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CNQX and AMPA inhibit electrical synaptic transmission: a potential interaction between electrical and glutamatergic synapses

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Abstract

Electrical synapses play an important role in signaling between neurons and the synaptic connections between many neurons possess both electrical and chemical components. Although modulation of electrical synapses is frequently observed, the cellular processes that mediate such changes have not been studied as thoroughly as plasticity in chemical synapses. In the leech (*Hirudo sp.*), the competitive AMPA receptor antagonist CNQX inhibited transmission at the rectifying electrical synapse of a mixed glutamatergic/electrical synaptic connection. This CNQX-mediated inhibition of the electrical synapse was blocked by concanavalin A (Con A) and dynamin inhibitory peptide (DIP), both of which are known to inhibit endocytosis of neurotransmitter receptors. CNQX-mediated inhibition was also blocked by pep2-SVKI (SVKI), a synthetic peptide that prevents internalization of AMPA-type glutamate receptor. AMPA itself also inhibited electrical synaptic transmission and this AMPA-mediated inhibition was partially blocked by Con A, DIP and SVKI. Low frequency stimulation induced long-term depression (LTD) in both the electrical and chemical components of these synapses and this LTD was blocked by SVKI. GYKI 52466, a selective non-competitive antagonist of AMPA receptors, did not affect the electrical EPSP, although it did block the chemical component of these synapses. CNQX did not affect non-rectifying electrical synapses in two different pairs of neurons. These results suggest an interaction between AMPA-type glutamate receptors and the gap junction proteins that mediate electrical synaptic transmission. This putative interaction between glutamate receptors and gap junction proteins represents a novel mechanism for regulating the strength of synaptic transmission.

Keywords

Gap junction; innexin; leech; synaptic plasticity; glutamate receptor

1. Introduction

Electrical synapses provide a direct pathway for ionic and biochemical communication between cells and play a critical role in neuronal signaling. In neural networks which contain large numbers of cells, such as in cortical circuits or the retina, electrical synapses play a critical

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role in synchronizing activity between interconnected neurons (Roerig & Feller 2000; Bennett & Zukin 2004; Meier & Dermietzel 2006). Many synapses possess both electrical and chemical components and it is now apparent that plasticity in the chemical synaptic component can lead to changes in the electrical component (Johnson et al. 1994; Smith & Pereda 2003; Pereda et al. 2004). In addition, a number of recent studies have shown that gap junctions are closely associated with postsynaptic densities (PSD) (Sotelo et al. 1978; Rash et al. 2000; Lynn et al. 2001; Zoidl et al. 2007). This proximity of the electrical and chemical synaptic components may allow for an interaction between the two modes of transmission either by direct protein-protein interaction or by short intracellular signaling pathways.

The leech (*Hirudo sp*) provides a useful system to study the interaction between electrical and chemical synaptic transmission. The leech CNS is well characterized (see review by Kristan et al. 2005) and there are a number of synapses between readily-identifiable cells known to have both electrical and chemical components. For example, the mechanosensory touch cells (T cells), of which there are three bilateral pairs, form synaptic connections with each other that have both an electrical and chemical component (Nicholls & Baylor 1968; Baylor and Nicholls 1969). In this study, the relationship between electrical and glutamatergic transmission in the T-to-T synaptic connection was investigated. Electrical synaptic transmission was found to be inhibited by both CNQX (a competitive antagonist of AMPA/Kainate glutamate receptors) and AMPA (a selective agonist of AMPA/Kainate receptors). The CNQX/AMPA-mediated inhibition can be at least partially blocked by concanavalin A (Con A) or dynamin inhibitory peptide (DIP), which inhibits clathrin-dependent endocytosis, and by pep2-SVKI, a synthetic peptide that inhibits internalization of AMPA-type glutamate receptors. These results indicate an interaction between glutamate receptors and the gap junction proteins that mediate electrical synaptic transmission.

2. Results

Properties of the T-to-T electrical synapse

As first described by Baylor and Nicholls (1969), the T-to-T synapse has a monosynaptic electrical component and a polysynaptic chemical component (Figure 1A). It is impossible to distinguish the electrical and chemical components of the T-to-T synapse in normal saline at room temperature (Figure 1B, top). However, the electrical EPSP can be isolated by recording in 15mM Mg^{2+} saline solution to selectively block chemical synaptic transmission (Fig. 1B, top; Del Castillo & Katz 1954; Baylor & Nicholls 1969). To confirm that 15mM Mg^{2+} saline solution removes the entire chemical component of the T-to-T EPSP, the ganglion was cooled to approximately 15°C, delaying the onset of the chemical EPSP just enough to allow the electrical and chemical components to be distinguished (Figure 1B, bottom; also see Nicholls & Purves 1972). In these low temperature recordings the later chemical component was completely abolished when 15mM Mg^{2+} saline was applied, leaving only the earlier electrical component. All subsequent intracellular recordings were conducted at room temperature in 15mM Mg^{2+} saline to eliminate chemical synaptic transmission unless otherwise stated.

Normally, electrical synaptic function is studied by measuring the coupling coefficient between two neurons linked by electrical synapses. However, no current flows from the presynaptic to the postsynaptic cell in the T-to-T electrical synapse unless an action potential is initiated in the presynaptic neuron (Fig. 1C, D; Baylor and Nicholls 1969; Acklin, 1988). The dependence of the T-to-T electrical connection on action potential firing indicates that this electrical synapse has a voltage threshold which shifts from a non-conducting to a conducting state upon the arrival of an action potential, similar to what has been reported in giant motor synapses of the crayfish (Furshpan & Potter, 1959). This property of T-to-T electrical synapses is likely due to the voltage-sensitive properties of the gap junction proteins (innexins) that mediate electrical synaptic transmission between T cells and is consistent with observations from

vertebrate connexins that have a relatively high voltage threshold (-30 to 0 mV; Werner et al. 1989; Ebihara et al. 1999; White et al. 1999). The latency of the EPSP to the action potential was very brief, ≤ 0.6 msec and persisted in 15 mM Mg^{2+} saline, consistent with an electrical synapse (Del Castillo and Katz 1954). Sustained current flow between two T cells could be observed when a long, suprathreshold depolarizing current pulse was injected into one T cell (Fig. 1D), indicating that the gap junctions remain open for a time following the activating action potential. Hyperpolarizing current did not flow between the coupled T cells (Fig. 1C). All of these observations are consistent with earlier findings from Baylor and Nicholls (1969). Despite the fact that it was possible to measure sustained current flow between the coupled T cells, most of the subsequent experiments examining changes in the strength of the T-to-T electrical connection were made by measuring the electrical EPSP elicited by the T cell action potential. This was done because the electrical EPSP represented the normal, physiologically-relevant function of the T-to-T electrical synapse.

Effect of CNQX on the T-to-T electrical synapse

When the T-to-T synapse was recorded with $20 \mu M$ CNQX in normal saline, the EPSP amplitude decreased (Fig. 2A) indicating that the chemical component of this synaptic connection was glutamatergic. As part of a control experiment, the effect of CNQX on the T-to-T synapse was tested in the presence of 15 mM Mg^{2+} saline, when only the electrical synapse would be active (Del Castillo & Katz 1954; Baylor & Nicholls 1969). Surprisingly, the T-to-T electrical EPSP was significantly reduced in CNQX-treated ganglia when compared to a control group in which CNQX was omitted (Fig. 2B Two-way ANOVA treatment effect $F_{1,36}=36.8$, $P<0.0001$, time effect $F_{3,36}=11.2$, $P<0.0001$, interaction effect $F_{3,36}=4.3$, $P<0.05$. Newman Keuls post hoc test showed significant differences at both 5 min and 15 min recordings, $P<0.05$). No changes in input resistance were observed in either the CNQX-treated or saline control groups making it unlikely that the CNQX effect was due to shunting of current out of the cell (Fig. 2B Bar graph One-way ANOVA $P>0.05$). Inhibition of the T-to-T electrical synapse by CNQX was concentration-dependent (Fig. 2C, D) with significant inhibition observed at 10 , 20 and $200 \mu M$ CNQX (Fig. 2C, D One-way ANOVA $P<0.0001$). GYKI 52466 (GYKI; $20 \mu M$), which is a non-competitive antagonist of AMPA receptors (Donevan & Rogawski 1993; Zappala et al. 2001), also blocked the chemical component of the T-to-T synapse when the EPSP was recorded in normal saline, just like CNQX, but had no effect on the electrical EPSP recorded in 15 mM Mg^{2+} saline (data not shown, compared with control group Two-way ANOVA $P>0.05$).

