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27. We thank C. Sano for help in DNA construction, N. Dawkins-Pisani for technical assistance, and H. Schulman, M. Hollmann, and S. F. Heineman for cDNA clones. Y.H. was supported by Japan Society for the Promotion of Science and Uehara Memorial Foundation, J.A.E. by Alzheimer Association and National Alliance for Research on Schizophrenia and Depression, and J.-C.P. by the Human Frontier Science Program Organization. This study was supported by NIH and the Mathers Foundation (to R.M).

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Response of Schwann Cells to Action Potentials in Development

Beth Stevens and R. Douglas Fields*

Sensory axons become functional late in development when Schwann cells (SC) stop proliferating and differentiate into distinct phenotypes. We report that impulse activity in premyelinated axons can inhibit proliferation and differentiation of SCs. This neuron-glial signaling is mediated by adenosine triphosphate acting through P2 receptors on SCs and intracellular signaling pathways involving Ca^{2+}, Ca^{2+}/calmodulin kinase, mitogen-activated protein kinase, cyclic adenosine 3′,5′-monophosphate response element binding protein, and expression of *c-fos* and *Krox-24*. Adenosine triphosphate arrests maturation of SCs in an immature morphological stage and prevents expression of O4, myelin basic protein, and the formation of myelin. Through this mechanism, functional activity in the developing nervous system could delay terminal differentiation of SCs until exposure to appropriate axon-derived signals.

Neural impulse activity has a critical influence on development of the nervous system at late stages of prenatal development and early postnatal life. By regulating neuronal survival, outgrowth, synaptic organization, and gene expression, impulse activity in developing neural circuits helps tailor nervous system structure in accordance with functional requirements (*1*). Much less is known regarding possible activity-dependent regulation of nonneuronal cells (glia) during development. These cells provide essential structural and functional support for developing and adult neurons and undergo marked changes in proliferation, lineage progression, and differentiation during late stages of development when neural impulse activity could provide an instructive influence. The objective of the present study was to determine whether SCs can detect impulses from premyelinated axons and, if so, to identify the signaling pathways responsible and their functional consequences.

Time-lapse confocal microscopy was used to monitor changes in intracellular Ca^{2+} in SCs in response to electrical stimulation of dorsal root ganglion (DRG) neurons (*2*). Calcium imaging has been used to detect activity-dependent axon-SC communication in the adult nervous system at the nodes of Ranvier (*3*) and synaptic terminals (*4*) in association with K^+ buffering and neurotransmitter secretion. However, it is not known whether SCs can detect impulse activity in extrasynaptic regions and in premyelinated axons before formation of nodes of Ranvier. This was investigated by culturing SCs (*5*) on DRG (*6*) axons in a preparation equipped with stimulating electrodes (*7*). Calcium levels increased immediately in neurons in response to action potential firing and activation of voltage-sensitive Ca^{2+} channels. Fifteen to 150 s after stimulation at 10 Hz (Fig. 1, A and B), intracellular Ca^{2+} increased to high levels in multiple SCs associated with the axons. The Ca^{2+} response in SCs varied proportionately with stimuli between 1 and 10 Hz and could be elicited repeatedly by electrical stimulation delivered several minutes after Ca^{2+} recovery to basal levels (Fig. 1B) (*8*).

The delay between the neuronal and the SC response suggests involvement of a soluble signaling molecule released from nonsynaptic regions because synapses do not form in pure DRG cultures (*9*). The evidence suggests that the Ca^{2+} response of SCs is mediated by adenosine triphosphate (ATP) re-

Laboratory of Developmental Neurobiology, National Institutes of Health, National Institute of Child Health and Human Development, Building 49, Room 5A38, 49 Convent Drive, Bethesda, MD 20892, USA.

*To whom correspondence should be addressed. E-mail: fields@helix.nih.gov

leased from electrically active DRG neurons. SCs express P2Y-purinergic receptors, and ATP application has been shown to elicit Ca^{2+} responses in SCs of myelinated and unmyelinated nerves and in culture (*10*). Direct application of ATP (10 nM to 1 mM) to 2- to 3-day-old SC monocultures induced an immediate and large increase in intracellular Ca^{2+} ($\Delta F/F_0$ = 2.9 ± 0.168) followed by sustained low-frequency oscillations.

