uisite for latching, because no increase in tissue stiffness or stress was observed without phosphorylation when the level of Ca^{2+} was increased from $1.8 \times 10^{-8} M$.

The Ca^{2+} binding site regulating latching is unknown. The regulatory light chains of all myosins have Ca^{2+} binding sites, some of which have a regulatory role in many invertebrate muscles (*12*). Such sites are attractive candidates because phosphorylation of the regulatory light chain could alter its conformation and Ca^{2+} binding characteristics. It is also possible that cross-bridge attachment to the thin filament after phosphorylation enables a thin-filament protein to bind Ca^{2+} (*1*), leading to maintained cross-bridge attachment. A small hysteresis in the stress-Ca^{2+} relation in skinned striated muscle has been attributed to an increased affinity of the regulatory site for Ca^{2+} because of active stress in the myofilaments (*13*).

Vascular smooth muscle normally opposes stress due to blood pressure with a rate of energy consumption some one three-hundredths of that required by frog striated muscle to maintain a comparable stress (*14*). Stimulation of arterial smooth muscle by agonists produces a transient increase followed by a decrease in myoplasmic Ca^{2+}, as estimated from light emission by aequorin-loaded cells (*15*) and from myosin phosphorylation (*3*, *4*). In previous studies (*3*) it was suggested that stress maintenance by nonphosphorylated cross-bridges is associated with reduced cycling rates, as estimated from isotonic shortening velocities. On the basis of this study, we suggest that the high economy of stress maintenance in smooth muscle is due to an unidentified regulatory mechanism with a high sensitivity to Ca^{2+}. This system appears to function only after the initial activation and Ca^{2+} transient that lead to myosin phosphorylation and stress development. Relaxation is the result of further reductions in cell Ca^{2+} below the threshold for latching (*16*).

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Electrical Synapse Formation Depends on Interaction of Mutually Growing Neurites

Abstract. *A neuron's competence to form electrical synapses depends on its growth status. Experiments in situ and in cell culture with identified neurons of the snail* Helisoma *demonstrate that active neurite outgrowth from both potential partners must be spatially and temporally coincident for electrical synapse formation to occur.*

Growing neurons can establish highly predictable sets of connections during both development and adult neuronal plasticity. Equally important, many potential connections are never formed. Specific connectivity could depend on many mechanisms, including those governed by intrinsic properties of neurons or by the nature of interactions between neurons. Because highly reproducible sets of new electrical synapses can be triggered to form between neurons of the snail *Helisoma* (*1*, *2*), it is now possible to determine why certain identified neurons reliably form connections while others do not.

We tested the hypothesis that formation of new electrical synapses requires spatially and temporally coincident neurite outgrowth from each of the potential partners. This growth-dependence hypothesis was initially suggested by experiments in which nerve crushing evoked neurite growth in buccal ganglia in situ (*3*). We now report two higher resolution experimental approaches which independently demonstrate that only growing neurons readily form electrical connections.

Our first approach was to restrict treatment to individual neurons while preserving the in situ environment. We used a cultured ganglionic preparation known to have little or no central neuronal sprouting in the absence of axotomy near the cell body (*3–6*). Individual axons were selectively severed by localized photoirradiation of injected fluorescent dye ("zap axotomy") (*6*). When neurons 5R and 5L were zap-axotomized, they grew profusely and formed electrical connections that were qualitatively indistinguishable from those formed when bilateral nerve crushing evoked growth (*6*). To test the require-