How might CNQX mediate depression of the electrical synapse? In addition to being a competitive antagonist, CNQX could induce internalization of AMPA-type glutamate receptors (Lin et al. 2000). It was possible that there was an interaction between glutamate receptors and the gap junction proteins at these synapses, such that AMPA receptors internalization produced a decrease in electrical synaptic transmission. There were no known antibodies that recognize leech versions of glutamate receptors or innexins, therefore we explored potential involvement of receptor internalization by applying CNQX in the presence of concanavalin A (Con A), which had been used to block clathrin-dependent internalization of neurotransmitter receptors (Paatero et al. 1988; Mayer et al. 1989; Xiang et al. 2002; Kim et al. 2004; Arttamangkul et al. 2006). When Con A ($600 \mu g/ml$) was co-applied with CNQX, the CNQX-mediated depression of the electrical synapse was completely blocked (Figure 3A, D Two-way ANOVA between Con A + CNQX and CNQX group, $P<0.01$). Con A application by itself had no effect on the T-to-T electrical synapse.

Dynamin inhibitory peptide (DIP), a blocker of endocytosis (Marks & McMahon 1998; Wigge & McMahon 1998; Carroll et al. 1999; Morishita & Marie 2005) was also tested. DIP ($50 \mu M$) was not as effective as Con A, but nevertheless did interfere with CNQX-mediated depression

of the electrical synapse. At the beginning of the CNQX treatment, DIP did block depression of the electrical synapse (Figure 3B, 5min). Two-way ANOVA showed three groups were significantly different from each other (Treatment effect $F_{2,40}=18.0$, $P<0.0001$, time effect $F_{3,40}=9.1$, $P<0.001$, interaction effect $F_{6,40}=2.7$, $P<0.05$). The DIP + CNQX group was significantly different from the CNQX group at 5min and 10min (Newman Keuls post hoc, $P<0.05$). However, by the end of CNQX treatment there was no significant difference between the DIP+CNQX and CNQX groups (Figure 3B Newman Keuls post hoc, $P>0.05$). The above experiments were repeated using 100 μ M DIP, but this concentration was no more effective than 50 μ M DIP (data not shown).

Pep2-SVKI (SVKI) is a synthetic peptide that blocks the interaction between glutamate receptor subunits and PDZ domain-containing proteins such as glutamate receptor-interacting proteins (GRIPs) and protein interacting with C kinase (PICK), prevented AMPA receptor internalization (Li et al. 1999; Daw et al. 2000; Thalhammer et al. 2002). To date, SVKI has not been used on invertebrate neurons, but invertebrate AMPA-type glutamate receptors do contain PDZ-binding domains and the mechanisms involved in glutamate receptor trafficking appear to be conserved between vertebrates and invertebrates (Chang and Rongo 2005; Walker et al. 2006). Treatment of the postsynaptic T cell with 100 μ M SVKI completely blocked CNQX-mediated inhibition of the T-to-T electrical synapse (Fig. 3C, D Two-way ANOVA between CNQX+SVKI and control group $P>0.1$; between CNQX + SVKI and CNQX group $P<0.05$). SVKI alone did not affect electrical synaptic transmission. Taken together, the Con A, DIP and SVKI results support the hypothesis that the CNQX-mediated inhibition of the electrical synapse involves glutamate receptor internalization.

Effect of AMPA on the T-to-T electrical synapse

AMPA itself is also known to induce internalization of AMPA-type glutamate receptors and is more effective at stimulating internalization than CNQX (Man et al. 2000; Lin et al. 2000). Therefore, the effects of AMPA on T-to-T electrical synaptic transmission were also examined. 100 μ M AMPA completely abolished the T-to-T electrical EPSP by the end of a 15min treatment (Figure 4A, B Two-way ANOVA, treatment effect $F_{2,24}=112.1$ $P<0.0001$; time effect $F_{3,24}=58.7$; $P<0.0001$; interaction effect $F_{6,24}=13.7$ $P<0.0001$). The effect of 10 μ M AMPA was similar to 100 μ M AMPA, but was slower to develop and did not completely eliminate the electrical EPSP (Fig. 4A, B). Con A, DIP and SVKI were able to inhibit the initial 10 μ M AMPA-induced depression of the electrical synapse, but could not prevent depression at later time points in the AMPA treatment (Fig. 4C Two-way ANOVA, treatment effect $F_{2,20}=17.7$ $P<0.001$, time effect $F_{3,20}=17.7$ $P<0.001$, interaction effect $F_{6,20}=3.7$ $P<0.01$; Newman Keuls post hoc test 5min Con A + AMPA group was significantly different from AMPA group $P<0.01$ but not significantly different from control group $P>0.1$; Fig. 4D Two-way ANOVA, $F_{2,36}=23.2$ $P<0.0001$, $F_{3,36}=8.2$ $P<0.001$, $F_{6,36}=3.64$ $P<0.01$; Newman Keuls post hoc test 5min DIP + AMPA group was significantly different from AMPA group $P<0.05$ but not significantly different from control group $P>0.05$; Fig. 4E Two-way ANOVA, treatment effect $F=17.1$ $P<0.001$, interaction effect $F_{6,20}=3.1$ $P<0.01$; Newman Keuls post hoc test 5min AMPA+SVKI group was significantly different from AMPA group $P<0.05$ but not significantly different from control group $P>0.1$). Neither concentration of AMPA had a significant effect on input resistance, indicating that AMPA's effect on the electrical EPSP was not due to a shunting of currents out of the cell (data not shown, one-way ANOVA $P>0.05$). This is not surprising given that AMPA receptors rapidly desensitize. Again, these results are consistent with the hypothesis that there is an interaction between AMPA receptors and innexins at the T-to-T synapse in which ligand binding to the AMPA receptor induces inhibition of the electrical synapse that involves AMPA receptor internalization.

The effect of AMPA on chemical synaptic transmission was also tested by recording T-to-T synaptic transmission in normal saline since AMPA-induced internalization of AMPA receptors would be expected to reduce the chemical/glutamatergic component of this synapse. The decrease in the T-to-T EPSP during the first 5min of 10 μ M AMPA treatment in normal saline was substantially greater ($\approx$ 80%) when compared to the level of inhibition during the same time point 10 μ M AMPA treatment in 15mM Mg²⁺ saline ($\approx$ 40%; Fig. 4A, F). This is consistent with both the chemical and electrical components of the T-to-T synapse being inhibited by AMPA treatment. Con A prevented the AMPA-mediated inhibition of the T-to-T synapse during the first 5min of the AMPA treatment in normal saline (Fig. 4F Two-way ANOVA treatment effect $F_{2,24}=32.5$ $P<0.0001$, time effect $F_{3,24}=20.7$ $P<0.0001$, interaction effect $F_{6,24}=5.9$ $P<0.001$; Newman Keuls post hoc test 5min Con A + AMPA group was significantly different from AMPA group $P<0.05$ but not significantly different from control group $P>0.05$), identical to the results observed in AMPA + Con A experiments conducted in 15mM Mg²⁺ saline, but was unable to prevent AMPA-mediated depression at later time points (Newman Keuls post hoc test 15min Con A + AMPA group was not significantly different from AMPA group $P>0.1$).

