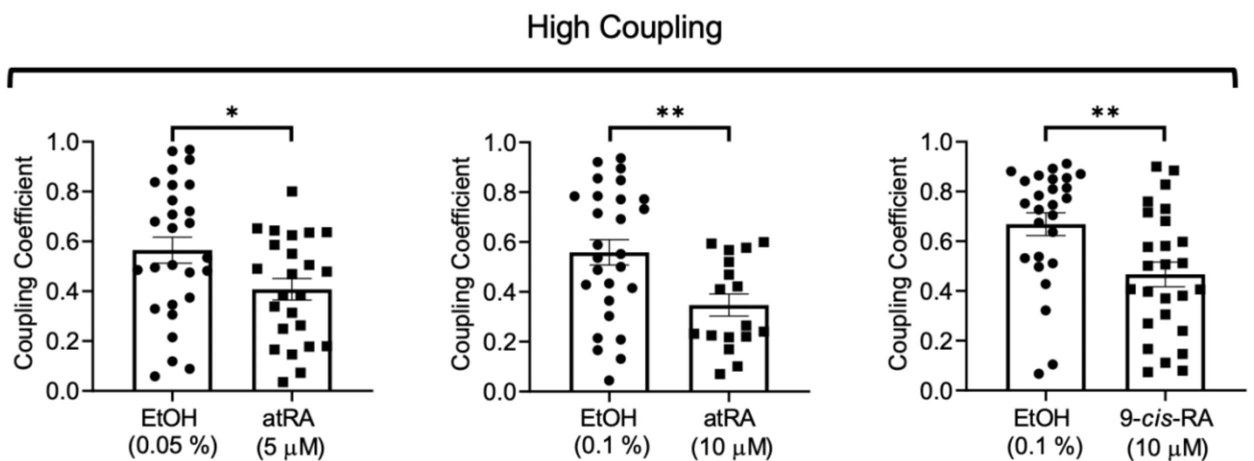

Figure 13. Long-term exposure to RA reduces synaptic strength of PeA-PeA pairs in the high-coupling direction. Incubation (48 h) in retinoids reduces baseline synaptic strength relative to vehicle (EtOH) controls. 5 µM (**A**) and 10 µM (**B**) concentrations of atRA, as well as 10 µM 9-cis RA (**C**) significantly ($P < 0.05$) reduces the coupling coefficient of PeA-PeA pairs in the high coupling direction; *$P = 0.05$, **$P = 0.01$. All data represent mean and SEM.

Likewise, in the low coupling direction, the average coupling coefficient of PeA-PeA pairs incubated in 5 μM atRA (n = 24; 0.30 ± 0.03) was significantly reduced (*P* = 0.04) relative to pairs incubated in 0.05% EtOH (n = 27; 0.43 ± 0.05; Fig. 14A). Again, the average coupling coefficients of PeA-PeA pairs incubated in the higher concentration of 10 μM atRA (n = 17; 0.22 ± 0.03) were also reduced compared to pairs incubated in 0.1 % EtOH (n = 27; 0.41 ± 0.05; Fig. 14B) (*P* = 0.008). Finally, the average coupling coefficient for PeA-PeA pairs incubated in 10 μM 9-*cis*-RA (n =26; 0.33 ± 0.04) was reduced relative to vehicle 0.1% EtOH-incubated pairs (n = 27; 0.50 ± 0.04; Fig. 14C) (*P* = 0.01).

These data indicate that, regardless of initial classification (high vs. low coupling), the coupling coefficients were reduced for all retinoid-incubation conditions (relative to their respective controls).

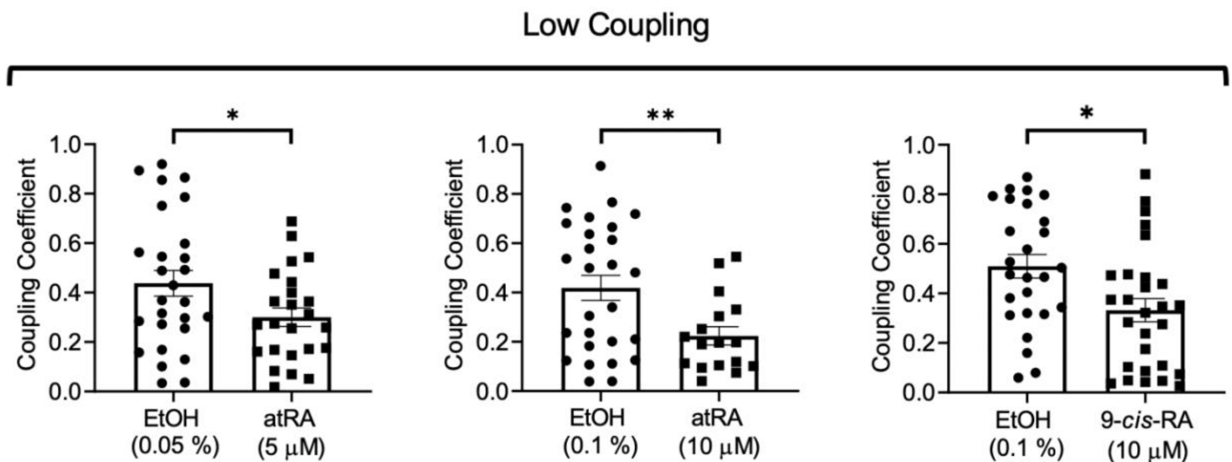

Figure 14. Long-term exposure to RA reduces synaptic strength of PeA-PeA pairs in the low coupling direction. Separate analysis in the low coupling direction shows that 48 h incubation in retinoids from the time of culture, reduces PeA coupling. Incubation in 5 μM (**A**) and 10 μM atRA (**B**) or 10 μM 9-cis RA (**C**) significantly reduce the average coupling coefficient compared to their respective vehicle controls (0.05 and 0.1% EtOH). Data represent mean and SEM. *$P < 0.05$ and **$P < 0.01$.

Long-term Retinoid Exposure Induces Capping Effect on PeA Coupling

Finally, I performed a pooled analysis of bidirectional coupling coefficients measured from PeA-PeA pairs across each of the six conditions. As expected, unpaired t-tests found that the strength of coupling was significantly reduced ($P < 0.05$) across each retinoid condition, relative to their respective control (Fig. 15). However, further examination of these pooled datasets also highlighted an apparent "capping" effect. In EtOH-incubated cell pairs ~ 1/3 of the measured coupling coefficients were > 0.60 CC (0.05% EtOH: 21 out of 56; 0.1% EtOH [atRA control]: 21 out of 54; 0.1% EtOH [9-*cis* RA control]: 30 out of 52). In contrast, the proportion of atRA-incubated cell pairs exhibiting coupling coefficients > 0.60 was significantly reduced in 5 µM atRA (8 out of 48; $P = 0.02$), 10 µM atRA (0 out of 34; $P < 0.0001$) and 10 µM 9-cis-RA (13 out of 52; $P = 0.001$).

In summary, these data suggest that in PeA-PeA pairs that formed an electrical synapse after long-term retinoid exposure, the proportion of cells that exhibited coupling coefficients greater than 0.60 was significantly reduced.

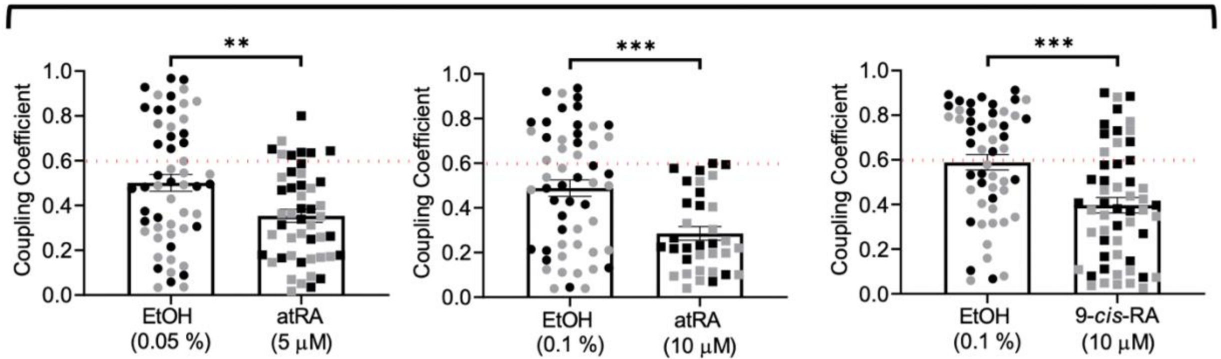

Figure 15. Long-term exposure (48 h) to RA reduces synaptic strength of PeA-PeA pairs (pooled directions). Bi-directional coupling coefficients from each PeA-PeA pair across retinoid treatment conditions. Pooled high (black) and low (grey) baseline coupling coefficients. Separate unpaired t-tests found that the average coupling coefficient across conditions was significantly reduced following exposure to 5 µM (**$P < 0.01$), 10 µM atRA (***$P < 0.001$), and 10 µM 9-*cis* RA (***$P < 0.01$). Dashed red line represents coupling coefficient threshold (0.60) used to determine the proportion of cells exhibiting an average coupling coefficient > 0.60; this was significantly reduced in all retinoid-incubated PeA-PeA pairs (relative to EtOH-treated cells). Data represent mean and SEM.

Chapter 4: Discussion

The concept that behavioral changes indicative of learning and memory are the result of plasticity within the neural circuits driving those behaviors, is a cornerstone of modern neurobiology. In contrast to chemical synapses, the contributions of electrical synapses in shaping cognitive processes (such as learning and memory), have largely been ignored, despite evidence of their involvement. In mice, intraperitoneal injection of gap junctional blockers prior to classical fear conditioning disrupts context-dependent fear learning, memory and extinction (Bissiere et al., 2011). Deficits in spatial learning in the Morris Water Maze were observed in rats exposed to gap junctional blockers (Hosseinzadeh et al., 2005), and deletion of Cx36, the most highly expressed neuronal connexin in rats, also impairs spatial learning and memory (Frisch et al., 2005). In invertebrates, knockdown of innexins, *Inx5* and *Inx7*, which participate in the formation of heterotypic gap junction channels in the mushroom body of the *Drosophila* brain, impairs memory formation (Wu et al., 2011). These studies provide evidence that any reductions in electrical synapses can lead to deficits in learning and memory. Moreover, electrical synapses exhibit a capacity for plasticity induced by network activity or post-translational modifications. For instance, high-frequency stimulation can potentiate electrical synapses in fish Mauther neurons (Haas et al., 2016; Pereda & Faber, 1996; Yang et al., 1990) and phosphorylation of gap junction channels or gap junction-associated proteins can modulate channel conductance (Moreno & Lau, 2007). Akin to chemical transmission, such changes in electrical transmission likely dynamically shape interneuronal transmission and network activity underlying learning and memory. Thus, although there is a link between electrical synapses and learning and memory, exactly how electrical synapses contribute to these cognitive processes has yet to be resolved. In part, this is likely due to our poor

understanding of electrical synapses and how they are regulated. Broadly speaking, electrical synapses are thought to be under the influence of two main forms of regulation: *the activity of the circuit they are localized to and the action of neuromodulators*. Thus, basic research into the intrinsic and extrinsic factors which influence plasticity at electrical synapses is a necessary step to bring about a more comprehensive understanding of not only their role in basic cellular processes but also higher-order cognitive processes.