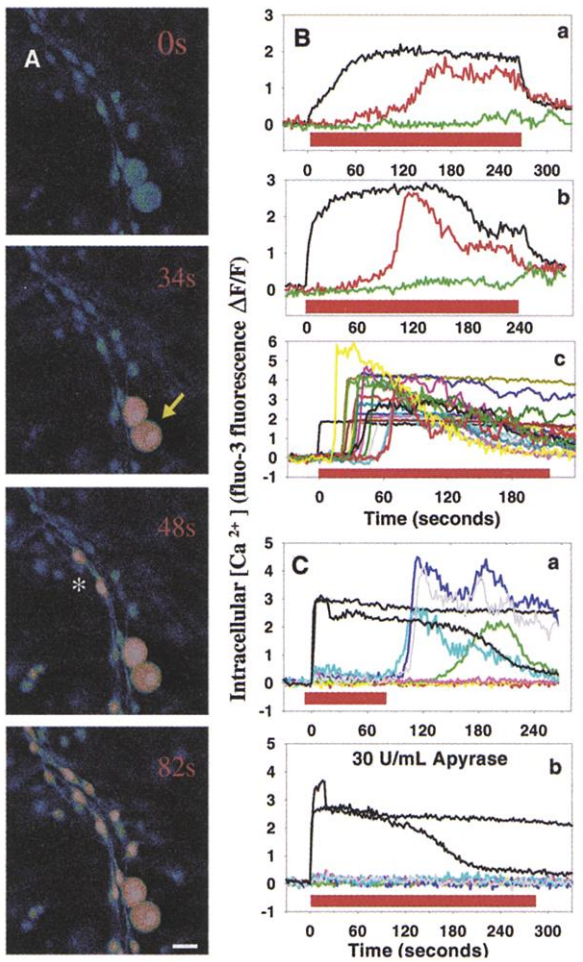

Fig. 1. Action potentials in premyelinated axons increase intracellular Ca^{2+} in SCs (*2*). (**A**) Scanning laser confocal microscopy was used to monitor changes in intracellular Ca^{2+} in SCs and DRG neurons in coculture, with the fluorescent Ca^{2+} indicator fluo-3/AM. Action potentials induced an immediate influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels in DRG neurons (arrow) (34 s). This was followed 15 to 150 s later by an increase in intracellular Ca^{2+} concentration in multiple SCs (*) (48 and 82 s). Bar, 20 μm. The Ca^{2+} responses for individual cells in (A) are plotted in (**B**) after stimulation at different frequencies (1 to 10 Hz). Ca^{2+} increased in DRG neurons (black traces) immediately upon stimulation (indicated by red bars), but responses in SCs (color traces) were delayed and proportionate to the stimulus frequencies, which were 1 , 3, and 10 Hz for (a), (b), and (c), respectively (*8*). (**C**) In SCs, the action potential-induced increase in intracellular Ca^{2+} (a) was blocked by electrical stimulation in the presence of apyrase (30 U/ml) (b), but Ca^{2+} responses in DRG neurons were not affected. Colors are as in (B). Stimulus frequency in (a) and (b) is 10 Hz; the duration of stimulation is indicated by the red bars. A time-lapse video of this figure may be viewed on *Science* Online at www.sciencemag.org/feature/data/1046675.shl.