Effect of AMPA and CNQX on sustained current flow between T cells

The preceding experiments tested the effects of CNQX and AMPA on the transient electrical EPSP. To confirm that AMPA and CNQX were inhibiting electrical coupling, experiments were conducted in which the sustained current flow between the T cells was monitored. This was accomplished by applying a 250msec depolarizing current pulse to the presynaptic T cell that was sufficient to elicit a single action potential and then measuring the level of sustained depolarization in the postsynaptic T cell following the initial EPSP, approximately 200msec from the beginning of the current pulse (see Fig. 1D). A coupling coefficient was then calculated using the ratio of the postsynaptic membrane potential over the presynaptic membrane potential. Care was taken to only measure T-to-T current flow following a single action potential in the presynaptic cell since the current flow between the T cells may be affected by the number and frequency of presynaptic action potentials. All recordings were carried out in 15mM Mg²⁺ saline and the sustained current flow between T cells was compared prior to and 15min after treatment with AMPA, CNQX or saline. 100 μ M AMPA completely eliminated post-action potential current flow between the T cells (Fig. 5A, D One-way ANOVA $p<0.05$; Newman Keuls post hoc AMPA-treated group significantly different from the control group $p<0.05$), a result identical to the effect of this concentration of AMPA on the electrical EPSP. 200 μ M CNQX also reduced the level of sustained current flow between the recorded T cells, although this effect was not statistically significant (Fig. 5B, D). The CNQX-treated coupling was not significantly different from the control preparations tested, but was also not significantly different from the AMPA-treated group (post hoc $P>0.05$). Earlier results indicate that CNQX is not as effective as AMPA in inhibiting the electrical EPSP (see Fig. 2 & Fig 4) and it is also likely that since the current injected into the presynaptic T cell was minimal amount necessary to elicit a single action potential, that a “basement” effect for CNQX was observed. Nevertheless, these results show that treatments that inhibit the electrical EPSP also reduce sustained current flow between the coupled T cells.

Modification of the T-to-T synapse during long term depression

In order to examine whether decreases in the T-to-T electrical EPSP has a functional role, low frequency stimulation (LFS) was used to induce long term depression (LTD) in the T-to-T synapse. In all of the experiments made in normal saline, which contains 1mM Mg²⁺, it was impossible to distinguish the electrical and chemical components of the T-to-T EPSP. However in recordings by Baylor and Nicholls (1969), the chemical component of the EPSP was sufficiently delayed so that the electrical component of the EPSP could be readily observed. One difference between these earlier experiments and our own recordings is that Baylor and

Nicholls (1969) used Mg^{2+} -free saline. Recordings made in Mg^{2+} -free saline reproduced the findings of Baylor and Nicholls (1969), showing a delay in the chemical component of the EPSP that was not observed in recordings made in 1mM Mg^{2+} saline (Fig. 6A). There were no differences in the size of the T-to-T EPSP recorded in 1mM Mg^{2+} (2.6 ± 0.1 mV) and those recorded in Mg^{2+} -free saline (2.5 ± 0.3 mV). It is not known why eliminating Mg^{2+} delays the chemical EPSP. One possibility is that there is an increase in inhibitory synaptic input in the Mg^{2+} -free saline, delaying the firing of the unknown neuron that mediates the polysynaptic EPSP.

In both normal saline and Mg^{2+} -free saline, 900 stimuli LFS (1Hz) induced identical levels of LTD in the T-to-T synapse (Figure 6B, C normal saline group student t test $P < 0.001$; 0mM Mg^{2+} group student t test $P < 0.01$). Furthermore, in experiments carried out in Mg^{2+} -free saline LTD, it was possible to observe depression in both the electrical and chemical component of the EPSP (this later component is dominated by the chemical EPSP, but electrical EPSP has not fully decayed so it is referred to as the “total EPSP”). This LFS-induced LTD of the T-to-T synapse was blocked by SVKI (Fig. 6D electrical EPSP student t-test between 900s LFS +SVKI group and 900s LFS group, $P < 0.001$; student t-test between SVKI group and control groups, $P > 0.05$) indicating a dependence for AMPA receptor internalization. No changes in input resistance were observed in T cells that underwent LFS in normal saline ($95.2 \pm 4.8\%$ of initial input resistance), T cells in 0mM Mg^{2+} saline ($93.0 \pm 1.7\%$) or the pep-SVKI-treated T cells ($94.6 \pm 6.9\%$).

Effect of CNQX on the T-to-S electrical/chemical synapse

The effects of CNQX were examined on a second mixed glutamatergic/electrical synapse that between the T and S cells (Muller & Scott 1981). The S cell is an interneuron thought to be critical for learning in the whole-body shortening reflex (Sahley et al. 1994; Modney et al. 1997; Burrell et al. 2003; Burrell & Sahley 2005) Action potentials elicited in a T cell produce a 1–2mV, short latency electrical EPSP followed by a larger 4–6mV chemical EPSP (Figure 7B; also see Muller & Scott 1981). There is a substantial delay between the start of the electrical EPSP and the start of the chemical EPSP; therefore, the two components can be readily distinguished. CNQX completely blocked the chemical EPSP, indicating that this component of the T-to-S synapse was glutamatergic, and also significantly reduced the T-to-S electrical EPSP by approximately 40% (Fig. 7B Two-way ANOVA, Treatment effect $F_{1,20}=30.6$, $P < 0.0001$). These results are identical to the observed effects of CNQX at the T-to-T synapse. No change in input resistance in the postsynaptic S cells was observed between the control and CNQX groups.

Effect of CNQX on non-rectifying electrical synapses

The effect of CNQX was also tested on two different non-rectifying synapses: between the paired Retzius (R) neurons (Figure 7C; Stuart 1970; Lent 1977; De-Miguel et al. 2001). In this electrical synapse, both positive and negative currents flow equally well in both directions between the coupled neurons. To examine the effect of CNQX on electrical transmission between R cells, the coupling ratio was calculated as the postsynaptic membrane potential/presynaptic membrane potential and was tested prior to and following CNQX treatment. As shown in Figure 7C and D, the coupling ratio between paired R cells was not affected by a 15min application of 20 μ M CNQX (One-way ANOVA, $P > 0.05$). Comparison of CNQX-treated R cell pairs with initial recordings in normal saline solution (Figure 7C top trace) showed that CNQX did not inhibit non-rectifying electrical synapses (Figure 7C bottom trace). Another non-rectifying electrical synapse between paired longitudinal motor neurons (L cells) was tested and the coupling ratio was also not affected by CNQX treatment (data not shown, One-way ANOVA, $P > 0.05$).

3. Discussion

In this study, we have shown that both CNQX and AMPA inhibited electrical synaptic transmission in the CNS of the medicinal leech. Both Con A and DIP, which inhibit endocytosis, blocked CNQX-mediated depression of the electrical synapse and attenuated AMPA-mediated depression. SVKI, an inhibitor of AMPA-type receptor internalization, also blocked CNQX-induced depression of the electrical synapse and partially blocked AMPA-mediated depression. SVKI also blocked LTD of the electrical and chemical components of the T-to-T synapse. GYKI 52466, a non-competitive glutamate receptor antagonist, did not affect electrical synaptic transmission. CNQX-mediated depression of the electrical EPSP was also observed at the T-to-S synapse, but CNQX had no effect on coupling at the non-rectifying electrical synapses between paired Retzius cells or paired longitudinal motor neurons.