As electrical synapses are found within all metazoan nervous systems, accounting for ~20 % of all synapses in mature neural circuits (Connors & Long, 2004; Lasseigne et al., 2021; Martin et al., 2020), the findings of this report contribute to the basic understanding of these ubiquitous biological structures. Herein, I provide novel evidence for the role of retinoids in the formation *and* modulation of invertebrate electrical synapses. Specifically, I demonstrate that long-term retinoid exposure inhibits electrical synaptogenesis (and results in subsequent reduced synaptic strength), and that acute exposure to retinoids rapidly reduces electrical coupling between the central neurons of the pond snail, *Lymnaea stagnalis.*

Acute Effects of Retinoids on Electrical Synapses

Following acute exposure (1 h) to atRA, electrical coupling between PeA neurons is significantly reduced. Similarly, in Bass retinal horizontal cells, atRA also rapidly (< 30 min) induces an uncoupling effect (Zhang & McMahon, 2000). There is also some indirect evidence that atRA inhibits electrical coupling in the rat CNS. Specifically, gap junction communication is linked to the spread of seizures, as gap junction blockers (carboxolene) prevent seizure activity, while gap junction openers (trimethylamine) potentiate it (Gajda et al., 2003, 2006; Jahromi et al., 2002). Injection of atRA into the basolateral amygdala of

rats rapidly (within 15 mins) mimics the anti-seizure effect of carboxelene and prevents the proconvulsant effect of trimethylamine (Sayyah et al., 2007). The authors propose that atRA's anti-seizure effect is possibly related to inhibiting electrical coupling within a large network of neurons in the basolateral amygdala. The rapid nature of these effects suggests that they are likely mediated by a nongenomic mechanism.

RA is traditionally thought to drive its effects via activation of the nuclear retinoid receptors (RARs/RXRs), which control the transcription of a number of genes (Mey & McCaffery, 2004; Tanoury et al., 2013). The importance of ancillary, nongenomic mechanisms cannot be understated, however. There are examples demonstrating that RA can directly activate other enzymes (Khatib et al., 2019; Ochoa et al., 2003; Radominska-Pandya et al., 2000), or that it can bind to and activate non-nuclear retinoid receptors, which direct signaling outside the nucleus (Poon & Chen, 2008; Thapliyal et al., 2022). These findings extend the "reach" of the RA signaling pathway to outside of the nucleus. Genomic signaling proper is restricted to the nucleus and requires time (hours) for gene transcription to occur. In contrast, nongenomic RA signaling can occur throughout the cell, wherever effectors are localized, and is generally faster (can occur in the order of minutes). This ability (of RA) to elicit both genomic and nongenomic mechanisms, undoubtably contributes to the wide variety of cellular processes regulated by retinoids. One interesting example of nongenomic retinoid signaling is the role of non-nuclear RARα in the synaptic dendrites of rat hippocampal neurons, where it can function as a RNA-binding protein, repressing translation of glutamate receptor (GluR1) mRNA (Poon & Chen, 2008). Once bound by RA, RARα disassociates from GluR1 mRNA allowing for translation of this

receptor. This example illustrates that in addition to being able to control transcriptional activity, retinoid signaling can also regulate translational activity.

In this study, application of the protein synthesis inhibitor, anisomycin, did not influence atRA's ability to reduce electrical coupling between PeA cell pairs, indicating that atRA's acute uncoupling effect is likely independent of translational activity. Likewise, atRA mediates a number of rapid electrophysiological effects in *Lymnaea* neurons that cannot be blocked by inhibiting protein synthesis (Vesprini et al., 2015). These include the capacity for atRA to rapidly (< 1 h) induce atypical firing patterns, influence action potential shape/duration and induce cell silencing (Vesprini & Spencer, 2014), as well as reduce $[Ca^{2+}]_i$ (< 15 mins) (Vesprini et al., 2015). In contrast, incubation in anisomycin prevents RA-mediated chemoattraction in intact and transected neurites (isolated from the cell body), suggesting its nongenomic effects on growth cone guidance are regulated by protein synthesis (Farrar et al., 2009). Thus, it is clear that cellular processes affected by nongenomic retinoid signaling in *Lymnaea* neurons involve distinct underlying mechanisms.

In *Lymnaea* neurons, both the *Lym*RAR and *Lym*RXR have been localized only to non-nuclear compartments, such as the cytoplasm, neurites/growth cones and the plasma membrane (Carter et al., 2010, 2015). These observations indicate that *Lymnaea's* retinoid receptors likely mediate certain neuronal processes requiring nongenomic retinoid signaling. However, my data indicate that the acute effect of atRA on electrical coupling appears independent of the extra-nuclear activation of the retinoid receptors, as neither the vertebrate RAR (LE540) nor RXR (HX531) pan-antagonists prevented the rapid RA-induced uncoupling effect. HX531 and LE540 inhibit the chemoattractive effects of atRA

on regenerating *Lymnaea* neurons (Carter et al., 2010; Rand, 2012), yet receptor antagonism, again, has produced mixed results in terms of its ability to block some of the electrophysiological effects of atRA. Neither HX531 nor LE540, prevent atRA-induced cell silencing or $[Ca^{2+}]_I$ reductions, but HX531 does partially inhibit atRA-induced atypical firing (Vesprini et al., 2015; Vesprini & Spencer, 2014). Moreover, these two antagonists, alone, produce rapid voltage-dependent inhibition of voltage-gated Ca^{2+} channels (a process that dynamically shapes synaptic release), albeit via separate mechanisms. Although HX531 is purported to affect Ca^2 channels independently of G protein activation, LE540, requires a G protein-dependent mechanism (de Hoog et al., 2019). Thus, akin to nongenomic retinoid signaling in vertebrates, activation of extra-nuclear retinoid receptors likely mediates different cellular/molecular processes in *Lymnaea* neurons.

The synthetic retinoid, EC23, a vertebrate RAR pan-agonist mimicked the uncoupling effect of atRA in this study, but curiously, its effects could also not be blocked by RXR/RAR receptor antagonism. These findings also suggest that the RA-induced uncoupling in PeA neurons does not involve retinoid receptor activation. Of note, the previous account (in Bass retinal horizontal cells) demonstrating that selective RAR_{β} (CD2314) and RAR_{γ} (CD666) agonists, mimicked the uncoupling effect of atRA, concluded that an extracellular $RAR_{\beta/\gamma}$-like binding site was likely responsible for gating gap junctions (Zhang & McMahon, 2000). However, as the authors did not determine whether RAR antagonists could block these retinoid-induced uncoupling effects, it is uncertain whether this effect is mediated by activation of extra-nuclear RARs or some other substrate with an RAR-like binding site. Hence, it is possible that the retinoid-induced

uncoupling observed in my study, is also driven via a mechanism that is dependent on the activation of a substrate with an RAR-like binding site (Zhang & McMahon, 2000).

It is also possible that the antagonist concentrations used in my study were insufficient to block the effects of either 10 μM atRA or 1 μM EC23. Although much less is known about LE540 compared to HX531, whether either act as competitive or noncompetitive antagonists for their respective receptors is not clear from the literature. Specifically, HX531, the most widely used RXR antagonist *in vivo,* is thought to inhibit the activation of RAR/RXR heterodimers and/or RXR homodimers (Ebisawa et al., 1999; Kanayasu-Toyoda et al., 2005; Takahashi et al., 2002; Vivat et al., 1997; Watanabe & Kakuta, 2018).

On the other hand, at least in vertebrates, EC23 directly binds to the ligand binding domain of the RARα, β and γ and exhibits a higher affinity for the binding pocket of these receptor isoforms than atRA itself (Haffez et al., 2017), though it does not bind to vertebrate RXRs (Gambone et al., 2002). As our lab recently demonstrated that EC23 can also apparently activate the ligand binding domain of the *Lym*RXR (de Hoog et al., 2022), it is likely that EC23 is not as selective for the *Lym*RAR as was previously thought. Due to their highly hydrophobic nature, synthetic diphenylacetylene-based retinoids, such as EC23, likely exhibit a propensity for off-target interactions with other proteins (Hudhud et al., 2022). Therefore, it is plausible that EC23 may interact with another intermediate molecule(s) that affects electrical coupling. One possibility is through nongenomic activation of protein kinases, which can lead to post-translational modifications of gap junction channels or gap junction-associated proteins. In this regard, both EC23 and atRA activate ERK1/2 phosphorylation to induce neurite outgrowth in human neuroblastoma

(SH-SY5Y) cells (Khatib et al., 2019). It is thus conceivable that PeA coupling is influenced by the action of these retinoids affecting the phosphorylation status of gap junctions.

Two PKC inhibitors, H7 and Gö6976 failed to block atRA's acute uncoupling effect, indicating that the effect is likely independent of PKC activation. Although PKC-mediated phosphorylation has previously been shown to affect gap junction communication, the effects of PKC differ across different species and cell types. For example, PKC-mediated phosphorylation of Cx43 leads to decreases in gap junction communication in human cardiac cells (Lampe & Lau, 2000; Solan & Lampe, 2009) but increases communication in guinea pig cardiomyocytes and HeLa cells (Weng et al., 2002). In the mollusc, *Aplysia* (which is closely related to *Lymnaea*), PKC activation actually enhances junctional conductance at electrical synapses between neuroendocrine bag cell neurons (Beekharry et al., 2018). This observation highlights that although regulatory control of gap junctions is often conserved across species and cell types, the functional outcome of that control can vary. Interestingly, RA's acute uncoupling effect in Bass retinal horizontal cells was also not blocked by protein kinase inhibitors, including those targeting PKC. This finding may suggest that retinoid-mediated reductions in electrical coupling involves nongenomic pathways independent of phosphorylating kinases. However, there is evidence that RA can modulate the phosphorylation status of gap junction channels. For instance, atRA increases intercellular gap junction communication in human endometrial stromal cells through phosphatase-dependent dephosphorylation of Cx43 (Wu et al., 2013). Thus, given the mixed effects produced by phosphorylating/dephosphorylating proteins

across different species and cell types, it is plausible that atRA's acute uncoupling effect is mediated by other kinases/phosphatases not investigated in this study.

Similar to vertebrate connexins, innexin-based channels of invertebrates contain phosphorylation sites and their activity is modulated by phosphorylating proteins. Of the 12 innexin-based channels identified in the CNS of the terrestrial slug, *L. valentianus*, putative phosphorylation sites for at least nine different protein kinases have been identified, including PKC (Sadamoto et al., 2021). As the innexin-encoding genes in *Lymnaea* have only recently been identified, further phylogenetic analysis and predictive modelling at the peptide sequence level is needed to determine potential phosphorylation sites contained within *Lymnaea* innexin paralogs. Such information would prove useful in guiding future research aimed at identifying signaling pathways that modulate gap junction communication in *Lymnaea.*

Long-Term Effects of Retinoids on Electrical Synapses

In this study, long-term (48 h) retinoid exposure not only reduced the proportion of PeA cell pairs (plated in a soma-soma configuration) that formed electrical synapses, but reduced the average strength of coupling in cell pairs which formed an electrical synapse. Although retinoid signaling is known to affect gap junction function in a wide variety of vertebrate cell types, the majority of evidence for this role comes from studies that used nonneuronal cells. Aside from the aforementioned examples indicating that retinoid signaling can rapidly modulate gap junction communication (i.e. following acute retinoid exposure [< 1 h]), the majority of studies examined the effects of retinoids on gap junction function following long-term retinoid exposure (> 12 h) and these likely involved

transcriptional effects. Whereas it is unlikely that the *Lym*RAR or *Lym*RXR are involved in the rapid RA-induced uncoupling effect (as receptor antagonism did not prevent this effect), their participation in gap junction formation and the resulting reduction in synaptic strength following long-term exposure to RA cannot be ruled out. One obvious possibility is through transcriptional regulation via the retinoid receptors acting in their "canonical" role as nuclear receptors. This transcriptional regulation could influence the expression of proteins involved in gap junction formation, such as certain innexin isoforms, scaffolding/regulatory proteins, and/or assembly/trafficking machinery.

In previous studies, long-term exposure of human hepatocellular carcinoma cells to atRA for 48 h (Yang et al., 2014), and canine primary lens epithelial cells for 24 h (Long et al., 2010), enhanced Cx43 expression and gap junction communication. There is also evidence that retinoid signaling selectively influences the expression of certain neuronal specific connexins; Cx36 (but not Cx45) transcripts are upregulated in human neuroblastoma cells (SH-SY5Y) following 2 to 10 day incubations in atRA (Sidhu et al., 2013). The latter example indicates that not all connexin isoforms expressed within a cell are regulated by retinoids, which might be an important consideration for regulation of innexin proteins in *Lymnaea* (discussed below). This point is further emphasized by the fact that only certain connexin-encoding genes in vertebrates contain DNA-binding domains for RXRα. For instance, RXRα binding motifs have only been identified in Cx36 (Cicirata et al., 2000) and Cx43 (Gu et al., 2016) genes. Together these findings suggest that long-term retinoid exposure might influence gap junction function via the canonical retinoid signaling pathway, possibly involving the RXRα acting as a transcriptional regulator.