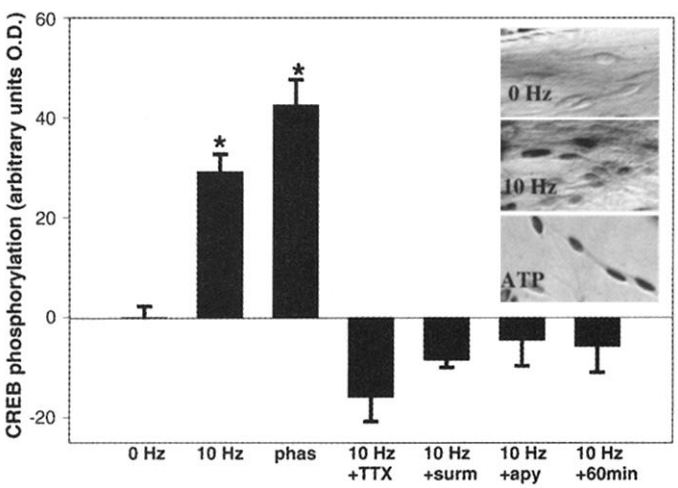

Fig. 2. The transcription factor CREB is phosphorylated in SCs by activity-dependent release of ATP from DRG neurons (*15*). (**Inset**) Levels of phosphorylated CREB (P-CREB) in the nucleus of SCs were increased on DRG axons stimulated for 30 min at 10 Hz to fire action potentials or in monocultures of SCs treated with 10 nM to 100 μM ATP for 15 min. The activity-dependent increase in P-CREB was blocked in the presence of 1 μM tetrototoxin (10 Hz + TTX), a 30 μM concentration of the P2 receptor antagonist suramin (10 Hz + surm), or apyrase (27 U/ml) (10 Hz + apy) . Levels of P-CREB returned to baseline 60 min after the 10 Hz stimulation (10 Hz + 60 min) stopped. Pulsed stimulation (phas) was applied for 0.5 s on intervals of 2 s. Error bars indicate SEM ($P < 0.0001$, one-way ANOVA; $n = 58$; *$P < 0.001$ versus control) (*35*).

Activity-dependent release of ATP has been detected from synaptic terminals, but ATP release from nonsynaptic regions of axons has not previously been shown. To investigate this, we assayed culture medium from stimulated and unstimulated cultures of pure DRG neurons for ATP content (*11*). The measurements showed an activity-dependent increase in the concentration of ATP in conditioned medium (191% increase from control), which was dependent on activation of sodium-dependent action potentials (*11*). Electrical stimulation in the presence of apyrase (30 U/ml), an enzyme that rapidly degrades extracellular ATP (*12*), blocked the Ca^{2+} response in SCs but had no effect on the Ca^{2+} response in DRG neurons (Fig. 1C). Other factors may be released in response to impulse activity in DRG neurons; however, the blockade by apyrase is compelling evidence that ATP is the activity-dependent signaling molecule in these experiments.

Axon-derived signals have been shown to modulate several transcription factors related to the differentiation of SCs (*13*). We next investigated whether action potential-induced Ca^{2+} responses in SCs were of sufficient magnitude to activate signaling pathways regulating genes that control long-term adaptive responses of SCs. The cyclic adenosine 3′,5′-monophosphate (cAMP) response element binding protein (CREB) is an important transcription factor mediating Ca^{2+}-dependent gene expression (*14*), but activation of CREB in SCs by ATP has not been shown. Immunocytochemical staining showed that CREB was activated by phosphorylation at Ser^{133} in SCs in response to electrical stimulation of DRG neurons or by direct application of ATP to SCs in monoculture (*15*) (Fig. 2). ATP-induced CREB phosphorylation was directly proportional to ATP concentration within the range of 10 nM to 1 mM, and no staining was seen when SCs were stimulated electrically in cultures without neurons (*16*). Electrical stimulation of co-cultures in the presence of apyrase, the purinergic receptor antagonist suramin, or tetrototoxin (TTX) blocked the activity-dependent phosphorylation of CREB in SCs (Fig. 2). Electrical stimulation in the presence of the Ca^{2+}/calmodulin (CaM) kinase inhibitor KN62 (30 μM) or the MEK-1 inhibitor PD098059 (50 μM) significantly inhibited CREB phosphorylation in SCs ($P < 0.0001$) relative to controls stimulated at 10 Hz (60% reduction in KN62, $n = 16$; 100% reduction in PD098059; $n = 14$). These results implicate at least two Ca^{2+}-dependent signaling pathways in the ATP-mediated phosphorylation of CREB.