AMPA receptors are constantly cycled between intracellular stores and the cell surface (Malinow and Malenka 2002; Sheng and Kim 2002) and internalization of AMPA receptors is believed to be the major mechanism mediating long-term depression (LTD) of glutamatergic synaptic transmission (Man et al. 2000; Lin et al. 2000; Kim et al. 2001). Previous studies have demonstrated that CNQX and AMPA can induce internalization of glutamate receptors in cultured hippocampal neurons with AMPA being much more effective than CNQX (Lin et al. 2000). It is thought that AMPA-mediated internalization is initiated by two processes; one that depends on current flux through the activated AMPA receptor channel and a second process that depends on direct protein-to-protein interactions between the AMPA receptor and one or more unknown molecules (Lin et al. 2000). The CNQX-mediated internalization of AMPA receptors is thought to be weaker, at least in part, because CNQX can only activate the latter mechanism and does not elicit current flow through the receptor. GYKI 52466 is not able to elicit AMPA receptor internalization presumably because it is not a competitive antagonist and therefore does not bind to the glutamate-binding site (Lin et al. 2000). Although CNQX/AMPA-mediated internalization of glutamate receptors has not been examined in invertebrates, these receptors do undergo trafficking as a result of LTP, serotonergic modulation and metabotropic glutamate receptor activation (Antonov et al. 2007; Chitwood et al. 2001; Ji and Hawkins 2003; Li et al. 2005; Grey & Burrell 2008; Pan & Broadie 2007). Furthermore, there is evidence of conservation in the cellular machinery used to mediate glutamate receptor trafficking between vertebrates and invertebrates (Chang and Rongo 2005; Walker et al. 2006).

Our electrical synapse data parallel the AMPA/CNQX-mediated depression of glutamatergic synapses in a number of interesting ways. In both AMPA is much more effective than CNQX and appears to depend on occupation of the glutamate receptor binding site since GYKI 52466 had no effect. That inhibition of the electrical EPSP involved endocytosis is based on the findings that Con A and DIP blocked CNQX-mediated depression and partially disrupted AMPA-mediated depression. These results suggest an interaction between AMPA-type glutamate receptors and the gap junction proteins that mediate electrical synaptic transmission. Specifically, CNQX- or AMPA-induced internalization of AMPA receptors may produce a decrease in electrical synaptic transmission due to an unidentified linkage between the AMPA receptors and the gap junction proteins. Such a mechanism is supported by the fact that SVKI, which specifically inhibits AMPA receptor internalization, blocks CNQX-mediated depression of the electrical synapse, attenuates AMPA-mediated depression and blocks LTD of both the chemical and electrical components of the T-to-T synapse. This ability of SVKI to block LTD in the leech is consistent with some forms of LTD observed in the vertebrate brain which is thought to be expressed via the internalization of glutamate receptors (Kim et al. 2001). It is not known at this time whether this LTD requires activation of NMDA receptors, but NMDA receptor-dependent LTP and LTD have been observed at other synapses in the leech CNS (Burrell & Sahley 2004; Li & Burrell unpublished data).

How might internalization of glutamate receptors lead to a decrease in electrical synaptic transmission? One possibility is that the gap junction proteins (innexins in the leech and other protostomal invertebrates) are co-internalized along with the glutamate receptors. Increasingly, molecular and electrophysiological evidence supports the idea that there can be an intimate association between the chemical and electrical components of a synapse. Axosomatic and axodendritic gap junctions have been found to cluster at active zones opposed to postsynaptic densities (PSDs), suggesting that membrane receptors and gap junction proteins can interact with each other either by short-range intercellular signaling or by direct protein-protein interactions (Sotelo et al. 1978; Rash et al. 2000; Lynn et al. 2001; Zoidl et al. 2007). Alternatively, depression of the electrical EPSP may not involve trafficking of the gap junction proteins themselves, but instead involve inhibition of gap junction function, e.g. by reducing current flow through the gap junctions. Unfortunately, it is impossible to directly monitor the trafficking of either innexins or glutamate receptors in the leech CNS since there are no known antibodies that recognize the leech version of these proteins.

Although CNQX-mediated inhibition of the electrical EPSP appears to be mediated by a purely endocytosis-dependent process (based on the effects of Con A, DIP and SVKI), AMPA-mediated inhibition of the electrical synapse was only partially blocked by drugs that blocked endocytosis. This suggests that AMPA application inhibited the electrical synapse via two distinct mechanisms, one linked to internalization of the AMPA-type glutamate receptors and one or more additional processes that are independent of receptor trafficking. Examples of these latter processes include increased intracellular Ca^{2+} , which is known to inhibit electrical coupling (Connors and Long 2004), or activation of other modulatory neurons in the leech brain as a result of AMPA application. Previous work has shown that serotonin inhibits electrical synapses in the leech CNS (Colombaioni and Brunelli 1988; Beck et al. 2002; Moss et al. 2005) and neuronal gap junctions have been shown to be modified by a variety of modulatory transmitters or hormones including serotonin, ecdysone, dopamine and acetylcholine (Piccolino et al. 1984; Teranishi et al. 1983; Moreno et al. 1991; Johnson et al. 1993, 1994; Rorig and Sutor 1996; Velazquez et al. 1997; Teshiba et al. 2001; Antonsen and Edwards 2007).

It is also possible that the CNQX/AMPA induces depression of the electrical EPSP by directly binding to the innexins or some other protein that then acts on the innexins. This explanation cannot be excluded given that no direct observation of glutamate receptor or innexin trafficking could be made. However, there have been no other reports of either CNQX or AMPA directly acting on either vertebrate or invertebrate gap junction proteins and these drugs had no effect on the non-rectifying electrical synapses between the paired Retzius and longitudinal motor neurons. Furthermore, the ability of SVKI to block CNQX-mediated depression and attenuate AMPA-mediated depression suggests a direct role by the glutamate receptors.

CNQX and AMPA did not inhibit electrical coupling between the paired Retzius interneurons and paired longitudinal motor neurons. As in most invertebrates, gap junctions in the leech are composed of innexin proteins which are encoded by a multi-gene family with 12 innexin genes (Hm-inx) currently identified in the medicinal leech (Dyke et al. 2004; Dykes and Macagno 2006). Three types of gap junction proteins are observed in the animal kingdom; connexins and pannexins in the deuterostomes (e.g. vertebrates and invertebrate chordates) and innexins in the protostomal invertebrates, (e.g. arthropods, annelids and mollusks; Barbe & Monye 2006). As with vertebrate electrical synapses, innexin subtypes determine the functional properties of the leech gap junction, such as whether current flows equally well in both directions between the coupled neurons (non-rectifying electrical synapse) or exhibit a clear directionality of current flow (rectifying electrical synapse) and whether both positive and negative current can pass through the gap junctions. It is possible that the inability of CNQX or AMPA to inhibit coupling between the Retzius or longitudinal motor neurons may be due

to a different compliment of innexin subtypes that make up these connections. Another possibility is that the R-to-R and L-to-L gap junctions may not be in close enough proximity to the glutamate receptors to be affected by the CNQX/AMPA treatments.

AMPA receptor internalization plays a critical role in some forms of long-term depression (LTD; Luscher et al. 1999; Seifert et al. 2000) and a linkage between this process and depression of the electrical EPSP would not be surprising in synapses that have both a glutamatergic chemical and electrical component. If the glutamatergic component of a synapse is altered then it would be advantageous for the electrical component to change in a parallel manner. The results presented here suggest that a linkage between the glutamatergic and electrical synaptic components does exist and it is not the first evidence an interaction. LTP of the glutamatergic component of Mauthner cell synapses is accompanied by potentiation of the electrical component (Smith & Pereda 2003) and this potentiation of the electrical EPSP requires the activation of nearby chemically receptive zone(s) in the same synapse. This coordinated modulation of both electrical and glutamatergic synaptic transmission may play an important role in reshaping neural pathways for a number of processes including sensory processing, learning and memory, neural development and homeostatic processes. AMPA-mediated depression of the glutamatergic and electrical synapses may also provide a protective mechanism against glutamate-induced excitotoxicity.