Comparing studies utilizing acute exposure to retinoids (< 1 h) with long-term exposure (> 12 h), it is apparent that retinoid signaling affecting gap junction structure and function likely involves both genomic and nongenomic mechanisms. Given this, perhaps the biggest challenge that emerges from my findings is discerning whether the effects of long-term exposure to RA involve (1) the same underlying nongenomic mechanisms driving the acute effects, (2) distinct genomic mechanisms or (3) a combination of both.

As atRA can activate transcriptional activity of the *Lym*RXR (de Hoog et al., 2022), it is possible that the effects on PeA synapse formation and subsequent coupling strength following long-term exposure to atRA, is directly tied to transcriptional control of certain gene products via the *Lym*RXR. It would be useful to determine whether any of *Lymnaea's* innexin-encoding genes contain *Lym*RXR DNA binding motifs (similar to the RXRα domain found in the vertebrate Cx36/Cx43 genes; Cicirata et al., 2000; Gu et al., 2016). This would provide support for the role of the *Lym*RXR as a transcriptional regulator of innexin expression in *Lymnaea*. The involvement of the *Lym*RAR in the long-term effects of atRA on electrical synapses also cannot be ruled out, though whether this receptor can bind RA and activate transcription in *Lymnaea,* has not yet been determined.

It is currently unknown whether retinoid signaling can influence the expression of *Lymnaea* innexin paralogs. Addressing this question would be a first step towards determining whether transcriptional level changes are involved in mediating the long-term effects of atRA on PeA electrical synapses. Of note, qPCR data indicate that neither 1 h nor 12 h incubation in 0.5 μM atRA influences the expression of three innexin paralogs in *Lymnaea's* CNS (Hoelscher, 2021). However, as this concentration is below the threshold found to have any significant effects on PeA electrical synapses (either following acute or

long-term exposure to atRA), this should also be reassessed using the 5-10 μM concentrations shown to affect synapse formation and strength of coupling.

Isomer-Selective Effects of Retinoid Signaling

Whereas only acute exposure to the atRA isomer significantly reduced coupling between PeA pairs, long-term exposure to both all-*trans* and 9-*cis* isomers led to analogous effects (i.e. reduced synaptogenesis and synaptic strength). There are a number of instances showing that RA can produce both isomer-independent and -dependent effects. In *Lymnaea* neurons, both all-*trans* and 9-*cis* isomers elicit similar effects on neurite outgrowth and in growth cone turning assays (Dmetrichuk et al., 2006, 2008). However, atRA also exhibits a number of isomer-selective effects on electrophysiological properties, including inducing atypical firing patterns, influencing action potential shape/duration, reducing $[Ca^{2+}]_i$ and cell silencing. Similarly, atRA, but not 9-*cis* RA, reduces Ca^{2+} influx via inhibition of voltage-gated Ca^{2+} channels (de Hoog et al., 2018). Although the isomer-selective acute effects on electrical coupling likely reflect atRA's selective capacity to influence specific nongenomic mechanisms, at this time it is not clear whether the isomer-independent effects of atRA and 9-*cis* RA on synapse formation result from similar or separate mechanisms.

In previous studies, long-term exposure to either atRA or 9-*cis* RA isomers led to mixed effects on gap junction function, seemingly involving nongenomic and genomic mechanisms, depending on the species and cell type under investigation. For instance, in rat gliomal cells, 72 h exposure to atRA enhanced gap junction communication without having any significant effect on Cx43 expression (Zhang et al., 2002), whereas 24 h exposure to the 9-*cis* isomer downregulated Cx43 expression in immortal cells lines (e.g.

human embryonic kidney cells (HEK-293), HeLa cells, and murine cardiac cells (HL-1) (Gu et al., 2016). Yet, in human prostate cancer cells, both 9-*cis* RA and atRA (after 12 to 48 h) both upregulated the expression and integration of Cx32 into functional gap junctions (Kelsey et al., 2012). Historically atRA has been deemed the natural ligand of RARs in vertebrates, though 9-*cis* RA can also bind. However, RXRs were predominantly thought to bind the 9-cis RA isomer. There is now evidence, however, that both atRA and 9-*cis* RA can also bind to and activate vertebrate RXRs (Tsuji et al., 2015). Both atRA and 9-*cis* RA isomers can bind to and activate transcriptional activity of the *Lym*RXR to a similar extent at micromolar concentrations (de Hoog et al., 2022). These observations may support the notion that both RA isomers are capable of regulating electrical synapse formation and communication via genomic mechanisms. However, the occurrence of spontaneous isomerization of 9-*cis* RA to atRA (Sass et al., 1995) or vice versa (Levin et al., 1992) might also account for the similar effects observed here following long-term exposure to both isomers. Whether either of the RA isomers can affect the expression of genes involved in electrical synaptogenesis or gap junction communication in *Lymnaea* is currently unknown, though this question could be partially addressed by future qPCR analysis. However, it will also be necessary to determine whether any changes in the expression of innexin-encoding genes leads to any change in the proteome or whether these changes result in any functional consequences to electrical communication, in order to elucidate the particular mechanisms underlying RA's effect (following long-term exposure) on PeA electrical coupling.

Cell-Specific Effects of Retinoids

One observation from previous findings by Rothwell et al. (2017), was that not all neurons cultured in a soma-soma configuration were sensitive to the effects of atRA. Two of the cell pairs, VD4-RPeD2 and VD4-LPeD1, used in the previous study form chemical not electrical synapses *in vivo*, yet in culture they displayed a propensity to form electrical synapses, at least initially. Only the electrical synapses formed between cultured VD4-RPeD2 pairs were sensitive to atRA. This finding, in part, suggests that innexin expression in *Lymnaea*'s CNS is heterogeneous across different cell types, and might also indicate that the cell specific effects of RA on coupling might result from its action on specific innexin isoforms. Although it is not conclusively known whether the electrical synapses in *Lymnaea* are derived from homotypic or heterotypic channels, the rectification seen at PeA electrical synapses implies that the gap junctions are constituted by heterotypic channels (Marder, 2009). Such examples of rectification are observed within invertebrate nervous systems (Johnson et al., 1993; Kristan et al., 2005). Although 10 innexin-encoding genes have been detected in *Lymnaea* (*Lst Inx1-10*) there is evidence they are not equally expressed across its CNS. For example, *Lst Inx1* displays heterogenous ganglionic expression, being more highly expressed in the left pedal ganglia compared to the right pedal ganglia. Mersman et al. (2020) found *Lst Inx1* transcripts within neuronal clusters with functionally defined electrically coupled cell-types (e.g. PeA and cerebral A), yet, its expression was noticeably absent in other cells which form electrical synapses (e.g. RPD2 and VD1). This finding is notable when considering that both Rothwell et al. (2017) and I found that RA affects coupling in cultured PeA-PeA pairs, whereas others have shown that RA did not alter coupling between RPD2 and VD1 cells, at least *in vivo* (unpublished

observation). Thus, it is plausible that the cell-specific nature of the RA-induced uncoupling effect at PeA-PeA or VD4-RPeD2, but not VD4-LPeD1 and RPD2-VD1 synapses, might be a consequence of differential expression of specific 'RA sensitive' innexin paralogs across these different cell types. This hypothesis is supported by the notion that only some vertebrate connexins are regulated by retinoid signaling (Sidhu et al., 2013). My observation that long-term retinoid exposure, in both a dose- and isomer-independent manner, led to what I defined as a "capping effect" between PeA pairs, perhaps also supports the idea that only specific innexins subtypes might be RA sensitive. For instance, the capping effect might reflect RA's capacity to control the integration of certain high or low conducting innexin isoforms into gap junctions. Nonetheless, it is tantalizing to speculate that *Lst Inx1* might be one of these 'RA sensitive' innexin isoforms, given that, as of yet, RA's uncoupling effect was only observed in *Lst Inx1* expressing cell-types which form electrical synapses *in vivo*. Future studies utilizing different cell-types and culture conditions (plating cells in soma-soma vs neurite-neurite configuration) will be useful in identifying potential 'RA sensitive' innexin paralogs expressed across different cell types or cellular compartments.

Physiological Role of Retinoid-Mediated Uncoupling?

In this study, the acute effect of RA was most prominent at 10 µM, but at lower concentrations a modest, but significant, effect was observed at both 1 h and 2 h timepoints. While average physiological concentrations of RA are thought to range between 10^{-6} to 10^{-9} M in vertebrates (Kane et al., 2005; Napoli, 1986) and > 10^{-7} M in *Lymnaea's* CNS (Dmetrichuk et al., 2008), this, however, does not rule out the possibility of

“microdomains”, where RA is locally synthesized and present at concentrations similar to those used in this study. It is well established in vertebrates that during development, the rate of RA synthesis is known to undergo dynamic changes in a spatiotemporal manner, which alters the expression of specific genes that control patterning and cell fate. One example comes from the developing retina in chicks where the concentration of RA fluctuates across different developmental stages (McCaffery et al., 1999; Mey et al., 1997, 2001). Although the factors that influence changes in RA synthesis across different tissues during adulthood are not well understood, it is thought RA synthesis is relatively constrained within a certain optimal homeostatic window dictated by each tissue/cell type. One example of activity-dependent regulation of RA levels in adult neurons comes from mice, where decreases in $[Ca^{2+}]_i$ can upregulate RA synthesis in the synaptic dendrites of hippocampal neurons (Wang et al., 2011). Thus, the potent effects of 10 μM atRA might reflect the fact that RA’s capacity to influence electrical synapses might be context-dependent, possibly under circumstances where RA synthesis is locally upregulated. To understand which contexts might give rise to upregulated RA synthesis in *Lymnaea* neurons, it is perhaps important to consider the function of the PeA neurons my investigation focused on.

PeA cells are a contingent of ~ 60 serotonergic motorneurons evenly dispersed across the medial portions of both the left and right pedal ganglia, and which control locomotion in *Lymnaea* (Syed & Winlow, 1989). *In vivo*, PeA motorneurons form electrical synapses with their ipsilateral and contralateral counterparts. Although contralateral PeA coupling must be neurite-neurite in nature, whether ipsilateral PeA cells form soma-soma or neurite-neurite synapses with each other is not currently known.

Changes in the electrical properties of PeA neurons have previously been correlated with *Lymnaea's* behavioral status. Food deprivation (for 24 h) can produce long lasting changes in PeA activity including increased cell firing and depolarization of the RMP (Dyakonova et al., 2015a). Conversely, the same research group also showed that isolated PeA neurons cells are sensitive to glucose, and exhibit reduced firing following its application (Dyakonova et al., 2015b). Together these two findings suggest that the activity of PeA neurons change in accordance with *Lymnaea's* nutritional status. Likewise, given the locomotory function of these PeA neurons, it is reasonable that increased excitability within the PeA network may in turn lead to increased foraging behavior to meet *Lymnaea's* nutritional demands. Notably, vitamin A (and thus RA) is only derived from dietary means and so, like glucose, its biosynthesis fluctuates as a function of the animal's nutritional status. Indeed, changes in RA synthesis and signaling associated with satiety levels have been shown in the hepatic cells of mice (Klyuyeva et al., 2021). There is also evidence that RA is an important molecule regulating glucose and fatty acid metabolism (Blaner, 2019; Chen & Chen, 2014). Hence, it is plausible that the rate of RA synthesis is influenced by the transition from food deprivation to satiety. In this context, the relative bioavailability of RA might serve as a proxy indicator or 'biosensor' of nutritional status. Hence, the RA-induced uncoupling seen at PeA neurons at high concentrations might be reflective of physiological conditions/states, such as satiety, where the synthesis of RA is highly upregulated. The functional consequence might be negative feedback onto the locomotion circuitry by reducing synchronicity (and hence output) between PeA motorneurons. Coordinated control of motor programs is thought to rely heavily upon precisely timed spiking between motorneurons, thus decreased electrical coupling within this network may

disrupt motor output. However, at which level of *Lymnaea's* locomotory network, plasticity might play a role in producing behavioral modifications is not currently known. It can also not be ruled out that RA's uncoupling effect does not carry out any physiological function within this particular network *in vivo*.