Two immediate early genes, c-*fos* and *Krox-24*, have been implicated in adaptive responses of cells to extracellular stimulation. c-*fos* is regulated in part by CREB binding to a CRE

element in the promoter (*14*). The zinc-finger transcription factor *krox-24* (*Zif268, NGFI-A, Erg-1*) is expressed in immature and nonmyelinating SCs, where it has been implicated in the control SC differentiation (*17*). Electrical stimulation also increased expression of both c-*fos* and *Krox-24* in SCs; expression was blocked when stimulation was performed in the presence of apyrase. Direct application of ATP to purified SCs also induced c-*fos* and *Krox-24* mRNA and protein expression in SC monoculture (Fig. 3), indicating a novel mechanism for inducing these genes.

We then determined whether this activity-dependent axon-SC communication resulted in functional consequences that may be relevant to SC development. In the perinatal period, SCs undergo a sharp reduction in proliferation and differentiate into either myelinating or nonmyelinating phenotypes (*18*). This developmental stage coincides with the onset of active spontaneous and stimulus-evoked impulse activity in DRG axons (*19*). Therefore, SC proliferation was compared on axons firing at different frequencies. The rate of bromodeoxyuridine (BrdU) incorporation into mitotic nuclei was significantly reduced in SCs cultured on axons firing at 10 Hz (*20*) (Fig. 4), a frequency well within the normal physiological range of firing in DRG axons during the perinatal period (*19*) when proliferation of SCs declines precipitously (*21*). Moreover, 24 hours after direct application of 300 μM ATP, the proliferation rate of SCs was reduced significantly in co-culture ($P < 0.0009$, $n = 76$) or monoculture ($P < 0.009$, $n = 22$), and electrical stimulation in the presence of apyrase blocked the activity-dependent reduction in SC proliferation rate (Fig. 4).

After SCs stop proliferating, they begin differentiating into myelinating and nonmyelinating phenotypes in vivo (*18*). SC differentiation can be initiated in culture by the addition of ascorbic acid (*22*), which results in marked changes in morphology and in gene expression associated with myelination. The normal maturation of SCs from a spindle-shaped to a more rounded and flattened morphology in vitro (*23*) was completely prevented by ATP treatment over 4 days (Fig. 5, A and C). In addition, expression of the O4 antigen, a marker of SC lineage progression, was strongly inhibited by ATP in a dose-dependent manner (Fig. 5, B and D) and by a 7- to 10-day phasic stimulation of neurons in co-culture (*24*). There were no differences in the total number of SCs, or evidence of apoptosis in these co-cultures as determined by cell counts or TUNEL (terminal deoxytransferase–mediated deoxyuridine triphosphate nick end labeling) assay after 1-hour or 7-day ATP treatment (*25*). In the rat sciatic nerve, O4 expression begins before differentiation into myelinating or nonmyelinating phenotype (*26*), suggesting that impulse activity could prevent or delay differentiation into either myelinating or nonmyelinating phenotypes.

The developmental role of impulse activity in regulating glial responses such as myelination is controversial. Some studies suggest that impulse activity inhibits myelination (*27*) and others indicate that impulse activity promotes myelination (*28*) or has no effect (*29*). The present findings suggest that impulse activity could influence myelination by inhibiting the