4. Experimental Procedure

Hirudo medicinalis, weighing 3g, were obtained from a commercial supplier (Leeches USA, Westbury, N.Y.) and kept in pond water [0.52 g/L H₂O Hirudo salt (Leeches USA Ltd.)] at 18°C. Individual ganglia were dissected from the animal and placed in a recording chamber (1.5 mL) with constant perfusion (~2 ml/min). The dissections and recordings were carried out in leech saline containing: 115 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, and 10 HEPES. In high Mg²⁺ saline experiments, 15mM MgCl₂ was used, which replaced NaCl mole for mole.

Dual intracellular recordings were made by impaling neurons with intracellular glass microelectrodes using a micropositioner (Model 1480; Siskiyou Inc., Grants Pass, OR). Electrodes were pulled from borosilicate capillary tubing (1.0mm outer diameter, 0.75mm inner diameter; FHC Bowdoinham, ME) to a resistance of 25–35 MΩ (Sutter Instruments P-97; Novato, CA) and filled with 3 M potassium acetate. Current pulses were delivered to the neurons using a two-channel stimulator with stimulus isolation units (S88 and SIU5, respectively; Astromed-Grass, West Warwick, RI). Signals were amplified with a bridge amplifier (BA-1S; NPI, Tamm, Germany) and then digitally converted (Digidata 1322A A/D converter) for viewing and subsequent analysis (Axoscope 10; Molecular Devices, Sunnyvale, CA). Individual neurons were identified based on their position, size and action potential shape. EPSPs were elicited by current injections into the presynaptic cells at regular (5 min) intervals. The resting membrane potential of the postsynaptic neuron (T cell or S cell) was approximately –40mV and was hyperpolarized to –50mV to prevent the initiation of action potentials. Since a decrease in input resistance can cause an apparent decrease in synaptic signaling, input resistance was measured throughout each experiment by injecting negative current pulse (0.5nA, 500msec).

In temperature control experiments, a Peltier device and a temperature control system (HCPPS; ALA Scientific, Westbury, NY and TC-10; NPI, Tamm, Germany) were used to precool the perfusion solution just before it entered the recording chamber. A thermal probe was placed in the recording chamber to monitor the solution temperature in the recording chamber. These low temperature recordings were used to delay the chemical EPSP in the T-to-T synapses to permit measurement of the electrical EPSP in normal saline.

For T-to-T experiments, synaptic transmission between ipsilateral T cells was tested every 5 min. To isolate the electrical component of the T-to-T EPSP, the ganglion was perfused with 15mM Mg^{2+} saline to eliminate chemical synaptic transmission (Zipser et al. 1979) for 15min before the first test of the electrical synaptic transmission. Following an initial test of the electrical EPSP, the ganglion was perfused for 15 min with CNQX or AMPA (Sigma; St. Louis, MO) dissolved in 15mM Mg^{2+} , followed by a 15min washout in 15mM Mg^{2+} saline. In some experiments, CNQX/AMPA treatments were made in the presence of concanavalin A (Con A; Sigma), dynamin inhibitory peptide (DIP) or pep2-SVKI (Tocris; Ellisville, MO). In the case of DIP or pep2-SVKI, the peptides were dissolved in the electrode filling solution and injected into the cell via iontophoresis (1nA of negative current) for 10min prior to the start of the experiment.

To examine the effect of CNQX on electrical coupling between paired Retzius cells (R cells) or paired longitudinal motor neurons (L cells), 500 msec current pulses ($-0.25, -0.5, -0.75, -1, -2, -3$ nA, $0.25, 0.5, 0.75, 1.0$ nA,) were injected into one Retzius cell (R1) or L cell (L1) and the resulting changes in membrane potential in R1/L1 cell (V1) and the contralateral R2/L2 cell (V2) were recorded and measured. Electrical coupling between these paired neurons was calculated as the ratio V2/V1. Electrical coupling was measured prior to and following a 15 min treatment with 20 μ M CNQX dissolved in leech normal saline solution.

To determine whether synaptic activity could produce modulation of the chemical and electrical components of the T-to-T synapse, low frequency stimulation (LFS) was used to induce long-term depression (LTD) at T-to-T synapse. LFS-induced LTD was carried out in normal saline and in 0mM Mg^{2+} saline which allowed for separation between electrical and chemical EPSP (see Results; Baylor & Nicholls 1969). Following an initial pre-LFS recording of the EPSP, the T-to-T synapse underwent LFS training by stimulating the presynaptic T cell to elicit a single action potential 900 times at 1Hz, a common procedure for inducing LTD (Anwyl 2006). The T-to-T EPSP was re-tested 40min after LFS.

EPSP amplitude and input resistance measurements were normalized to their initial values (% of baseline) and presented as the mean $\pm$ SE. In experiments conducted in saline with 15mM Mg^{2+} the baseline measurement for EPSP amplitude and input resistance were taken after the ganglion had been bathed in the high Mg^{2+} saline for 15min. Statistical analyses were performed using two-way ANOVA and Newman-Keuls post-hoc tests (Statistica analysis software; Statsoft).

Abbreviations

AMPA, Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid; CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione; CNS, Central Nervous System; Con A, Concanavalin A; DIP, Dynamin Inhibitory Peptide; EPSP, Excitatory Postsynaptic Potential; LTD, Long Term Depression; PSD, Post Synaptic Density.

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References

Acklin SE. Electrical properties and anion permeability of doubly rectifying junctions in the leech central nervous system. *J Exp Biol* 1988;137:1–11.