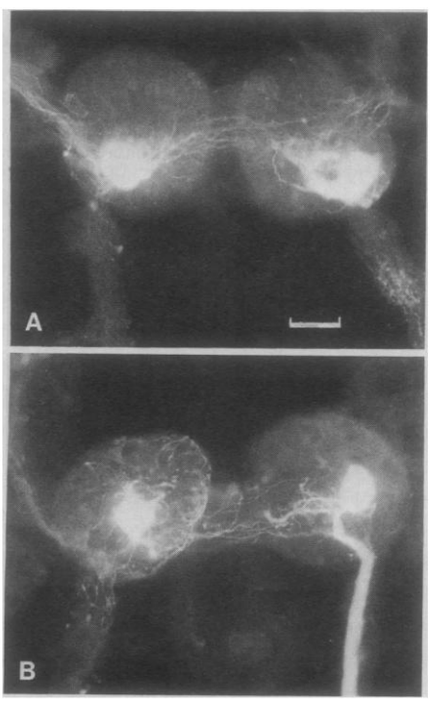

Fig. 1. Buccal ganglia with Lucifer yellow–injected (*14*) pairs of neuron 5's, shown after selective "zap axotomy" (where axon exits ganglion) of both neurons (A) or a single neuron (B). Mean coupling coefficients are 0.29 ± 0.05 ($N = 8$) and 0.03 ± 0.01 ($N = 8$) for (A) and (B), respectively. The unperturbed neuron 5 (on the right in B) retained its normal morphology. Cultures were 5 days old (*4*, *5*). Scale bar, 100 μm.

ment for mutual growth, we restricted zap axotomy to a single neuron 5. With one neuron 5 growing and the other untreated (Fig. 1B), only very weak connections formed, and coupling coefficients (*7*) were significantly lower ($P < 0.001$, *t*-test) than when both neurons were growing (Fig. 1A). Sprouting and apparent contact were extensive, suggesting that strong electrical coupling depends on conjoint growth rather than on the opportunity for physical contact. The weak coupling that did form with unilaterally induced growth could have been associated with a slight degree of undetected growth from "stable" neurons in the absence of zap axotomy (*8*). Consequently, we used cell culture (*5, 9*), in which growth can be more directly observed, to further test our hypothesis.

Individual neuron 5's that were removed from buccal ganglia (*10*) and positioned within 500 μm of each other in culture as spheres consistently sprouted and produced overlapping neurite outgrowth (Fig. 2A). After growth, 100 percent of such simultaneously plated neurons with neurite contact became electrically coupled to one another (mean coupling coefficient, 0.39 ± 0.06; $N = 19$) (Fig. 2, B and C). Such coupling was not unique to neuron 5, but could form between a variety of neurons in culture (*11*).

Helisoma neurons in cell culture undergo a characteristic sequence of sprouting, active growth, and cessation of outgrowth and are electrophysiologically viable for up to 3 weeks. A morphological steady state is approached within 4 days of initial plating (*5, 12*). These characteristics allowed assessment of the relation between the growth status of a particular neuron and its competence to make new electrical connections.

Neurons could be added to existing cultures (Fig. 3A) when the first-plated neurons were either growing (day 2) or in a stable morphological state (day 7). When neurons were judged to be morphologically stable at the time of addition of the "secondary" neurons, coupling to the growing secondary neurons was absent or extremely weak [0.02 ± 0.01 ($N = 15$)] despite extensive overlapping of neurites. In contrast, when the original neurons were growing at the time of the addition of secondary neurons, mean coupling coefficients were significantly higher [0.56 ± 0.09 ($N = 9$)]. Furthermore, zero coupling values were more frequent for cases with stable original neurons (7 of 15 zero values compared to 0 of 9; $P < 0.05$, chi-square test). Thus coupling between original and secondary neurons was growth-dependent. Also, since coupling was strong among original neurons and among secondary neurons (Fig. 3B), it is clear that all neurons were competent to form connections at their respective times of growth.

Although the occurrence of electrical synapses is widespread (*13*), the constraints on their formation have been little studied. In all our experiments, spatially and temporally coincident neurite outgrowth was necessary and sufficient for formation of strong electrical coupling, whether growth was examined by selective nerve crushing (*3–5*), zap