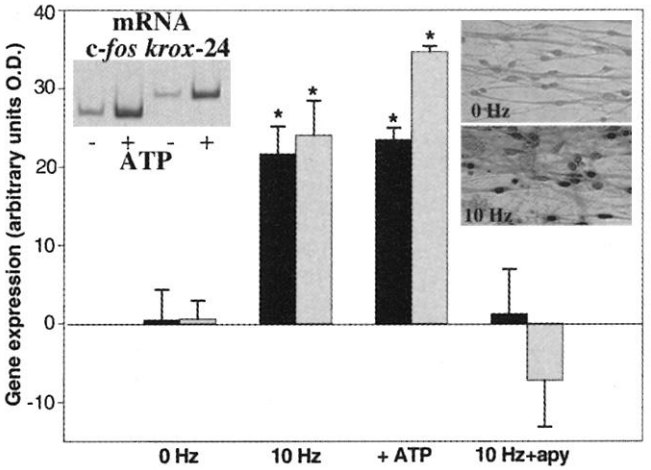

Fig. 3. Action potentials induce gene expression in SCs by the release of ATP (*15*). Immunocytochemical staining for c-Fos (black bars) and Krox-24 (gray bars) showed an increase in nuclear expression of these genes in SCs on axons stimulated for 30 min at 10 Hz (10 Hz) compared with unstimulated axons (0 Hz). Direct application of 100 μM ATP for 15 min stimulates expression of both genes (+ ATP), and electrical stimulation in the presence of apyrase (27 U/ml) blocks the response (10 Hz+apy). No increase in expression of a gene associated with myelinating phenotype, Krox-20, was detected in SCs in response to electrical stimulation or ATP application (*16*). Error bars indicate SEM. Krox-24, $P = 0.000$, one-way ANOVA; $n = 28$; $^*P < 0.005$ versus control. c-Fos, $P < 0.006$, one-way ANOVA; $n = 19$; $^*P < 0.05$ versus control (*35*). (Inset, right) Immunocytochemical staining for Krox-24 in SCs in control (0 Hz) and stimulated (10 Hz) co-cultures. (Inset, left), The ATP-induced increase in mRNA transcripts for both genes is shown by RT-PCR.

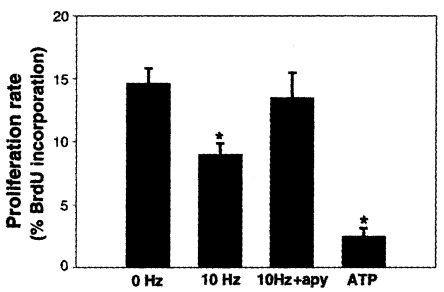

Fig. 4. Regulation of SC proliferation by activity-dependent release of ATP from DRG neurons (*20*). The proliferation rate of SCs was decreased on axons stimulated for 1 hour at 10 Hz (10 Hz) compared with SCs on unstimulated axons (0 Hz). Stimulation in the presence of apyrase (27 U/ml) (10 Hz+apy) prevented the reduction in proliferation rate, and direct application of 300 μM ATP for 24 hours significantly inhibited SC proliferation on unstimulated axons. Error bars indicate SEM ($P < 0.0001$, one-way ANOVA; $n = 126$; $^*P < 0.005$ versus control) (*35*).