- Antonsen BL, Edwards DH. Mechanisms of serotonergic facilitation at a command neuron. *J. Neurophysiol* 2007;98:3494–3504. [PubMed: 17898136]
- Antonov I, Ha T, Antonova I, Moroz LL, Hawkins RD. Role of nitric oxide in classical conditioning of siphon withdrawal in *Aplysia*. *J Neurosci* 2007;27:10993–11002. [PubMed: 17928440]
- Anwyll R. Induction and expression mechanisms of postsynaptic NMDA receptor-independent homosynaptic long-term depression. *Prog Neurobiol* 2006;78:17–37. [PubMed: 16423442]
- Arttamangkul S, Torrecilla M, Kobayashi K, Okano H, Williams JT. Separation of muopioid receptor desensitization and internalization: endogenous receptors in primary neuronal cultures. *J. Neurosci* 2006;26:4118–4125. [PubMed: 16611829]
- Barbe MT, Monyer H, Bruzzone R. Cell-cell communication beyond connexins: the pannexin channels. *Physiology* 2006;21:103–114. [PubMed: 16565476]
- Baylor DA, Nicholls JG. Chemical and electrical synaptic connexions between cutaneous mechanoreceptor neurones in the central nervous system of the leech. *J. Physiol* 1969;203:591–609. [PubMed: 4319015]
- Beck A, Lohr C, Berthold H, Deitmer JW. Calcium influx into dendrites of the leech Retzius neuron evoked by 5-hydroxytryptamine. *Cell Calcium* 2002;31:137–134. [PubMed: 12027387]
- Bennett MV, Zukin RS. Electrical coupling and neuronal synchronization in the mammalian brain. *Neuron* 2004;41:495–511. [PubMed: 14980200]
- Berlin RD, Oliver JM, Walter RJ. Surface functions during Mitosis I: phagocytosis, pinocytosis and mobility of surface-bound Con A. *Cell* 1978;15:327–341. [PubMed: 719746]
- Burrell BD, Sahley CL, Muller KJ. Progressive recovery of learning during regeneration of a single synapse in the medicinal leech. *J. Comp. Neurol* 2003;457:67–74. [PubMed: 12541325]
- Burrell BD, Sahley CL. Serotonin Mediates Learning-Induced Potentiation of Excitability. *J. Neurophysiol* 2005;94:4002–4010. [PubMed: 16120666]
- Carroll RC, Beattie EC, Xia H, Luscher C, Altschuler Y, Nicoll RA, Malenka RC, Zastrow M. Dynamin-dependent endocytosis of ionotropic glutamate receptors. *Proc. Natl. Acad. Sci. U S A* 1999;96:14112–14117. [PubMed: 10570207]
- Chang HC-H, Rongo C. Cystolic tail sequences and subunit interactions are critical for synaptic localization of glutamate receptors. *J. Cell Sci* 2005;118
- Chitwood RA, Li Q, Glanzman DL. Serotonin facilitates AMPA type responses in isolated motor neurons in *Aplysia* in culture. *J Physiol* 2001;534:501–510. [PubMed: 11454967]
- Connors BW, Long MA. Electrical synapses in the mammalian brain. *Annu. Rev. Neurosci* 2004;27:393–418. [PubMed: 15217338]
- Colombaioni L, Brunelli M. Neurotransmitter-induced modulation of an electrotonic synapse in the CNS of *Hirudo medicinalis*. *Exp. Biol* 1988;47:139–144. [PubMed: 2838317]
- Daw MI, Chittajallu R, Bortolotto ZA, Dev KK, Duprat F, Henley JM, Collingridge GL, Isaac JT. PDZ proteins interacting with C-terminal GluR2/3 are involved in a PKC dependent regulation of AMPA receptors at hippocampal synapses. *Neuron* 2000;28:873–886. [PubMed: 11163273]
- Del Castillo J, Katz B. The effect of magnesium on the activity of motor nerve endings. *J. Physiol* 1954;124:553–559. [PubMed: 13175198]
- De-Miguel FF, Vargas-Caballero M, Garcia-Perez E. Spread of synaptic potentials through electrical synapses in Retzius neurons of the leech. *J. Exp. Biol* 2001;204:3241–3250. [PubMed: 11606598]
- Dierkes PW, Hochstrate P, Schlue WR. Distribution and functional properties of glutamate receptors in the leech central nervous system. *J Neurophysiol* 1996;75:2312–2321. [PubMed: 8793744]
- Donevan SD, Rogawski MA. GYKI 52466, a 2,3-benzodiazepine, is a highly selective, noncompetitive antagonist of AMPA/kainate receptor responses. *Neuron* 1993;10:51–59. [PubMed: 7678966]
- Dykes IM, Freeman FM, Bacon JP, Davies JA. Molecular basis of gap junctional communication in the CNS of the leech *Hirudo medicinalis*. *J. Neurosci* 2004;24:886–894. [PubMed: 14749433]
- Dykes IM, Macagno ER. Molecular characterization and embryonic expression of innexins in the leech *Hirudo medicinalis*. *Dev. Genes* 2006;216:85–197.
- Ebihara L, Xu X, Oberti C, Beyer EC, Berthoud VM. Co-expression of lens fiber connexins modifies hemi-gap-junctional channel behavior. *Biophys J* 1999;76:198–206. [PubMed: 9876134]

- Furshpan EJ, Potter DD. Transmission at the giant motor synapses of the crayfish. *J Physiol* 1959;145:289–325. [PubMed: 13642302]
- Garcia-Perez E, Vargas-Caballero M, Velazquez-Ulloa N, Minzoni A, De-Miguel FF. Synaptic integration in electrically coupled neurons. *J. Biophys* 2004;86:646–655.
- Grey KB, Burrell BD. Forskolin induces NMDA receptor-dependent potentiation at a central synapse in the leech. *J. Neurophysiol* 2008;99:2719–2724. [PubMed: 18337371]
- Ji I, Hawkins RD. Presynaptic and postsynaptic mechanisms of a novel form of homosynaptic potentiation at aplysia sensory motor neuron synapses. *J Neurosci* 2003;23:7288–7297. [PubMed: 12917362]
- Johnson BR, Peck JH, Harris-Warrick RM. Amine modulation of electrical coupling in the pyloric network of the lobster stomatogastric ganglion. *J. Comp. Physiol* 1993;172:715–732. [PubMed: 8350285]
- Johnson BR, Peck JH, Harris-Warrick RM. Differential modulation of chemical and electrical components of mixed synapses in the lobster stomatogastric ganglion. *J. Comp. Physiol* 1994;175:233–249. [PubMed: 8071898]
- Kim C-H, Chung HJ, Lee H-K, Haganir RL. Interaction of the AMPA receptor subunit GluR2/3 with PDZ domains regulates hippocampal long-term depression. *PNAS* 2001;98:11725–11730. [PubMed: 11573007]
- Kim SJ, Kim MY, Lee EJ, Ahn YS, Baik JH. Distinct regulation of internalization and mitogen-activated protein kinase activation by two isoforms of the dopamine D2 receptor. *Mol. Endocrinol* 2004;18:640–652. [PubMed: 14684845]
- Kristan WB, Calabrese RL, Friesen WO. Neuronal control of leech behavior. *Prog. Neurobiol* 2005;76:279–327. [PubMed: 16260077]
- Lent CM. The Retzius cells within the central nervous system of leeches. *Prog. Neurobiol* 1977;8:81–117. [PubMed: 320629]
- Li Q, Roberts AC, Glanzman DL. Synaptic facilitation and behavioral dishabituation in Aplysia: dependence on release of Ca^{2+} from postsynaptic intracellular stores, postsynaptic exocytosis, and modulation of postsynaptic AMPA receptor efficacy. *J Neurosci* 2005;25:5623–5637. [PubMed: 15944390]
- Li P, Kerchner GA, Sala C, Wei F, Huettner JE, Sheng M, Zhuo M. AMPA receptor-PDZ interactions in facilitation of spinal sensory synapses. *Nat. Neurosci* 1999;2:972–977. [PubMed: 10526335]
- Lin JW, Ju W, Foster K, Lee SH, Ahmadian G, Wyszynski M, Wang YT, Sheng M. Distinct molecular mechanisms and divergent endocytotic pathways of AMPA receptor internalization. *Nat. Neurosci* 2000;3:1282–1290. [PubMed: 11100149]
- Luscher C, Xia H, Beattie EC, Carroll RC, von Zastrow M, Malenka RC, Nicoll RA. Role of AMPA receptor cycling in synaptic transmission and plasticity. *Neuron* 1999;24:649–658. [PubMed: 10595516]
- Lynn BD, Rempel JL, Nagy JJ. Enrichment of neuronal and glial connexins in the postsynaptic density subcellular fraction of rat brain. *Brain Res* 2001;898:1–8. [PubMed: 11292443]
- Malinow R, Malenka RC. AMPA receptor trafficking and synaptic plasticity. *Annu. Rev. Neurosci* 2002;25:103–126. [PubMed: 12052905]
- Man HY, Lin JW, Ju WH, Ahmadian G, Liu L, Becker LE, Sheng M, Wang YT. Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization. *Neuron* 2000;25:649–662. [PubMed: 10774732]
- Man HY, Sheng M, Wang YT. Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization. *Neuron* 2000;25:649–662. [PubMed: 10774732]
- Marks B, McMahon HT. Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals. *Curr. Biol* 1998;8:740–749. [PubMed: 9651678]
- Mayer ML, Vyklicky L. Concanavalin A selectively reduces desensitization of mammalian neuronal quisqualate receptor. *PNAS* 1989;86:1411–1415. [PubMed: 2537497]
- Meier C, Dermietzel R. Electrical synapses--gap junctions in the brain. *Results Probl. Cell Differ* 2006;43:99–128.
- Modney BK, Sahley CL, Muller KJ. Regeneration of a central synapse restores nonassociative learning. *J. Neurosci* 1997;17:6478–6482. [PubMed: 9236255]