Conclusion

While significant progress has been made in understanding electrical synapses, the extent to which various neuromodulators, specifically RA, affect these synapses, remains largely unknown. In this study, I present original findings that demonstrate the effects of retinoid signaling in the formation and modulation of electrical synapses in the mollusc *Lymnaea stagnalis*. These findings also add to the growing body of research indicating that retinoid signaling is conserved across both vertebrate and invertebrate lineages. Further exploration of the effects of retinoid signaling on electrical synapses has the potential to reveal novel insights into synaptic control within the nervous system, which could enhance our basic understanding of brain function and behavior.

References

Aggarwal, S., Kim, S.-W., Cheon, K., Tabassam, F. H., Yoon, J.-H., & Koo, J. S. (2006). Nonclassical action of retinoic acid on the activation of the cAMP response element-binding protein in normal human bronchial epithelial cells. *Molecular Biology of the Cell*, *17*(2), 566–575. https://doi.org/10.1091/mbc.e05-06-0519

Albalat, R., & Cañestro, C. (2009). Identification of Aldh1a, Cyp26 and RAR orthologs in protostomes pushes back the retinoic acid genetic machinery in evolutionary time to the bilaterian ancestor. *Chemico-Biological Interactions*, *178*(1–3), 188–196. https://doi.org/10.1016/j.cbi.2008.09.017

Aoto, J., Nam, C. I., Poon, M. M., Ting, P., & Chen, L. (2008). Synaptic signaling by all-*trans* retinoic acid in homeostatic synaptic plasticity. *Neuron*, *60*(2), 308–320. https://doi.org/10.1016/j.neuron.2008.08.012

Baker, R., & Llinás, R. (1971). Electrotonic coupling between neurones in the rat mesencephalic nucleus. *The Journal of Physiology*, *212*(1), 45–63. https://doi.org/10.1113/jphysiol.1971.sp009309

Beblo, D. A., & Veenstra, R. D. (1997). Monovalent cation permeation through the connexin40 gap junction channel. Cs, Rb, K, Na, Li, TEA, TMA, TBA, and effects of anions Br, Cl, F, acetate, aspartate, glutamate, and NO3. *The Journal of General Physiology*, *109*(4), 509–522. https://doi.org/10.1085/jgp.109.4.509

Beekharry, C. C., Gu, Y., & Magoski, N. S. (2018). Protein Kinase C Enhances Electrical Synaptic Transmission by Acting on Junctional and Postsynaptic Ca2+ Currents. *The Journal of Neuroscience*, *38*(11), 2796–2808. https://doi.org/10.1523/JNEUROSCI.2619-17.2018

Begemann, G., & Meyer, A. (2001). Hindbrain patterning revisited: Timing and effects of retinoic acid signalling. *BioEssays*, *23*(11), 981–986. https://doi.org/10.1002/bies.1142

Beierlein, M., Gibson, J. R., & Connors, B. W. (2000). A network of electrically coupled interneurons drives synchronized inhibition in neocortex. *Nature Neuroscience*, *3*(9), 904–910. https://doi.org/10.1038/78809

Benardo, L. S., & Foster, R. E. (1986). Oscillatory behavior in inferior olive neurons: Mechanism, modulation, cell aggregates. *Brain Research Bulletin*, *17*(6), 773–784. https://doi.org/10.1016/0361-9230(86)90089-4

Bennett, M. (1966). Physiology of electrotonic junctions. *Annals of the New York Academy of Sciences*, *137*(2), 509–539. https://doi.org/10.1111/j.1749-6632.1966.tb50178.x

Bennett, M., Crain, S. M., & Grundfest, H. (1959). Electrophysiology of supramedullary neurons in Spheroides maculatus. III. Organization of the supramedullary neurons. *The Journal of General Physiology*, *43*, 221–250. https://doi.org/10.1085/jgp.43.1.221

Bennett, M., & Zukin, S. (2004). Electrical Coupling and Neuronal Synchronization in the Mammalian Brain. *Neuron*, *41*(4), 495–511. https://doi.org/10.1016/S0896-6273(04)00043-1

Beyer, E. C., & Berthoud, V. M. (2018). Gap junction gene and protein families: Connexins, innexins, and pannexins. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1860*(1), 5–8. https://doi.org/10.1016/j.bbamem.2017.05.016

Bissiere, S., Zelikowsky, M., Ponnusamy, R., Jacobs, N. S., Blair, H. T., & Fanselow, M. S. (2011). Electrical Synapses Control Hippocampal Contributions to Fear Learning and Memory. *Science (New York, N.Y.)*, *331*(6013), 87–91. https://doi.org/10.1126/science.1193785

Blaner, W. S. (2019). Vitamin A signaling and homeostasis in obesity, diabetes, and metabolic disorders. *Pharmacology & Therapeutics*, *197*, 153–178. https://doi.org/10.1016/j.pharmthera.2019.01.006

Blomhoff, R., & Blomhoff, H. K. (2006). Overview of retinoid metabolism and function. *Journal of Neurobiology*, *66*(7), 606–630. https://doi.org/10.1002/neu.20242

Boassa, D., Ambrosi, C., Qiu, F., Dahl, G., Gaietta, G., & Sosinsky, G. (2007). Pannexin1 Channels Contain a Glycosylation Site That Targets the Hexamer to the Plasma Membrane*. *Journal of Biological Chemistry*, *282*(43), 31733–31743. https://doi.org/10.1074/jbc.M702422200

Bonnet, E., Touyarot, K., Alfos, S., Pallet, V., Higueret, P., & Abrous, D. N. (2008). Retinoic acid restores adult hippocampal neurogenesis and reverses spatial memory deficit in vitamin A deprived rats. *PLOS ONE*, *3*(10), e3487. https://doi.org/10.1371/journal.pone.0003487

Bouton, D., Escriva, H., Mendonça, R. L. de, Glineur, C., Bertin, B., Noël, C., Robinson-Rechavi, M., Groot, A. de, Cornette, J., Laudet, V., & Pierce, R. J. (2005). A conserved retinoid X receptor (RXR) from the mollusk *Biomphalaria glabrata* transactivates transcription in the presence of retinoids. *Journal of Molecular Endocrinology*, *34*(2), 567–582. https://doi.org/10.1677/jme.1.01766

Calkins, T. L., Woods-Acevedo, M. A., Hildebrandt, O., & Piermarini, P. M. (2015). The molecular and immunochemical expression of innexins in the yellow fever mosquito, Aedes aegypti: Insights into putative life stage- and tissue-specific functions of gap junctions. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, *183*, 11–21. https://doi.org/10.1016/j.cbpb.2014.11.013

Campo-Paysaa, F., Marlétaz, F., Laudet, V., & Schubert, M. (2008). Retinoic acid signaling in development: Tissue-specific functions and evolutionary origins. *Genesis*, *46*(11), 640–656. https://doi.org/10.1002/dvg.20444

Carpenter, S., Rothwell, C. M., Wright, M. L., de Hoog, E., Walker, S. E., Hudson, E., & Spencer, G. E. (2016). Extending the duration of long-term memories: Interactions between environmental darkness and retinoid signaling. *Neurobiology of Learning and Memory*, *136*, 34–46. https://doi.org/10.1016/j.nlm.2016.09.008

Carter, C. J., Farrar, N., Carlone, R. L., & Spencer, G. E. (2010). Developmental expression of a molluscan RXR and evidence for its novel, nongenomic role in growth cone guidance. *Developmental Biology*, *343*(1–2), 124–137. https://doi.org/10.1016/j.ydbio.2010.03.023

Carter, C. J., Rand, C., Mohammad, I., Lepp, A., Vesprini, N., Wiebe, O., Carlone, R., & Spencer, G. E. (2015). Expression of a retinoic acid receptor (RAR)-like protein in the embryonic and adult nervous system of a protostome species. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution*, *324*(1), 51–67. https://doi.org/10.1002/jez.b.22604

Castro, L. F. C., Lima, D., Machado, A., Melo, C., Hiromori, Y., Nishikawa, J., Nakanishi, T., Reis-Henriques, M. A., & Santos, M. M. (2007). Imposex induction is mediated

through the retinoid X receptor signalling pathway in the neogastropod *Nucella lapillus*. *Aquatic Toxicology*, *85*(1), 57–66. https://doi.org/10.1016/j.aquatox.2007.07.016

Chen, N., & Napoli, J. L. (2008). All-trans-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RARalpha. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *22*(1), 236–245. https://doi.org/10.1096/fj.07-8739com

Chen, W., & Chen, G. (2014). The Roles of Vitamin A in the Regulation of Carbohydrate, Lipid, and Protein Metabolism. *Journal of Clinical Medicine*, *3*(2), 453–479. https://doi.org/10.3390/jcm3020453

Chiang, M. Y., Misner, D., Kempermann, G., Schikorski, T., Giguère, V., Sucov, H. M., Gage, F. H., Stevens, C. F., & Evans, R. M. (1998). An essential role for retinoid receptors RARβ and RXRγ in long-term potentiation and depression. *Neuron*, *21*(6), 1353–1361. https://doi.org/10.1016/s0896-6273(00)80654-6

Christie, M. J., Williams, J. T., & North, R. A. (1989). Electrical coupling synchronizes subthreshold activity in locus coeruleus neurons in vitro from neonatal rats. *Journal of Neuroscience*, *9*(10), 3584–3589. https://doi.org/10.1523/JNEUROSCI.09-10-03584.1989

Cicirata, F., Parenti, R., Spinella, F., Giglio, S., Tuorto, F., Zuffardi, O., & Gulisano, M. (2000). Genomic organization and chromosomal localization of the mouse Connexin36 (mCx36) gene. *Gene*, *251*(2), 123–130. https://doi.org/10.1016/S0378-1119(00)00202-X

Clagett-Dame, M., McNeill, E. M., & Muley, P. D. (2006). Role of all-*trans* retinoic acid in neurite outgrowth and axonal elongation. *Journal of Neurobiology*, *66*(7), 739–756. https://doi.org/10.1002/neu.20241

Cocco, S., Diaz, G., Stancampiano, R., Diana, A., Carta, M., Curreli, R., Sarais, L., & Fadda, F. (2002). Vitamin A deficiency produces spatial learning and memory impairment in rats. *Neuroscience*, *115*(2), 475–482. https://doi.org/10.1016/s0306-4522(02)00423-2

Connors, B. W., & Long, M. A. (2004). Electrical synapses in the mammalian brain. *Annual Review of Neuroscience*, *27*, 393–418. https://doi.org/10.1146/annurev.neuro.26.041002.131128

Corcoran, J., Shroot, B., Pizzey, J., & Maden, M. (2000). The role of retinoic acid receptors in neurite outgrowth from different populations of embryonic mouse dorsal root ganglia. *Journal of Cell Science*, *113*(14), 2567–2574. https://doi.org/10.1242/jcs.113.14.2567

Curti, S., & O'Brien, J. (2016). Characteristics and plasticity of electrical synaptic transmission. *BMC Cell Biology*, *17*(S1), 13. https://doi.org/10.1186/s12860-016-0091-y

Curtin, K. D., Zhang, Z., & Wyman, R. J. (2002). Gap Junction Proteins Expressed during Development Are Required for Adult Neural Function in the Drosophila Optic Lamina. *Journal of Neuroscience*, *22*(16), 7088–7096. https://doi.org/10.1523/JNEUROSCI.22-16-07088.2002

de Hoog, E., Lukewich, M. K., & Spencer, G. E. (2018). Retinoic acid inhibits neuronal voltage-gated calcium channels. *Cell Calcium*, *72*, 51–61. https://doi.org/10.1016/j.ceca.2018.02.001

de Hoog, E., Lukewich, M. K., & Spencer, G. E. (2019). Retinoid receptor-based signaling plays a role in voltage-dependent inhibition of invertebrate voltage-gated Ca2+ channels. *The Journal of Biological Chemistry*, *294*(26), 10076–10093. https://doi.org/10.1074/jbc.RA118.006444

de Hoog, E., Saba Echezarreta, V. E., Turgambayeva, A., Foran, G., Megaly, M., Necakov, A., & Spencer, G. E. (2022). Molluscan RXR Transcriptional Regulation by Retinoids in a Drosophila CNS Organ Culture System. *Cells*, *11*(16), 2493. https://doi.org/10.3390/cells11162493

de Hoog, E., & Spencer, G. E. (2022). Activity-dependent modulation of neuronal KV channels by retinoic acid enhances CaV channel activity. *The Journal of Biological Chemistry*, *298*(6), 101959. https://doi.org/10.1016/j.jbc.2022.101959