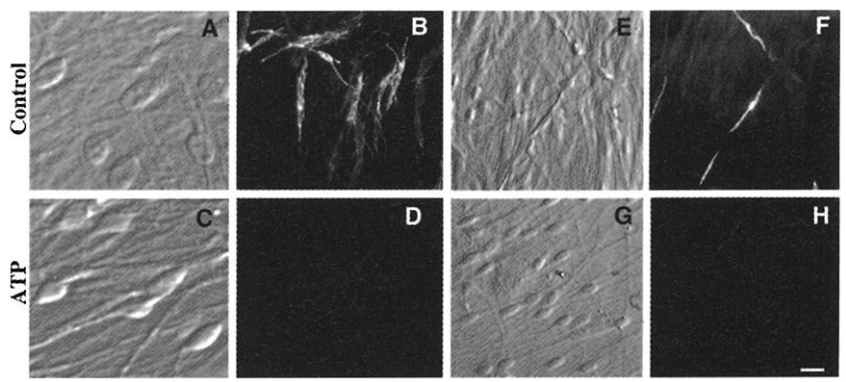

Fig. 5. ATP delays maturation and differentiation of SCs (*31, 32*). Chronic treatment of SCs with 300 μM ATP in co-culture with DRG neurons for 4 days prevented the normal development from spindle-shaped to rounded, flattened morphology (**A** and **C**) and prevented expression of the O4 antigen (**B** and **D**) just before differentiation into premyelinated or promyelinated phenotypes (18.2 ± 5.7 O4-positive cells per field control versus 0.03 ± 0.04 ATP; $n = 24$; *t* test, $P < 0.004$) (*26*). ATP treatment for 10 days prevented the close association of SCs with axons (**E** and **G**) and the formation of compact myelin and expression of MBP (**F** and **H**). Areas of the co-cultures containing no neuron cell bodies are shown (*6*). Bar in (H) indicates 20 μm; bar indicates 5 μm in (A) and (C), 10 μm in (B) and (D), and 20 μm in (E) through (F).

maturation and differentiation of SCs. Krox-20 has been associated with induction of genes characteristic of myelinating SCs (*30*). Expression of both Krox-20 (*30*) and galactocerebroside (Gal-C) (*23*), a myelin glycolipid, is reduced in SCs differentiating into the nonmyelinating phenotype. When long-term cultures were co-treated with ATP and ascorbic acid (*31*), the normal down-regulation of Krox-20 and Gal-C was inhibited, indicating failure to differentiate beyond the immature stage (89.7% versus 50% Krox-20$^+$ cells, ATP versus control, $P = 0.000$, χ^2 test, $n = 1691$ cells; and 7.2 $\pm$ 0.41 versus 0.5 $\pm$ 0.28 Gal-C positive profiles per field in ATP versus control, $n = 8$ cultures, $P < 0.0001$, t test). SCs remained spindle-shaped and were not aligned with axons even after 2 weeks of co-treatment with ascorbic acid and ATP (Fig. 5, E and G). Moreover, no compact myelin was detected in ATP-treated cultures, and myelin basic protein (MBP), a component of compact myelin, was not detected by immunocytochemistry (*32*) (Fig. 5, F and H).

The immunological and morphological evidence suggests that ATP arrests SC maturation before differentiation into either the myelinating or nonmyelinating phenotypes. Impulse activity may delay terminal differentiation of SCs until exposure to appropriate myelin-inducing signals. Many myelination signals are axon-specific and, like the caliber of the axon, are related to maturation of individual axons. Impulse activity may promote myelination by increasing the pool of SCs in a predifferentiated state available to respond to myelin-inducing signals.

SC proliferation, lineage progression, and differentiation are highly regulated by extrinsic and axonally derived factors. Firing frequency and pattern change with the developmental stage of the axon. Impulse activity may be one signal from the axon indicating the appropriate time for SCs to exit the cell cycle and become responsive to factors controlling differentiation into phenotypes necessary for SC functions related to neuronal excitability. A large number of factors contribute to regulation of SC development and proliferation. The present study indicates that electrical activity, acting through the release of ATP, can have a profound influence on SC development, proliferation, and gene expression.