- Moreno AP, Arellano RO, Rivera A, Ramon F. Humoral factors reduce gap junction sensitivity to cytoplasmic pH. II. In vitro manipulations. *Am. J. Physiol* 1991;260:1039–1045.
- Morishita W, Marie H, Malenka RC. Distinct triggering and expression mechanisms underlie LTD of AMPA and NMDA synaptic responses. *Nat. Neurosci* 2005;8:1043–1050. [PubMed: 16025109]
- Moss BL, Fuller AD, Sahley CL, Burrell BD. Serotonin modulates axo-axonal coupling between neurons critical for learning in the leech. *Neurophysiol* 2005;94:2575–2589.
- Muller KJ, Scott SA. Transmission at a 'direct' electrical connexion mediated by an interneuron in the leech. *J. Physiol* 1981;311:565–583. [PubMed: 6267257]
- Nicholls JG, Baylor DA. Specific modalities and receptive fields of sensory neurons in CNS of the leech. *J. Neurophysiol* 1968;31:740–756. [PubMed: 5711143]
- Nicholls JG, Purves D. A comparison of chemical and electrical synaptic transmission between single sensory cells and a motoneurone in the central nervous system of the leech. *J. Physiol* 1972;225:637–656. [PubMed: 4342522]
- Paatero GI, Wikstrom L, Isomaa B. Concanavalin A-induced redistribution of surface receptors in *Acanthamoeba castellanii* at different growth phases. *Eur. J. Cell Biol* 1988;47:112–120. [PubMed: 3229417]
- Pan L, Broadie KS. *Drosophila* fragile X mental retardation protein and metabotropic glutamate receptor A convergently regulate the synaptic ratio of ionotropic glutamate receptor subclasses. *J. Neurosci* 2007;27:12378–12389.
- Pereda AE, Rash JE, Nagy JI, Bennett MV. Dynamics of electrical transmission at club endings on the Mauthner cells. *Brain. Res. Rev* 2004;47:227–244. [PubMed: 15572174]
- Piccolino M, Neyton J, Gerschenfeld HM. Decrease of gap junction permeability induced by dopamine and cyclic adenosine 3':5'-monophosphate in horizontal cells of turtle retina. *J. Neurosci* 1984;4:2477–2488. [PubMed: 6092564]
- Rash JE, Staines WA, Yasumura T, Patel D, Furman CS, Stelmack GL, Nagy JI. Immunogold evidence that neuronal gap junctions in adult rat brain and spinal cord contain connexin-36 but not connexin-32 or connexin-43. *Proc. Natl. Acad. Sci. U S A* 2000;97:7573–7578. [PubMed: 10861019]
- Rela L, Szczupak L. In situ characterization of a rectifying electrical junction. *J. Neurophysiol* 2007;97:1405–1412. [PubMed: 17167060]
- Roerig B, Feller MB. Neurotransmitters and gap junctions in developing neural circuits. *Brain Res. Rev* 2000;32:86–114. [PubMed: 10751659]
- Rorig B, Sutor B. Serotonin regulates gap junction coupling in the developing rat somatosensory cortex. *Eur. J. Neurosci* 1996;8:1685–1695. [PubMed: 8921259]
- Sheng M, Kim MJ. Postsynaptic signaling and plasticity mechanisms. *Science* 2002;298:776–780. [PubMed: 12399578]
- Sahley CL, Modney BK, Boulis NM, Muller KJ. The S cell: an interneuron essential for sensitization and full dishabituation of leech shortening. *J. Neurosci* 1994;14:6715–6721. [PubMed: 7965072]
- Seifert G, Zhou M, Dietrich D, Schumacher TB, Dybek A, Weiser T, Wienrich M, Wilhelm D, Steinhäuser C. Developmental regulation of AMPA-receptor properties in CA1 pyramidal neurons of rat hippocampus. *Neuropharmacology* 2000;39:931–942. [PubMed: 10727703]
- Smith M, Pereda AE. Chemical synaptic activity modulates nearby electrical synapses. *PNAS* 2003;2003:4849–4854. [PubMed: 12668761]
- Sotelo C, Korn H. Morphological correlates of electrical and other interactions through low-resistance pathways between neurons of the vertebrate central nervous system. *Int. Rev. Cytol* 1978;55:67–107. [PubMed: 389866]
- Stuart AE. Physiological and morphological properties of motoneurons in the central nervous system of the leech. *J. Physiol* 1970;209:627–646. [PubMed: 5499800]
- Teshiba T, Shamsian A, Yashar B, Yeh SR, Edwards DH, Krasne FB. Dual and opposing modulatory effects of serotonin on crayfish lateral giant escape command neurons. *J. Neurosci* 2001;21:4523–4529. [PubMed: 11404440]
- Teranishi T, Negishi K, Kato S. Dopamine modulates S-potential amplitude and dyecoupling between external horizontal cells in carp retina. *Nature* 1983;301:243–246. [PubMed: 6401844]

- Thalhammer A, Everts I, Hollmann M. Inhibition by lectins of glutamate receptor desensitization is determined by the lectin's sugar specificity at kainate but not AMPA receptors. *Mol. Cell Neurosci* 2002;21:521–533. [PubMed: 12504587]
- Velazquez JL, Han D, Carlen PL. Neurotransmitter modulation of gap junctional communication in the rat hippocampus. *Eur. J. Neurosci* 1997;9:2522–2531. [PubMed: 9517457]
- Walker CS, Brockie PJ, Madsen DM, Francis MM, Zheng Y, Koduri S, Mellem JE, Strutz-Seebohm N, Maricq AV. Reconstitution of invertebrate glutamate receptor function depends on stargazing-like proteins. *PNAS* 2006;103:10781–10786. [PubMed: 16818877]
- Werner R, Levine E, Rabadan-Diehl C, Dahl G. Formation of hybrid cell-cell channels. *PNAS* 1989;86:5380–5384. [PubMed: 2546155]
- White TW, Deans MR, O'Brien J, Al-Ubaidi MR, Goodenough DA, Ripps H, Bruzzone R. Functional characteristics of skate connexin35, a member of the γ subfamily of connexins expressed in the vertebrate retina. *Eur J Neurosci* 1999;11:1883–1890. [PubMed: 10336656]
- Wigge P, McMahon HT. The amphiphysin family of proteins and their role in endocytosis at the synapse. *Trends Neurosci* 1998;21:339–344. [PubMed: 9720601]
- Xiang Y, Devic E, Kobilka B. The PDZ binding motif of the beta 1 adrenergic receptor modulates receptor trafficking and signaling in cardiac myocytes. *J. Biol. Chem* 2002;277:33783–33790. [PubMed: 12097326]
- Zappala M, Grasso S, Micale N, Polimeni S, De Micheli C. Synthesis and structure-activity relationships of 2,3-benzodiazepines as AMPA receptor antagonists. *Mini. Rev. Med. Chem* 2001;1:243–253. [PubMed: 12369971]
- Zipser B. Identifiable neurons controlling penile eversion in the leech. *J. Neurophysiol* 1979;42:455–464. [PubMed: 422973]
- Zoidl G, Petrasch-Parwez E, Ray A, Meier C, Bunse S, Habbes HW, Dahl G, Dermietzel R. Localization of the Pannexin1 protein at postsynaptic sites in the cerebral cortex and hippocampus. *Neuroscience* 2007;146:9–16. [PubMed: 17379420]