DeVries, S. H., Qi, X., Smith, R., Makous, W., & Sterling, P. (2002). Electrical coupling between mammalian cones. *Current Biology: CB*, *12*(22), 1900–1907. https://doi.org/10.1016/s0960-9822(02)01261-7

Dmetrichuk, J. M., Carlone, R. L., Jones, T. R. B., Vesprini, N. D., & Spencer, G. E. (2008). Detection of endogenous retinoids in the molluscan CNS and characterization of the trophic and tropic actions of 9-*cis* retinoic acid on isolated neurons. *The Journal of Neuroscience*, *28*(48), 13014–13024. https://doi.org/10.1523/JNEUROSCI.3192-08.2008

Dmetrichuk, J. M., Carlone, R. L., & Spencer, G. E. (2006). Retinoic acid induces neurite outgrowth and growth cone turning in invertebrate neurons. *Developmental Biology*, *294*(1), 39–49. https://doi.org/10.1016/j.ydbio.2006.02.018

Dmetrichuk, J. M., Spencer, G. E., & Carlone, R. L. (2005). Retinoic acid-dependent attraction of adult spinal cord axons towards regenerating newt limb blastemas in vitro. *Developmental Biology*, *281*(1), 112–120. https://doi.org/10.1016/j.ydbio.2005.02.019

Dyakonova, V., Hernádi, L., Ito, E., Dyakonova, T., Chistopolsky, I. A., Zakharov, I. S., & Sakharov, D. A. (2015). The Activity of Isolated Neurons and the Modulatory State of an Isolated Nervous System Represent a Recent Behavioural State. *Journal of Experimental Biology*, jeb.111930. https://doi.org/10.1242/jeb.111930

Dyakonova, V., Hernádi, L., Ito, E., Dyakonova, T., Zakharov, I., & Sakharov, D. (2015). The activity of isolated snail neurons controlling locomotion is affected by glucose. *Biophysics*, *11*, 55–60. https://doi.org/10.2142/biophysics.11.55

Ebisawa, M., Umemiya, H., Ohta, K., Fukasawa, H., Kawachi, E., Christoffel, G., Gronemeyer, H., Tsuji, M., Hashimoto, Y., Shudo, K., & Kagechika, H. (1999). Retinoid X receptor-antagonistic diazepinylbenzoic acids. *Chemical & Pharmaceutical Bulletin*, *47*(12), 1778–1786. https://doi.org/10.1248/cpb.47.1778

Eichner, C., Dalvin, S., Skern-Mauritzen, R., Malde, K., Kongshaug, H., & Nilsen, F. (2015). Characterization of a novel RXR receptor in the salmon louse (*Lepeophtheirus salmonis*, Copepoda) regulating growth and female reproduction. *BMC Genomics*, *16*, 81. https://doi.org/10.1186/s12864-015-1277-y

Falk, M. M., Kells, R. M., & Berthoud, V. M. (2014). Degradation of connexins and gap junctions. *FEBS Letters*, *588*(8), 1221–1229. https://doi.org/10.1016/j.febslet.2014.01.031

Farrar, N. R., Dmetrichuk, J. M., Carlone, R. L., & Spencer, G. E. (2009). A novel, nongenomic mechanism underlies retinoic acid-induced growth cone turning. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *29*(45), 14136–14142. https://doi.org/10.1523/JNEUROSCI.2921-09.2009

Feng, Z.-P., Klumperman, J., Lukowiak, K., & Syed, N. I. (1997). In Vitro Synaptogenesis between the Somata of Identified Lymnaea Neurons Requires Protein Synthesis But Not Extrinsic Growth Factors or Substrate Adhesion Molecules. *Journal of Neuroscience*, *17*(20), 7839–7849. https://doi.org/10.1523/JNEUROSCI.17-20-07839.1997

Frisch, C., Souza-Silva, M. A. D., Söhl, G., Güldenagel, M., Willecke, K., Huston, J. P., & Dere, E. (2005). Stimulus complexity dependent memory impairment and changes in motor performance after deletion of the neuronal gap junction protein connexin36 in mice. *Behavioural Brain Research*, *157*(1), 177–185. https://doi.org/10.1016/j.bbr.2004.06.023

Furshpan, E. J., & Potter, D. D. (1959). Transmission at the giant motor synapses of the crayfish. *The Journal of Physiology*, *145*(2), 289–325.

Gajda, Z., Gyengési, E., Hermesz, E., Ali, K. S., & Szente, M. (2003). Involvement of gap junctions in the manifestation and control of the duration of seizures in rats in vivo. *Epilepsia*, *44*(12), 1596–1600. https://doi.org/10.1111/j.0013-9580.2003.25803.x

Gajda, Z., Hermesz, E., Gyengési, E., Szupera, Z., & Szente, M. (2006). The functional significance of gap junction channels in the epileptogenicity and seizure susceptibility of juvenile rats. *Epilepsia*, *47*(6), 1009–1022. https://doi.org/10.1111/j.1528-1167.2006.00573.x

Galarreta, M., & Hestrin, S. (2001). Spike transmission and synchrony detection in networks of GABAergic interneurons. *Science (New York, N.Y.)*, *292*(5525), 2295–2299. https://doi.org/10.1126/science.1061395

Galarreta, M., & Hestrin, S. (2002). Electrical and chemical synapses among parvalbumin fast-spiking GABAergic interneurons in adult mouse neocortex. *Proceedings of the National Academy of Sciences*, *99*(19), 12438–12443. https://doi.org/10.1073/pnas.192159599

Gambone, C. J., Hutcheson, J. M., Gabriel, J. L., Beard, R. L., Chandraratna, R. A. S., Soprano, K. J., & Soprano, D. R. (2002). Unique property of some synthetic retinoids: Activation of the aryl hydrocarbon receptor pathway. *Molecular Pharmacology*, *61*(2), 334–342. https://doi.org/10.1124/mol.61.2.334

Ganfornina, M. D., Sánchez, D., Herrera, M., & Bastiani, M. J. (1999). Developmental expression and molecular characterization of two gap junction channel proteins expressed during embryogenesis in the grasshopper Schistocerca americana. *Developmental Genetics*, *24*(1–2), 137–150. https://doi.org/10.1002/(SICI)1520-6408(1999)24:1/2<137::AID-DVG13>3.0.CO;2-7

Gibson, J. R., Beierlein, M., & Connors, B. W. (1999). Two networks of electrically coupled inhibitory neurons in neocortex. *Nature*, *402*(6757), 75–79. https://doi.org/10.1038/47035

Gong, X. Q., & Nicholson, B. J. (2001). Size selectivity between gap junction channels composed of different connexins. *Cell Communication & Adhesion*, *8*(4–6), 187–192. https://doi.org/10.3109/15419060109080721

Goodenough, D. A., & Paul, D. L. (2009). Gap Junctions. *Cold Spring Harbor Perspectives in Biology*, *1*(1), a002576. https://doi.org/10.1101/cshperspect.a002576

Gu, R., Xu, J., Lin, Y., Zhang, J., Wang, H., Sheng, W., Ma, D., Ma, X., & Huang, G. (2016). Liganded retinoic acid X receptor α represses connexin 43 through a potential retinoic acid response element in the promoter region. *Pediatric Research*, *80*(1), Article 1. https://doi.org/10.1038/pr.2016.47

Gutierrez-Mazariegos, J., Nadendla, E. K., Lima, D., Pierzchalski, K., Jones, J. W., Kane, M., Nishikawa, J.-I., Hiromori, Y., Nakanishi, T., Santos, M. M., Castro, L. F. C., Bourguet, W., Schubert, M., & Laudet, V. (2014). A mollusk retinoic acid receptor (RAR) ortholog sheds light on the evolution of ligand binding. *Endocrinology*, *155*(11), 4275–4286. https://doi.org/10.1210/en.2014-1181

Haas, J. S., Greenwald, C. M., & Pereda, A. E. (2016). Activity-dependent plasticity of electrical synapses: Increasing evidence for its presence and functional roles in the mammalian brain. *BMC Cell Biology*, *17*(1), 14. https://doi.org/10.1186/s12860-016-0090-z

Haffez, H., Chisholm, D. R., Valentine, R., Pohl, E., Redfern, C., & Whiting, A. (2017). The molecular basis of the interactions between synthetic retinoic acid analogues and the retinoic acid receptors. *MedChemComm*, *8*(3), 578–592. https://doi.org/10.1039/C6MD00680A

Hanna, R. B., Keeter, J. S., & Pappas, G. D. (1978). The fine structure of a rectifying electrotonic synapse. *The Journal of Cell Biology*, *79*(3), 764–773. https://doi.org/10.1083/jcb.79.3.764

Hayward, D. C., Bastiani, M. J., Trueman, J. W., Truman, J. W., Riddiford, L. M., & Ball, E. E. (1999). The sequence of *Locusta* RXR, homologous to *Drosophila* Ultraspiracle, and its evolutionary implications. *Development Genes and Evolution*, *209*(9), 564–571. https://doi.org/10.1007/s004270050290

Hellemans, K., Grinko, I., Rombouts, K., Schuppan, D., & Geerts, A. (1999). All-trans and 9-cis retinoic acid alter rat hepatic stellate cell phenotype differentially. *Gut*, *45*(1), 134–142. https://doi.org/10.1136/gut.45.1.134

Hervé, J.-C., Bourmeyster, N., & Sarrouilhe, D. (2004). Diversity in protein–protein interactions of connexins: Emerging roles. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1662*(1), 22–41. https://doi.org/10.1016/j.bbamem.2003.10.022

Hinckley, C. A., & Ziskind-Conhaim, L. (2006). Electrical Coupling between Locomotor-Related Excitatory Interneurons in the Mammalian Spinal Cord. *The Journal of Neuroscience*, *26*(33), 8477–8483. https://doi.org/10.1523/JNEUROSCI.0395-06.2006

Hoelscher, B. (2021). *Identification and Regulation of the Gap Junction Encoding Gene Innexin in the Central Nervous System of Lymnaea stagnalis and Factors Affecting Vertebrate Neuronal Development* [Ph.D. Thesis]. St. Louis University.

Hormuzdi, S. G., Filippov, M. A., Mitropoulou, G., Monyer, H., & Bruzzone, R. (2004). Electrical synapses: A dynamic signaling system that shapes the activity of neuronal networks. *Biochimica Et Biophysica Acta*, *1662*(1–2), 113–137. https://doi.org/10.1016/j.bbamem.2003.10.023

Hormuzdi, S. G., Pais, I., LeBeau, F. E., Towers, S. K., Rozov, A., Buhl, E. H., Whittington, M. A., & Monyer, H. (2001). Impaired electrical signaling disrupts gamma frequency oscillations in connexin 36-deficient mice. *Neuron*, *31*(3), 487–495. https://doi.org/10.1016/s0896-6273(01)00387-7

Hosseinzadeh, H., Asl, M. N., Parvardeh, S., & Tagi Mansouri, S. M. (2005). The effects of carbenoxolone on spatial learning in the Morris water maze task in rats. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research*, *11*(3), BR88-94.