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2. Confocal microscopy (MRC 1024, Bio-Rad, Hercules, GA) with the Ca^{2+}-sensitive indicator fluo-3/AM (Molecular Probes, Eugene, OR) was used to measure changes in fluorescence intensity ($\Delta F/F_o$) due to Ca^{2+} transients in SCs and DRG neurons in a Hepes-buffered balanced salt solution (pH 7.2) at room temperature (*33*). Nikon ×40, 0.7 numerical aperture (NA) long working distance and 1.3 NA objectives were used for plastic culture dishes and cover slips, respectively. Confocal microscopy excludes fluorescent signals from cells outside the plane of focus and enables unambiguous distinction of Ca^{2+} responses in SCs from responses in neurons and axons. Ca^{2+} transients in response to bath application of ATP (Molecular Probes) were also measured in SCs grown in monoculture for 24 to 72 hours.
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5. SCs from mouse sciatic nerve (P2) were purified by Thy 1.1 complement-mediated lysis and AraC treatment [see (*27*) for detailed procedure]. More than 99% of the cells stained positive for the SC marker S-100 (*24*).
6. DRG neurons dissected from embryonic day 13.5 mice were cultured for 3 weeks in medium containing 5% horse serum (HS) as described (*34*) in three-compartment cell culture dishes with stimulating electrodes (*7*). Cultures were >70% large-type RT97$^+$ neurons and contained no contaminating SC as detected by reverse transcription–polymerase chain reaction (RT-PCR) for a SC-specific isoform of L1 (*34*). SC/DRG co-cultures were made by plating SC (*5*) on DRG neurons after 3 weeks in culture (*27*). Neurons and SCs were easily distinguishable by morphological differences and size. Cell bodies of DRG neurons are spherical and are ~25 μm in diameter, whereas SC cell bodies are small (~10 μm) and spindle-shaped. The validity of these criteria was confirmed by immunocytochemistry tests for S-100 and for the phosphorylated neurofilament protein RT97.
7. Action potentials were induced in DRG axons by electrical stimulation (200-ns biphasic pulses at 5 V) through platinum electrodes in three-compartment cell culture chambers [see (*34*) for methods]. Cultured DRG neurons are not spontaneously active but follow stimulus frequencies up to 10 Hz [H. Z. Sheng, R. D. Fields, P. G. Nelson, *J. Neurosci. Res.* **35**, 459 (1993)]. Electrophysiological recording in DRG neurons labeled with DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) show that only those neurons with axons traversing the barrier between central and side compartments are stimulated to fire action potentials (*33*). Neurons stimulated for 1 week exhibit no changes in survival, cell diameter, size or frequency distribution, or protein content (*34*).
8. At higher frequency stimulation, the initial slope of the Ca^{2+} increase was steeper [$P < 0.002$, analysis of variance (ANOVA)], more SCs in the field responded ($P < 0.009$, χ^2 test), the amplitude of the Ca^{2+} increase was higher ($P < 0.03$, ANOVA), and the latency between the neuronal and SC response was shorter ($P < 0.001$, ANOVA) than for lower frequency stimulation. Different stimulus frequencies were delivered randomly to the same cells after 30-min rest intervals.
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11. Medium collected from side compartments of pure DRG cultures (200-μl volume) after stimulation (30 min) was measured for ATP content with a luciferase assay (Molecular Probes) and compared with unstimulated controls. Growth medium (*34*) was replaced with serum-free medium 2 hours before the experiments. ATP concentration increased 191.5 $\pm$ 28% and 171.0 $\pm$ 27% in response to 10 Hz and phasic (0.5-s 10-Hz bursts at 2-s intervals) stimulation, respectively ($P <$ 0.003, one-way ANOVA; $n = 87$). No increase was detected after stimulation in the presence of 1 μM tetrototoxin (86.6 $\pm$ 10% of control, $n = 49$). A standard curve, calculated by linear regression of luciferase activity against a series of known ATP concentrations ($r^2 = 0.99$) indicated levels of ~14.5 nM ATP in stimulated cultures. This concentration represents a ~10^{-7}- to 10^{-8}-fold dilution of the ATP concentration that would be produced within the axon-SC intercellular space (SC-axon volume = 15 pL/side compartment). The 3-week-old DRG neuron cultures were free of contaminating SCs (*6*, *34*). In SC monoculture, no ATP was released after electrical stimulation or depolarization with 60 mM KCl.
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15. SC/DRG co-cultures were stimulated electrically for 30 min and SC monocultures were stimulated for 15 min with 100 μM ATP. Cultures were fixed immediately with 1% paraformaldehyde [for phosphorylated CREB (P-CREB) and phosphorylated MAPK (P-MAPK)] or after 1 hour (for c-Fos, Krox-24, and Krox-20). Cells were permeabilized with 0.2% Triton X-100 and incubated for 1 hour at room temperature with antibody to P-CREB (1:800; New England Biolabs, Beverly, MA), P-MAPK (1:800; New England Biolabs), c-Fos (1:200; Calbiochem, San Diego, CA), and Krox-20 and Krox-24 (1:200 and 1:1000, Santa Cruz Biotech, Santa Cruz, CA). The avidin-biotin complex (ABC)–peroxidase method was used to localize biotinylated goat antibody to rabbit immunoglobulin G (IgG) (Vector Labs, Burlingame, CA). Images were acquired by video microscopy from 10 to 15 randomly chosen fields per culture, and, after digitization, the staining intensity of each nucleus was quantified and averaged with all others to obtain the mean nuclear staining intensity for each culture (Image-1, Universal Imaging, West Chester, PA). Values were normalized with respect to control for pooling replicate experiments and were quantified on a scale of 0 to 55 optical density (O.D.) (unstained to pure black) as described by R. D. Fields, F. Eshete, B. Stevens, and K. Itoh [*J. Neurosci.* **17**, 7252 (1997)]. In statistical tests, n = number of cultures. mRNA transcripts were amplified by 30 cycles of RT-PCR with standard methods (*34*) from RNA extracted from SC cultures stimulated with 300 μM ATP for 30 min. Primer sequences for c-Fos, Krox-24, and cyclophilin (control) are given by H. Z. Sheng, P. X. Lin, and P. G. Nelson [*Anal. Biochem.* **222**, 123 (1994)].
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20. SCs were co-cultured with DRG neurons, and after 48 hours axons were stimulated (*7*) at 10 Hz for 1 hour. Cultures were incubated with BrdU (Boehringer Mannheim, Indianapolis, IN) for 1 hour and fixed and stained according to manufacturer instructions. Cultures were counterstained with Hoechst nuclear stain (Molecular Probes) at a 1:4000 dilution for 10 min. All SC nuclei stained with BrdU and Hoechst were counted in each microscope field, and the proliferation rate was calculated as the ratio of BrdU-positive to Hoechst-positive SC nuclei per microscope field. Ten to 15 randomly chosen fields were sampled to obtain a mean for each culture well; n = number of culture wells. Pure DRG cultures stained with Hoechst or BrdU contain no SC nuclei, and all cells counted in co-culture co-localized with the SC marker Gal-C (*24*, *32*).
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24. The proportion of O4-positive SCs was measured by computerized morphometry (Metamorph, Universal Imaging) and showed a dose-response relation to ATP concentration (1 to 100 μM; $P < 0.001$ linear regression against the log of ATP concentration; $n = 8$). The concentration of ATP in the extracellular medium declined to 7.5% of the starting level 24 hours after adding ATP. The number of O4-positive SCs was also reduced to 33.5% of controls ($P < 0.03$, $n = 21$) in cultures stimulated 24 to 48 hours after co-culture in a phasic pattern for 7 to 10 days without ascorbic acid. The number of SC nuclei was not different at this time point (104 $\pm$ 7.2 versus 108 $\pm$ 13 per field). SCs were stained without fixation and were then incubated for 30