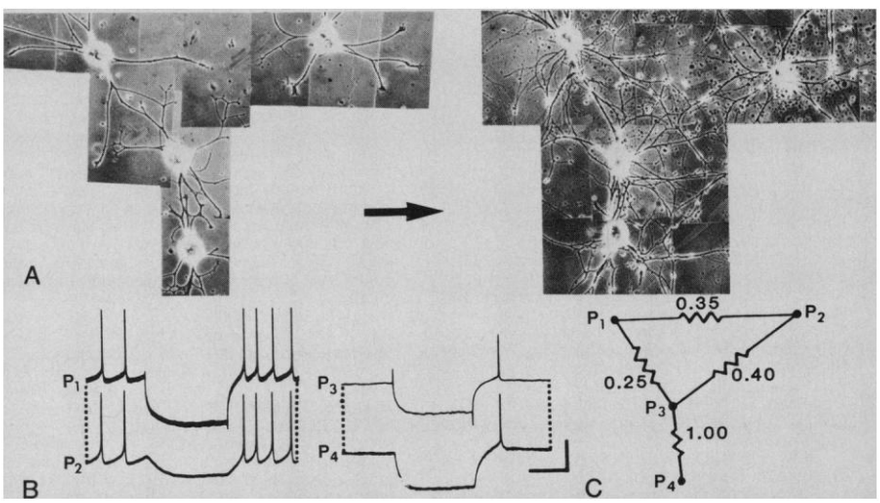

Fig. 2. (A) Group of neuron 5's in cell culture shown 1 day after plating (left) and 2 days after plating (right). (B) Passage of direct current and 1:1 propagation of action potentials between plated cells (P_1 to P_4) reveal strong electrical communication among the neurons at 2 days. (C) Schematic of the complex network, with pairwise coupling coefficients expressed near resistors symbolizing electrical junctions. Calibration, 20 mV (vertical) and 2 seconds (horizontal).

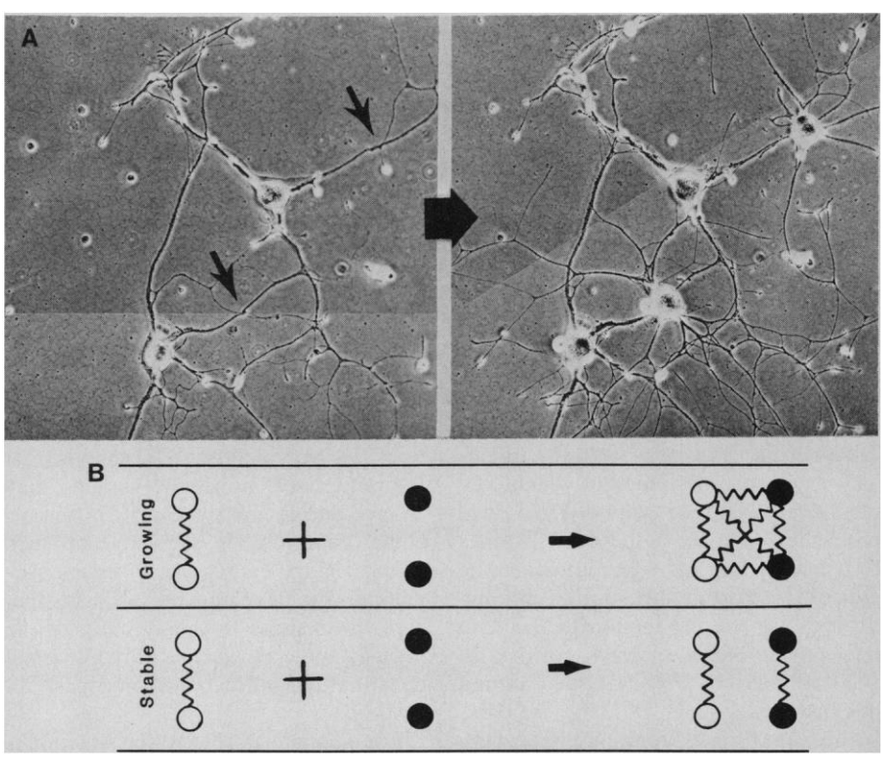

Fig. 3. Strong electrical connections resulting from temporal coordination of growth. (A) Established cultures of neurons (left) can have secondary neurons added (arrows); 1 day later, growth from secondary neurons produces networks of asynchronously plated neurons (right). (B) Coupling results differ according to the growth status of the original neurons (open circles) at the time of plating. If growing, the neurons couple with the growing secondary neurons. If neurons are morphologically stable when secondary neurons are added, then coupling with the growing secondary neurons fails to occur or is very weak.

axotomy, or cell culture. Our previous studies have shown that, once formed, certain of these electrical connections are selectively stabilized, while others are eliminated (*2, 3*). Thus while specialized mechanisms can refine neuronal circuits, growth-related processes can act as simple screening mechanisms (*3*) to set the stage for such refinement.