Hudhud, L., Chisholm, D. R., Whiting, A., Steib, A., Pohóczky, K., Kecskés, A., Szőke, É., & Helyes, Z. (2022). Synthetic Diphenylacetylene-Based Retinoids Induce DNA Damage in Chinese Hamster Ovary Cells without Altering Viability. *Molecules*, *27*(3), Article 3. https://doi.org/10.3390/molecules27030977

Jacobs, S., Lie, D. C., DeCicco, K. L., Shi, Y., DeLuca, L. M., Gage, F. H., & Evans, R. M. (2006). Retinoic acid is required early during adult neurogenesis in the dentate gyrus. *Proceedings of the National Academy of Sciences*, *103*(10), 3902–3907. https://doi.org/10.1073/pnas.0511294103

Jahromi, S. S., Wentlandt, K., Piran, S., & Carlen, P. L. (2002). Anticonvulsant actions of gap junctional blockers in an in vitro seizure model. *Journal of Neurophysiology*, *88*(4), 1893–1902. https://doi.org/10.1152/jn.2002.88.4.1893

Jiang, W., Yu, Q., Gong, M., Chen, L., Wen, E. Y., Bi, Y., Zhang, Y., Shi, Y., Qu, P., Liu, Y. X., Wei, X. P., Chen, J., & Li, T. Y. (2012). Vitamin A deficiency impairs postnatal cognitive function via inhibition of neuronal calcium excitability in hippocampus. *Journal of Neurochemistry*, *121*(6), 932–943. https://doi.org/10.1111/j.1471-4159.2012.07697.x

Johnson, A., de Hoog, E., Tolentino, M., Nasser, T., & Spencer, G. E. (2019). Pharmacological evidence for the role of RAR in axon guidance and embryonic development of a protostome species. *Genesis (New York, N.Y.: 2000)*, *57*(7–8), e23301. https://doi.org/10.1002/dvg.23301

Kanayasu-Toyoda, T., Fujino, T., Oshizawa, T., Suzuki, T., Nishimaki-Mogami, T., Sato, Y., Sawada, J., Inoue, K., Shudo, K., Ohno, Y., & Yamaguchi, T. (2005). HX531, a retinoid X receptor antagonist, inhibited the 9-cis retinoic acid-induced binding with steroid receptor coactivator-1 as detected by surface plasmon resonance. *The Journal of Steroid Biochemistry and Molecular Biology*, *94*(4), 303–309. https://doi.org/10.1016/j.jsbmb.2004.11.007

Kandarian, B., Sethi, J., Wu, A., Baker, M., Yazdani, N., Kym, E., Sanchez, A., Edsall, L., Gaasterland, T., & Macagno, E. (2012). The medicinal leech genome encodes 21 innexin genes: Different combinations are expressed by identified central neurons. *Development Genes and Evolution*, *222*(1), 29–44. https://doi.org/10.1007/s00427-011-0387-z

Kane, M. A., Chen, N., Sparks, S., & Napoli, J. L. (2005). Quantification of endogenous retinoic acid in limited biological samples by LC/MS/MS. *The Biochemical Journal*, *388*(Pt 1), 363–369. https://doi.org/10.1042/BJ20041867

Kelsey, L., Katoch, P., Johnson, K. E., Batra, S. K., & Mehta, P. P. (2012). Retinoids Regulate the Formation and Degradation of Gap Junctions in Androgen-Responsive Human Prostate Cancer Cells. *PLOS ONE*, *7*(4), e32846. https://doi.org/10.1371/journal.pone.0032846

Khatib, T., Marini, P., Nunna, S., Chisholm, D. R., Whiting, A., Redfern, C., Greig, I. R., & McCaffery, P. (2019). Genomic and non-genomic pathways are both crucial for peak induction of neurite outgrowth by retinoids. *Cell Communication and Signaling : CCS*, *17*, 40. https://doi.org/10.1186/s12964-019-0352-4

Klyuyeva, A. V., Belyaeva, O. V., Goggans, K. R., Krezel, W., Popov, K. M., & Kedishvili, N. Y. (2021). Changes in retinoid metabolism and signaling associated with metabolic remodeling during fasting and in type I diabetes. *The Journal of Biological Chemistry*, *296*, 100323. https://doi.org/10.1016/j.jbc.2021.100323

Kristan, W. B., & Calabrese, R. L. (1976). Rhythmic swimming activity in neurones of the isolated nerve cord of the leech. *The Journal of Experimental Biology*, *65*(3), 643–668. https://doi.org/10.1242/jeb.65.3.643

Lacchini, A. H., Davies, A. J., Mackintosh, D., & Walker, A. J. (2006). Beta-1, 3-glucan modulates PKC signalling in Lymnaea stagnalis defence cells: A role for PKC in H2O2 production and downstream ERK activation. *The Journal of Experimental Biology*, *209*(Pt 24), 4829–4840. https://doi.org/10.1242/jeb.02561

Lampe, P. D., & Lau, A. F. (2000). Regulation of gap junctions by phosphorylation of connexins. *Archives of Biochemistry and Biophysics*, *384*(2), 205–215. https://doi.org/10.1006/abbi.2000.2131

Landisman, C. E., Long, M. A., Beierlein, M., Deans, M. R., Paul, D. L., & Connors, B. W. (2002). Electrical synapses in the thalamic reticular nucleus. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *22*(3), 1002–1009.

Lasseigne, A. M., Echeverry, F. A., Ijaz, S., Michel, J. C., Martin, E. A., Marsh, A. J., Trujillo, E., Marsden, K. C., Pereda, A. E., & Miller, A. C. (2021). Electrical synaptic transmission requires a postsynaptic scaffolding protein. *ELife*, *10*, e66898. https://doi.org/10.7554/eLife.66898

Lawrence, T. S., Beers, W. H., & Gilula, N. B. (1978). Transmission of hormonal stimulation by cell-to-cell communication. *Nature*, *272*(5653), Article 5653. https://doi.org/10.1038/272501a0

Lefebvre, P., Benomar, Y., & Staels, B. (2010). Retinoid X receptors: Common heterodimerization partners with distinct functions. *Trends in Endocrinology & Metabolism*, *21*(11), 676–683. https://doi.org/10.1016/j.tem.2010.06.009

Lenz, M., Kruse, P., Eichler, A., Straehle, J., Beck, J., Deller, T., & Vlachos, A. (2021). All-trans retinoic acid induces synaptic plasticity in human cortical neurons. *ELife*, *10*, e63026. https://doi.org/10.7554/eLife.63026

Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., & Lovey, A. (1992). 9-cis retinoic acid stereoisomer binds and activates the nuclear receptor RXR alpha. *Nature*, *355*(6358), 359–361. https://doi.org/10.1038/355359a0

Llinas, R., Baker, R., & Sotelo, C. (1974). Electrotonic coupling between neurons in cat inferior olive. *Journal of Neurophysiology*, *37*(3), 560–571. https://doi.org/10.1152/jn.1974.37.3.560

Lohman, A. W., & Isakson, B. E. (2014). Differentiating connexin hemichannels and pannexin channels in cellular ATP release. *FEBS Letters*, *588*(8), 1379–1388. https://doi.org/10.1016/j.febslet.2014.02.004

Long, A. C., Bomser, J. A., Grzybowski, D. M., & Chandler, H. L. (2010). All-trans retinoic Acid regulates cx43 expression, gap junction communication and differentiation in primary lens epithelial cells. *Current Eye Research*, *35*(8), 670–679. https://doi.org/10.3109/02713681003770746

Lovat, P. E., Annicchiarico-Petruzzelli, M., Corazzari, M., Dobson, M. G., Malcolm, A. J., Pearson, A. D. J., Melino, G., & Redfern, C. P. F. (1999). Differential effects of retinoic acid isomers on the expression of nuclear receptor co-regulators in neuroblastoma. *FEBS Letters*, *445*(2), 415–419. https://doi.org/10.1016/S0014-5793(99)00162-3

Lynn, B. D., Li, X., & Nagy, J. I. (2012). Under construction: Building the macromolecular superstructure and signaling components of an electrical synapse. *The Journal of Membrane Biology*, *245*(5–6), 303–317. https://doi.org/10.1007/s00232-012-9451-5

Maden, M. (2007). Retinoic acid in the development, regeneration and maintenance of the nervous system. *Nature Reviews. Neuroscience*, *8*(10), 755–765. https://doi.org/10.1038/nrn2212

Maden, M., Keen, G., & Jones, G. E. (1998). Retinoic acid as a chemotactic molecule in neuronal development. *International Journal of Developmental Neuroscience: The Official Journal of the International Society for Developmental Neuroscience*, *16*(5), 317–322. https://doi.org/10.1016/s0736-5748(98)00046-x

Marder, E. (2009). Electrical synapses: Rectification demystified. *Current Biology: CB*, *19*(1), R34-35. https://doi.org/10.1016/j.cub.2008.11.008

Martin, A., Lasseigne, A. M., & Miller, A. C. (2020). Understanding the Molecular and Cell Biological Mechanisms of Electrical Synapse Formation. *Frontiers in Neuroanatomy*, *14*. https://www.frontiersin.org/articles/10.3389/fnana.2020.00012

Martin, A., & Pilar, G. (1963). TRANSMISSION THROUGH THE CILIARY GANGLION OF THE CHICK. *The Journal of Physiology*, *168*, 464–475. https://doi.org/10.1113/jphysiol.1963.sp007203

McCaffery, P., Wagner, E., O'Neil, J., Petkovich, M., & Dräger, U. C. (1999). Dorsal and ventral retinal territories defined by retinoic acid synthesis, break-down and nuclear receptor expression. *Mechanisms of Development*, *82*(1), 119–130. https://doi.org/10.1016/S0925-4773(99)00022-2

Meriney, S., & Fanselow, E. (2019). *Synaptic Transmission*. Academic Press.

Mersman, B. A., Jolly, S. N., Lin, Z., & Xu, F. (2020). Gap Junction Coding Innexin in Lymnaea stagnalis: Sequence Analysis and Characterization in Tissues and the Central Nervous System. *Frontiers in Synaptic Neuroscience*, *12*, 1. https://doi.org/10.3389/fnsyn.2020.00001

Mey, J., & McCaffery, P. (2004). Retinoic acid signaling in the nervous system of adult vertebrates. *The Neuroscientist*, *10*(5), 409–421. https://doi.org/10.1177/1073858404263520

Mey, J., McCaffery, P., & Dräger, U. C. (1997). Retinoic acid synthesis in the developing chick retina. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *17*(19), 7441–7449.

Mey, J., McCaffery, P., & Klemeit, M. (2001). Sources and sink of retinoic acid in the embryonic chick retina: Distribution of aldehyde dehydrogenase activities,

CRABP-I, and sites of retinoic acid inactivation. *Developmental Brain Research*, *127*(2), 135–148. https://doi.org/10.1016/S0165-3806(01)00127-4

Miller, A. C., & Pereda, A. E. (2017). The electrical synapse: Molecular complexities at the gap and beyond. *Developmental Neurobiology*, *77*(5), 562–574. https://doi.org/10.1002/dneu.22484

Misner, D. L., Jacobs, S., Shimizu, Y., de Urquiza, A. M., Solomin, L., Perlmann, T., De Luca, L. M., Stevens, C. F., & Evans, R. M. (2001). Vitamin A deprivation results in reversible loss of hippocampal long-term synaptic plasticity. *Proceedings of the National Academy of Sciences*, *98*(20), 11714–11719. https://doi.org/10.1073/pnas.191369798

Moreno, A. P., & Lau, A. F. (2007). Gap junction channel gating modulated through protein phosphorylation. *Progress in Biophysics and Molecular Biology*, *94*(1–2), 107–119. https://doi.org/10.1016/j.pbiomolbio.2007.03.004

Napoli, J. L. (1986). Quantification of physiological levels of retinoic acid. *Methods in Enzymology*, *123*, 112–124. https://doi.org/10.1016/s0076-6879(86)23015-3

Napoli, J. L. (2017). Cellular retinoid binding-proteins, CRBP, CRABP, FABP5: Effects on retinoid metabolism, function and related diseases. *Pharmacology & Therapeutics*, *173*, 19–33. https://doi.org/10.1016/j.pharmthera.2017.01.004

Nasser, T. I.-N. (2017). *Retinoic Acid and the Underlying Cellular Mechanisms involved in Neurite Outgrowth and Growth Cone Turning during Regeneration*. https://dr.library.brocku.ca/handle/10464/12908

Nowickyj, S. M., Chithalen, J. V., Cameron, D., Tyshenko, M. G., Petkovich, M., Wyatt, G. R., Jones, G., & Walker, V. K. (2008). Locust retinoid X receptors: 9-Cis-retinoic acid in embryos from a primitive insect. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.0712132105