Figure 1. Properties of the T-to-T synapse

(A) The T-to-T synapse has both a polysynaptic chemical component and a monosynaptic electrical component. (B) 15mM Mg^{2+} saline solution selectively blocks chemical synaptic transmission, revealing the electrical component of the T-to-T EPSP. The ganglion was cooled to 15°C so that the early electrical component and the later chemical component could be distinguished. The later component was completely abolished when 15mM Mg^{2+} was applied, leaving only the electrical component. (C) Subthreshold depolarizing current (*top pair of traces*) and hyperpolarizing current (*bottom pair of traces*) injected into the presynaptic T cell did not spread to the postsynaptic T cell. (D) Traces comparing the response of the postsynaptic T cell to a brief (5msec), suprathereshold current versus a long (250msec), suprathereshold current injection to the presynaptic T cell (black and gray traces, respectively). The brief current injection produced a transient electrical EPSP that rapidly decayed back to the resting potential, however sustained current flow between the two T cells could be observed during the 250msec current pulse if an action potential was elicited in the presynaptic T cell (compare these traces to those in 1C).

Figure 2. CNQX inhibited T-to-T electrical synaptic transmission and the effect of CNQX was concentration-dependent

(A) Representative traces in normal saline illustrating that the chemical component of the T-to-T synapse is inhibited by CNQX indicating that this synapse is glutamatergic. (B) In B–D, recordings were made in 15mM Mg^{2+} to eliminate chemical synaptic transmission. 20μM CNQX significantly reduced the T-to-T electrical EPSP (N=6). No change in input resistance was observed in either the CNQX-treated or saline control groups. (C) The inhibitory effect of CNQX increased in a concentration-dependent manner. No significant difference was observed between T cells treated with 2μM CNQX compared to the control group (N=3). 10μM (N=5), 20μM and 200μM (N=5) CNQX significantly reduced the amplitude of the electrical EPSP with 200μM being significantly more effective than 10μM and 20μM. 10μM and 20μM CNQX did not show a significant difference. The superimposed curved line is a sigmoidal fit meant to illustrate the trend in the data. (D) Representative traces showing the effect of increasing concentrations of CNQX on the T-to-T electrical synapse.

Figure 3. Con A, DIP and SVKI blocked CNQX-mediated inhibition of T-to-T electrical transmission

(A) 600 μ g/ml Con A completely blocked 20 μ M CNQX-mediated inhibition of the T-to-T electrical synaptic transmission (N=5). (B) 50 μ M DIP prevented the initial effect of 20 μ M CNQX on the T-to-T electrical EPSP (n=5). The DIP + CNQX group did not significantly differ from the control group at 5 min. At the end of 15 min, there are no significant difference between the DIP+CNQX group and the CNQX group. (C) 100 μ M SVKI completely blocked 20 μ M CNQX-mediated inhibition of T-to-T electrical synaptic transmission (N=5). (D) Representative traces from the 5 min post-treatment stage showing CNQX-mediated inhibition of the T-to-T electrical synapse (top) and the ability of Con A to block the effects of 20 μ M CNQX (bottom).

Figure 4. AMPA inhibited T-to-T electrical synaptic transmission and this effect was antagonized by Con A, DIP and SVKI

(A) Both 10 and 100μM AMPA significantly reduced the electrical EPSP at each time point compared with the control group (N=5 in each group). 100μM AMPA abolished the T-to-T electrical EPSP almost completely while the inhibition of 10μM AMPA was slower to develop and did not completely inhibit the electrical EPSP. (B) Representative traces from the 5 min post-treatment stage showing the effect of 10μM AMPA, (*top traces*) 100μM AMPA (*middle traces*) and 10μM AMPA + Con A (*bottom traces*). (C) Con A prevented the inhibitory effect of 10μM AMPA on electrical transmission at the 5min recording (N=4). Both 10min and 15min recordings in Con A + AMPA group still showed significant inhibition compared with the

control group. (D) 50 μ M DIP prevented the inhibitory effect of 10 μ M AMPA on electrical transmission at the first 5min recording compared with the control group (N=4). Again, DIP had no effect on AMPA-mediated inhibition at later time points. (E) 100 μ M Pep2-SVKI completely blocked the inhibitory effect of 10 μ M AMPA on electrical transmission at the first 5min recording compared with the control group, but had no effect on AMPA-mediated inhibition at later time points (N=4). (F) AMPA inhibited T-to-T synaptic transmission in normal saline indicating inhibition of both the chemical and electrical components of this synapse. As in 15mm Mg²⁺ saline, Con A attenuated this effect. The total EPSP was inhibited at the end of the 15min 10 μ M AMPA treatment in both AMPA and Con A + AMPA group (10 μ M AMPA group N=5; 10 μ M AMPA+ConA N=4; Con A group N=4). No significant difference was shown between the AMPA and Con A+ AMPA groups at this time point. However, Con A effectively prevented the inhibitory effect of 10 μ M AMPA on T-to-T synaptic transmission at the 5min recording compared with the control group.

Figure 5. AMPA and CNQX inhibited the level of sustained current flow between T cells

(A) Traces showing the level of sustained current flow between two ipsilateral T cells prior to (black traces) and after (grey traces) a 15min application of 100 μ M AMPA. (B) Traces showing the level of sustained current flow between two ipsilateral T cells prior to (black traces) and after (grey traces) a 15min application of 200 μ M CNQX. (C) Traces showing the level of sustained current flow between two ipsilateral T cells prior to (black traces) and after (grey traces) 15min in 15mM Mg²⁺ saline (control experiments). (D) Bar graph summarizing the effects of AMPA (N=3) and CNQX (N=5) on coupling between T cells.

Figure 6. The chemical and electrical components of the T-to-T synapse are depressed following low frequency stimulation-induced LTD. All the data are normalized to the baseline (EPSP post training/EPSP pre training)

(A) Representative traces showing the difference between T-to-T EPSP elicited in 0mM Mg^{2+} saline (*top traces*) versus normal saline with 1mM Mg^{2+} (*bottom trace*). Note that in 0mM Mg^{2+} saline, the chemical component of the EPSP is delayed just enough so that the electrical component can be observed. (B) Representative traces from pre-training recording (black trace) and post-training recording (gray trace). 900s LFS induce LTD in both chemical and electrical EPSP component of T-to-T synapse which can be totally blocked by Pep2-SVKI. (C) Vertical bar graph illustrating that 900s LFS (1Hz) was able to induce long term depression of T-to-T synapse in both normal saline group (N=4) and 0mM Mg^{2+} group (N=4). 900s LFS also induced a significant depression on Electrical EPSP in 0mM Mg^{2+} group (N=4). (D) Vertical bar graph showing SVKI (N=4) prevented 900s LFS-induced LTD relative to the control group (N=5).

Figure 7. CNQX reduces both the chemical and electrical component of the leech T-to-S synapse (A) Representative traces showing that 20μM CNQX reduced the electrical component and eliminated the chemical component of the T-to-S synapse. (B) Electrical EPSP data were measured and normalized to the initial level in 15mM Mg^{2+} saline. 20μM CNQX (N=6) inhibited the electrical component of the T-to-S synapse while no change was observed in the control group (N=6). Partial recovery of T-to-S electrical synaptic transmission was observed after a 10 min wash in normal saline solution. (C) Representative traces of R-to-R coupling prior to (top) and following (bottom) CNQX treatment. (D) Scatter plots showing that the coupling ratio between paired R (left) and L (right) cells was unaffected by a 15min application of 20μM CNQX. Coupling ratio was calculated as the presynaptic membrane potential / postsynaptic membrane potential.