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Minor Histone 2A Variants and Ubiquinated Forms in the Native H2A:H2B Dimer

Abstract. *Histone octamers from calf thymus were separated into $(H3:H4)_2$ tetramers and H2A:H2B dimers by chromatography through Sephadex G100. The tetramers and dimers were analyzed for variants, ubiquitin adducts, and proteolyzed forms. The minor histone variants H2A.X and H2A.Z were found to be associated with histone H2B as H2A.X:H2B and H2A.Z:H2B dimers, respectively. Ubiquitin adducts of the H2A's and H2B were also present in H2A:H2B dimers.*

Histones mediate the primary condensation of DNA in the eukaryotic chromosome. Brief digestion of chromatin with micrococcal nuclease yields particles called nucleosomes, each of which contains about 200 base pairs of DNA, two molecules of each of the core histones (H2A, H2B, H3, and H4), plus one molecule of H1. Further digestion of chromatin leads to the production of the core nucleosome particle, which consists of 146 base pairs of DNA wrapped around the histone octamer (*1*). The octamer, which is extracted from chromatin with 2*M* NaCl, is formed by the association of two H2A:H2B dimers with an $(H3:H4)_2$ tetramer (*2*). The primary sequences of histones within each class of core histone may have a subtle effect on the structure of the core nucleosome. In vertebrates, histones H3, H2B, and H2A can have nonallelic variants, which are related by simple amino acid substitutions (*3*). Newrock *et al.* (*4*) showed that the patterns of nonallelic variants may change during embryogenesis in sea urchins. Simpson (*5*) showed that such changes in the expression of histone variants during development are accompanied by changes in the stability of the nucleosome core particle during thermal denaturation and deoxyribonuclease digestion.

For mammalian H2A's, the situation is more complex. West and Bonner (*6*) identified eight protein species within the histone 2A family of proteins. The major variants are H2A.1 and H2A.2, and the minor variants are H2A.X and H2A.Z. The other four species are ubiquitin conjugates of each of these variants. These proteins are clearly separable on a two-dimensional electrophoretic system having an acid-urea-Triton (AUT) acrylamide gel in the first dimension and an acid-urea-cetyltrimethylammonium bromide (AUC) acrylamide gel in the second dimension (*7*). The H2A variants X and Z do not comigrate with H2A.1 or H2A.2 in sodium dodecyl sulfate (SDS)-polyacrylamide gels, but both contain a conserved peptide that has been found in all H2A's sequenced to date. H2A.Z is of particular interest because it is quite different from other H2A's as shown by peptide mapping, but it is more conserved during evolution (*8*). The synthesis of the major H2A variants, H2A.1 and H2A.2, as well as the H3 variants, H3.1 and H3.2, is restricted to the S phase of the cell cycle. In contrast, the minor variants of these two core histones—H2A.X, H2A.Z, and H3.3—are synthesized in small amounts throughout the S, G_2, and G_1 phases of the cell cycle (*9*). West and Bonner (*6*) showed that H2A.X and H2A.Z are associated with the nucleosome core particles released by digestion of nuclei with micrococcal nuclease. However, whether H2A.X and H2A.Z function like the major H2A variants in the nucleosome has not been directly tested. We now report that these minor variants of H2A are associated with the core histone octamer and that they subsequently fractionate with the H2A:H2B dimer. In addition, the ubiquitinated (ubiquitin-conjugated) forms of the H2A variants and H2B comigrate with the H2A:H2B dimer.

The proteins released by extraction of calf thymus chromatin with 2*M* NaCl, *p*H 7.5 (after an earlier extraction with 0.35*M* NaCl), were chromatographed through a Sephadex G100 column in the presence of 2*M* NaCl, 10 m*M* tris, and 1 m*M* EDTA, *p*H 7.5. The peak fractions were pooled and concentrated at reduced pressure to 15 mg/ml.

The histone octamer exists in equilibrium with its subunits, the $(H3:H4)_2$ tetramer and the H2A:H2B dimer, and the balance of the equilibrium is affected by the ionic strength, *p*H, temperature, and presence of urea (*2*). We were therefore able to use gel chromatography of "octamer" through Sephadex G100 in the same ionic strength (2*M* NaCl) conditions, but at a reduced *p*H (5.0), to fractionate the $(H3:H4)_2$ tetramer and the H2A:H2B dimer. The histones in each fraction were subjected to electrophoresis in a two-dimensional AUT-AUC gel system (*7*). Figure 1 shows the elution profile from the Sephadex G100