Ochoa, W. F., Torrecillas, A., Fita, I., Verdaguer, N., Corbalán-García, S., & Gomez-Fernandez, J. C. (2003). Retinoic acid binds to the C2-domain of protein kinase Cα. *Biochemistry*, *42*(29), 8774–8779. https://doi.org/10.1021/bi034713g

Otopalik, A. G., Lane, B., Schulz, D. J., & Marder, E. (2019). Innexin expression in electrically coupled motor circuits. *Neuroscience Letters*, *695*, 19–24. https://doi.org/10.1016/j.neulet.2017.07.016

Oyamada, M., Oyamada, Y., & Takamatsu, T. (2005). Regulation of connexin expression. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1719*(1), 6–23. https://doi.org/10.1016/j.bbamem.2005.11.002

Penuela, S., Bhalla, R., Gong, X.-Q., Cowan, K. N., Celetti, S. J., Cowan, B. J., Bai, D., Shao, Q., & Laird, D. W. (2007). Pannexin 1 and pannexin 3 are glycoproteins that exhibit many distinct characteristics from the connexin family of gap junction proteins. *Journal of Cell Science*, *120*(Pt 21), 3772–3783. https://doi.org/10.1242/jcs.009514

Peracchia, C. (1973). Low resistance junctions in crayfish. II. Structural details and further evidence for intercellular channels by freeze-fracture and negative staining. *The Journal of Cell Biology*, *57*(1), 54–65. https://doi.org/10.1083/jcb.57.1.54

Pereda, A. E. (2014). Electrical synapses and their functional interactions with chemical synapses. *Nature Reviews. Neuroscience*, *15*(4), 250–263. https://doi.org/10.1038/nrn3708

Pereda, A. E., & Faber, D. S. (1996). Activity-dependent short-term enhancement of intercellular coupling. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *16*(3), 983–992. https://doi.org/10.1523/JNEUROSCI.16-03-00983.1996

Perez Velazquez, J. L., & Carlen, P. L. (2000). Gap junctions, synchrony and seizures. *Trends in Neurosciences*, *23*(2), 68–74. https://doi.org/10.1016/S0166-2236(99)01497-6

Piskunov, A., Al Tanoury, Z., & Rochette-Egly, C. (2014). Nuclear and extra-nuclear effects of retinoid acid receptors: How they are interconnected. *Sub-Cellular Biochemistry*, *70*, 103–127. https://doi.org/10.1007/978-94-017-9050-5_6

Pogoda, K., Kameritsch, P., Retamal, M. A., & Vega, J. L. (2016). Regulation of gap junction channels and hemichannels by phosphorylation and redox changes: A revision. *BMC Cell Biology*, *17*(1), 11. https://doi.org/10.1186/s12860-016-0099-3

Poon, M. M., & Chen, L. (2008). Retinoic acid-gated sequence-specific translational control by RARα. *Proceedings of the National Academy of Sciences*, *105*(51), 20303–20308. https://doi.org/10.1073/pnas.0807740105

Potenza, N., del Gaudio, R., Luisa Chiusano, M., Maria Rosaria Russo, G., & Geraci, G. (2003). Specificity of Cellular Expression of C. variopedatus Polychaete Innexin in the Developing Embryo: Evolutionary Aspects of Innexins' Heterogeneous Gene Structures. *Journal of Molecular Evolution*, *57*(1), S165–S173. https://doi.org/10.1007/s00239-003-0023-2

Quinn, S. D., & De Boni, U. (1991). Enhanced neuronal regeneration by retinoic acid of murine dorsal root ganglia and of fetal murine and human spinal cord in vitro. *In Vitro Cellular & Developmental Biology: Journal of the Tissue Culture Association*, *27*(1), 55–62. https://doi.org/10.1007/BF02630895

Radominska-Pandya, A., Chen, G., Czernik, P. J., Little, J. M., Samokyszyn, V. M., Carter, C. A., & Nowak, G. D. (2000). Direct interaction of all-*trans*-retinoic acid with protein kinase C (PKC). Implications for PKC signaling and cancer therapy. *The Journal of Biological Chemistry*, *275*(29), 22324–22330. https://doi.org/10.1074/jbc.M907722199

Rand, C. (2012). *Elucidation of the Retinoid Signalling Pathway Involved in Axon Guidance in Lymnaea stagnalis and Xenopus laevis* [M.Sc. Thesis, Brock University]. https://dr.library.brocku.ca/handle/10464/4138

Rand, C., Spencer, G. E., & Carlone, R. L. (2017). Retinoic acid as a chemoattractant for cultured embryonic spinal cord neurons of the African Clawed Frog, Xenopus laevis. *Canadian Journal of Zoology*, *95*(9), 653–661. https://doi.org/10.1139/cjz-2016-0279

Redfern, C. P. F., Lovat, P. E., Malcolm, A. J., & Pearson, A. D. J. (1995). Gene expression and neuroblastoma cell Differentiation in response to retinoic acid: Differential effects of 9-cis and all-trans retinoic acid. *European Journal of Cancer*, *31*(4), 486–494. https://doi.org/10.1016/0959-8049(95)00066-R

Reitzel, A. M., Macrander, J., Mane-Padros, D., Fang, B., Sladek, F. M., & Tarrant, A. M. (2018). Conservation of DNA and ligand binding properties of retinoid X receptor from the placozoan *Trichoplax adhaerens* to human. *The Journal of Steroid Biochemistry and Molecular Biology*, *184*, 3–10. https://doi.org/10.1016/j.jsbmb.2018.02.010

Rekling, J. C., Shao, X. M., & Feldman, J. L. (2000). Electrical coupling and excitatory synaptic transmission between rhythmogenic respiratory neurons in the preBötzinger complex. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *20*(23), RC113. https://doi.org/10.1523/JNEUROSCI.20-23-j0003.2000

Rörig, B., & Sutor, B. (1996). Serotonin Regulates Gap Junction Coupling in the Developing Rat Somatosensory Cortex. *European Journal of Neuroscience*, *8*(8), 1685–1695. https://doi.org/10.1111/j.1460-9568.1996.tb01312.x

Rothwell, C. M., de Hoog, E., & Spencer, G. E. (2017). The role of retinoic acid in the formation and modulation of invertebrate central synapses. *Journal of Neurophysiology*, *117*(2), 692–704. https://doi.org/10.1152/jn.00737.2016

Rothwell, C. M., & Spencer, G. E. (2014). Retinoid signaling is necessary for, and promotes long-term memory formation following operant conditioning. *Neurobiology of Learning and Memory*, *114*, 127–140. https://doi.org/10.1016/j.nlm.2014.05.010

Sadamoto, H., Takahashi, H., Kobayashi, S., Kondoh, H., & Tokumaru, H. (2021). Identification and classification of innexin gene transcripts in the central nervous system of the terrestrial slug Limax valentianus. *PloS One*, *16*(4), e0244902. https://doi.org/10.1371/journal.pone.0244902

Sáez, J. C., Connor, J. A., Spray, D. C., & Bennett, M. V. (1989). Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-trisphosphate, and to calcium ions. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(8), 2708–2712. https://doi.org/10.1073/pnas.86.8.2708

Sakurai, A., & Katz, P. S. (2016). The central pattern generator underlying swimming in Dendronotus iris: A simple half-center network oscillator with a twist. *Journal of Neurophysiology*, *116*(4), 1728–1742. https://doi.org/10.1152/jn.00150.2016

Sánchez, A., Castro, C., Flores, D.-L., Gutiérrez, E., & Baldi, P. (2019). Gap Junction Channels of Innexins and Connexins: Relations and Computational Perspectives. *International Journal of Molecular Sciences*, *20*(10), Article 10. https://doi.org/10.3390/ijms20102476

Sarti, F., Schroeder, J., Aoto, J., & Chen, L. (2012). Conditional RARα knockout mice reveal acute requirement for retinoic acid and RARα in homeostatic plasticity. *Frontiers in Molecular Neuroscience*, *5*, 16. https://doi.org/10.3389/fnmol.2012.00016

Sarti, F., Zhang, Z., Schroeder, J., & Chen, L. (2013). Rapid suppression of inhibitory synaptic transmission by retinoic acid. *The Journal of Neuroscience*, *33*(28), 11440–11450. https://doi.org/10.1523/JNEUROSCI.1710-13.2013

Sasaki, Y. (2020). Local translation in growth cones and presynapses, two axonal compartments for local neuronal functions. In *Biomolecules*. https://doi.org/10.3390/biom10050668

Sass, J. O., Masgrau, E., Saurat, J. H., & Nau, H. (1995). Metabolism of oral 9-cis-retinoic acid in the human. Identification of 9-cis-retinoyl-beta-glucuronide and 9-cis-4-oxo-retinoyl-beta-glucuronide as urinary metabolites. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *23*(9), 887–891.

Sayyah, M., Rezaie, M., Haghighi, S., & Amanzadeh, A. (2007). Intra-amygdala all-trans retinoic acid inhibits amygdala-kindled seizures in rats. *Epilepsy Research*, *75*(2), 97–103. https://doi.org/10.1016/j.eplepsyres.2007.04.010

Schuldiner, M., Eiges, R., Eden, A., Yanuka, O., Itskovitz-Eldor, J., Goldstein, R. S., & Benvenisty, N. (2001). Induced neuronal differentiation of human embryonic stem cells. *Brain Research*, *913*(2), 201–205. https://doi.org/10.1016/s0006-8993(01)02776-7

Sidhu, M., Belliveau, D. J., Sidhu, M., & Belliveau, D. J. (2013). Connexin36 is a Negative Regulator of Differentiation in Human Neuroblastoma. In *Neuroblastoma*. IntechOpen. https://doi.org/10.5772/55643

Simões-Costa, M. S., Azambuja, A. P., & Xavier-Neto, J. (2008). The search for non-chordate retinoic acid signaling: Lessons from chordates. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution*, *310*(1), 54–72. https://doi.org/10.1002/jez.b.21139

Skerrett, I. M., & Williams, J. B. (2017). A structural and functional comparison of gap junction channels composed of connexins and innexins. *Developmental Neurobiology*, *77*(5), 522–547. https://doi.org/10.1002/dneu.22447

Söhl, G., & Willecke, K. (2004). Gap junctions and the connexin protein family. *Cardiovascular Research*, *62*(2), 228–232. https://doi.org/10.1016/j.cardiores.2003.11.013

Solan, J. L., & Lampe, P. D. (2009). Connexin43 phosphorylation: Structural changes and biological effects. *The Biochemical Journal*, *419*(2), 261–272. https://doi.org/10.1042/BJ20082319

Sosinsky, G. E., Boassa, D., Dermietzel, R., Duffy, H. S., Laird, D. W., MacVicar, B., Naus, C. C., Penuela, S., Scemes, E., Spray, D. C., Thompson, R. J., Zhao, H.-B., & Dahl, G. (2011). Pannexin channels are not gap junction hemichannels. *Channels*, *5*(3), 193–197. https://doi.org/10.4161/chan.5.3.15765

Spencer, G. E., Syed, N. I., van Kesteren, E., Lukowiak, K., Geraerts, W. P., & van Minnen, J. (2000). Synthesis and functional integration of a neurotransmitter receptor in isolated invertebrate axons. *Journal of Neurobiology*, *44*(1), 72–81. https://doi.org/10.1002/1097-4695(200007)44:1<72::aid-neu7>3.0.co;2-#

Starich, T., Sheehan, M., Jadrich, J., & Shaw, J. (2001). Innexins in C. elegans. *Cell Communication & Adhesion*, *8*(4–6), 311–314. https://doi.org/10.3109/15419060109080744

Stebbings, L. A., Todman, M. G., Phillips, R., Greer, C. E., Tam, J., Phelan, P., Jacobs, K., Bacon, J. P., & Davies, J. A. (2002). Gap junctions in Drosophila: Developmental expression of the entire innexin gene family. *Mechanisms of Development*, *113*(2), 197–205. https://doi.org/10.1016/S0925-4773(02)00025-4

Sukiban, J., Bräunig, P., Mey, J., & Bui-Göbbels, K. (2014). Retinoic acid as a survival factor in neuronal development of the grasshopper, Locusta migratoria. *Cell and Tissue Research*, *358*(2), 303–312. https://doi.org/10.1007/s00441-014-1957-y

Susswein, A. J., Hurwitz, I., Thorne, R., Byrne, J. H., & Baxter, D. A. (2002). Mechanisms Underlying Fictive Feeding in Aplysia: Coupling Between a Large Neuron With Plateau Potentials Activity and a Spiking Neuron. *Journal of Neurophysiology*, *87*(5), 2307–2323. https://doi.org/10.1152/jn.2002.87.5.2307

Syed, N. I., & Winlow, W. (1989). Morphology and electrophysiology of neurons innervating the ciliated locomotor epithelium in Lymnaea stagnalis (L.). *Comparative Biochemistry and Physiology Part A: Physiology*, *93*(3), 633–644. https://doi.org/10.1016/0300-9629(89)90024-8

Takahashi, B., Ohta, K., Kawachi, E., Fukasawa, H., Hashimoto, Y., & Kagechika, H. (2002). Novel Retinoid X Receptor Antagonists: Specific Inhibition of Retinoid Synergism in RXR−RAR Heterodimer Actions. *Journal of Medicinal Chemistry*, *45*(16), 3327–3330. https://doi.org/10.1021/jm0255320

Tanoury, A. Z., Piskunov, A., & Rochette-Egly, C. (2013). Vitamin A and retinoid signaling: Genomic and nongenomic effects: Thematic Review Series: Fat-Soluble Vitamins: Vitamin A. *Journal of Lipid Research*, *54*(7), 1761–1775. https://doi.org/10.1194/jlr.R030833

Thapliyal, S., Arendt, K. L., Lau, A. G., & Chen, L. (2022). Retinoic acid-gated BDNF synthesis in neuronal dendrites drives presynaptic homeostatic plasticity. *ELife*, *11*, e79863. https://doi.org/10.7554/eLife.79863

Theodosiou, M., Laudet, V., & Schubert, M. (2010). From carrot to clinic: An overview of the retinoic acid signaling pathway. *Cellular and Molecular Life Sciences*, *67*(9), 1423–1445. https://doi.org/10.1007/s00018-010-0268-z

Travis, G. H., Golczak, M., Moise, A. R., & Palczewski, K. (2007). Diseases caused by defects in the visual cycle: Retinoids as potential therapeutic agents. *Annual Review of Pharmacology and Toxicology*, *47*, 469–512. https://doi.org/10.1146/annurev.pharmtox.47.120505.105225

Tsuji, M., Shudo, K., & Kagechika, H. (2015). Docking simulations suggest that all-trans retinoic acid could bind to retinoid X receptors. *Journal of Computer-Aided Molecular Design*, *29*(10), 975–988. https://doi.org/10.1007/s10822-015-9869-9

Urushitani, H., Katsu, Y., Kagechika, H., Sousa, A. C. A., Barroso, C. M., Ohta, Y., Shiraishi, H., Iguchi, T., & Horiguchi, T. (2018). Characterization and comparison of transcriptional activities of the retinoid X receptors by various organotin compounds in three prosobranch gastropods; *Thais clavigera*, *Nucella lapillus* and *Babylonia japonica*. *Aquatic Toxicology (Amsterdam, Netherlands)*, *199*, 103–115. https://doi.org/10.1016/j.aquatox.2018.03.029

Urushitani, H., Katsu, Y., Ohta, Y., Shiraishi, H., Iguchi, T., & Horiguchi, T. (2011). Cloning and characterization of retinoid X receptor (RXR) isoforms in the rock shell, *Thais clavigera*. *Aquatic Toxicology*, *103*(1–2), 101–111. https://doi.org/10.1016/j.aquatox.2011.02.012

Urushitani, H., Katsu, Y., Ohta, Y., Shiraishi, H., Iguchi, T., & Horiguchi, T. (2013). Cloning and characterization of the retinoic acid receptor-like protein in the rock shell, *Thais clavigera*. *Aquatic Toxicology*, *142–143*, 403–413. https://doi.org/10.1016/j.aquatox.2013.09.008

Valenstein, E. S. (2005). *The War of the Soups and the Sparks: The Discovery of Neurotransmitters and the Dispute Over How Nerves Communicate* (p. 256 Pages). Columbia University Press.

Van Kesteren, R. E., Carter, C., Dissel, H. M. G., Van Minnen, J., Gouwenberg, Y., Syed, N. I., Spencer, G. E., & Smit, A. B. (2006). Local synthesis of actin-binding protein β-thymosin regulates neurite outgrowth. *Journal of Neuroscience*. https://doi.org/10.1523/JNEUROSCI.4164-05.2006

Van Minnen, J., Bergman, J. J., Van Kesteren, E. R., Smit, A. B., Geraerts, W. P., Lukowiak, K., Hasan, S. U., & Syed, N. I. (1997). De novo protein synthesis in isolated axons of identified neurons. *Neuroscience*, *80*(1), 1–7. https://doi.org/10.1016/s0306-4522(97)00137-1

Van Soest, P. F., Lodder, J. C., & Kits, K. S. (2000). Activation of Protein Kinase C by Oxytocin-Related Conopressin Underlies Pacemaker Current in Lymnaea Central Neurons. *Journal of Neurophysiology*, *84*(5), 2541–2551. https://doi.org/10.1152/jn.2000.84.5.2541

Vanden Abeele, F., Bidaux, G., Gordienko, D., Beck, B., Panchin, Y. V., Baranova, A. V., Ivanov, D. V., Skryma, R., & Prevarskaya, N. (2006). Functional implications of calcium permeability of the channel formed by pannexin 1. *The Journal of Cell Biology*, *174*(4), 535–546. https://doi.org/10.1083/jcb.200601115

Veenstra, R. D. (2001). Determining ionic permeabilities of gap junction channels. *Methods in Molecular Biology (Clifton, N.J.)*, *154*, 293–311. https://doi.org/10.1385/1-59259-043-8:293

Vesprini, N., Dawson, T. F., Yuan, Y., Bruce, D., & Spencer, G. E. (2015). Retinoic acid affects calcium signaling in adult molluscan neurons. *Journal of Neurophysiology*, *113*(1), 172–181. https://doi.org/10.1152/jn.00458.2014

Vesprini, N., & Spencer, G. E. (2014). Retinoic acid induces changes in electrical properties of adult neurons in a dose- and isomer-dependent manner. *Journal of Neurophysiology*, *111*(6), 1318–1330. https://doi.org/10.1152/jn.00434.2013

Vivat, V., Zechel, C., Wurtz, J.-M., Bourguet, W., Kagechika, H., Umemiya, H., Shudo, K., Moras, D., Gronemeyer, H., & Chambon, P. (1997). A mutation mimicking ligand-induced conformational change yields a constitutive RXR that senses allosteric effects in heterodimers. *The EMBO Journal*, *16*(18), 5697–5709. https://doi.org/10.1093/emboj/16.18.5697

Walker, S. E., Spencer, G. E., Necakov, A., & Carlone, R. L. (2018). Identification and characterization of microRNAs during retinoic acid-induced regeneration of a molluscan central nervous system. *International Journal of Molecular Sciences*, *19*(9), 2741. https://doi.org/10.3390/ijms19092741

Wang, H. Z., & Veenstra, R. D. (1997). Monovalent ion selectivity sequences of the rat connexin43 gap junction channel. *The Journal of General Physiology*, *109*(4), 491–507. https://doi.org/10.1085/jgp.109.4.491

Wang, H.-L., Zhang, Z., Hintze, M., & Chen, L. (2011). Decrease in calcium concentration triggers neuronal retinoic acid synthesis during homeostatic synaptic plasticity. *The Journal of Neuroscience*, *31*(49), 17764–17771. https://doi.org/10.1523/JNEUROSCI.3964-11.2011

Wang, Y., Song, J.-H., Denisova, J. V., Park, W.-M., Fontes, J. D., & Belousov, A. B. (2012). Neuronal Gap Junction Coupling Is Regulated by Glutamate and Plays Critical Role in Cell Death during Neuronal Injury. *The Journal of Neuroscience*, *32*(2), 713–725. https://doi.org/10.1523/JNEUROSCI.3872-11.2012

Watanabe, A. (1958). The interaction of electrical activity among neurons of lobster cardiac ganglion. *The Japanese Journal of Physiology*, *8*(4), 305–318. https://doi.org/10.2170/jjphysiol.8.305

Watanabe, M., & Kakuta, H. (2018). Retinoid X Receptor Antagonists. *International Journal of Molecular Sciences*, *19*(8), 2354. https://doi.org/10.3390/ijms19082354

Welzel, G., & Schuster, S. (2022). Connexins evolved after early chordates lost innexin diversity. *ELife*, *11*, e74422. https://doi.org/10.7554/eLife.74422

Weng, S., Lauven, M., Schaefer, T., Polontchouk, L., Grover, R., & Dhein, S. (2002). Pharmacological modification of gap junction coupling by an antiarrhythmic peptide via protein kinase C activation. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *16*(9), 1114–1116. https://doi.org/10.1096/fj.01-0918fje

White, T. W., Wang, H., Mui, R., Litteral, J., & Brink, P. R. (2004). Cloning and functional expression of invertebrate connexins from Halocynthia pyriformis. *FEBS Letters*, *577*(1–2), 42–48. https://doi.org/10.1016/j.febslet.2004.09.071

Wilczynska, A., & Bushell, M. (2015). The complexity of miRNA-mediated repression. *Cell Death & Differentiation*, *22*(1), Article 1. https://doi.org/10.1038/cdd.2014.112

Wingrove, J., de Hoog, E., & Spencer, G. E. (2023). Disruptions in network plasticity precede deficits in memory following inhibition of retinoid signaling. *Journal of Neurophysiology*, *129*(1), 41–55. https://doi.org/10.1152/jn.00270.2022

Wu, C.-L., Shih, M.-F. M., Lai, J. S.-Y., Yang, H.-T., Turner, G. C., Chen, L., & Chiang, A.-S. (2011). Heterotypic gap junctions between two neurons in the drosophila brain are critical for memory. *Current Biology*, *21*(10), 848–854. https://doi.org/10.1016/j.cub.2011.02.041

Wu, J., Taylor, R. N., & Sidell, N. (2013). Retinoic acid regulates gap junction intercellular communication in human endometrial stromal cells through modulation of the phosphorylation status of connexin 43. *Journal of Cellular Physiology*, *228*(4), 903–910. https://doi.org/10.1002/jcp.24241

Wuarin, L., Sidell, N., & de Vellis, J. (1990). Retinoids increase perinatal spinal cord neuronal survival and astroglial differentiation. *International Journal of Developmental Neuroscience: The Official Journal of the International Society for Developmental Neuroscience*, *8*(3), 317–326. https://doi.org/10.1016/0736-5748(90)90038-4

Yang, X. D., Korn, H., & Faber, D. S. (1990). Long-term potentiation of electrotonic coupling at mixed synapses. *Nature*, *348*(6301), 542–545. https://doi.org/10.1038/348542a0

Yang, Y., Qin, S.-K., Wu, Q., Wang, Z.-S., Zheng, R.-S., Tong, X.-H., Liu, H., Tao, L., & He, X.-D. (2014). Connexin-dependent gap junction enhancement is involved in the synergistic effect of sorafenib and all-trans retinoic acid on HCC growth inhibition. *Oncology Reports*, *31*(2), 540–550. https://doi.org/10.3892/or.2013.2894

Zhang, & McMahon. (2000). Direct gating by retinoic acid of retinal electrical synapses. *Proceedings of the National Academy of Sciences*, *97*(26), 14754–14759. https://doi.org/10.1073/pnas.010325897

Zhang, X., Ren, Z., Zuo, J., Su, C., Wang, R., Chang, Y., & Fang, F. (2002). The effect of all-trans retinoic acid on gap junctional intercellular communication and connexin 43 gene expression in glioma cells. *Chinese Medical Sciences Journal = Chung-Kuo I Hsueh K'o Hsueh Tsa Chih*, *17*(1), 22–26.