min with fluorescein isothiocyanate (FITC)–congugated goat antibody to mouse IgM (ICN/Cappel, Aurora, OH). Immunocytochemical detection of S-100 (1:500; Sigma) was detected with FITC-conjugated goat F(ab')2 fragment to mouse IgG (Cappel).
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31. Seven days after co-culture, SC differentiation and myelination were initiated by adding ascorbic acid (50 μg/ml) in medium containing 5% HS. For procedural details see (*27*). Morphology changes were observed by day 3 or 4, and myelin profiles were evident by phase contrast microscopy and MBP staining within 10 to 14 days of the changing of the medium. Cultures received daily changes of half their medium with or without 300 μM ATP (Sigma).
32. Co-cultures were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with monoclonal antibodies to myelin basic protein and Gal-C (Boehringer-Mannheim) at a dilution of 1:500 (MBP) and 1:50 (Gal-C) for 1 hour at room temperature. Primary antibodies were detected with FITC-conjugated goat F(ab')2 fragment to mouse IgG (ICN/Cappel) for MBP and rhodamine-conjugated Fc-specific goat antibody to mouse Gal-C (Jackson Immunoresearch, West Grove, PA).
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A BAC-Based Physical Map of the Major Autosomes of *Drosophila melanogaster*

Roger A. Hoskins,[1]* Catherine R. Nelson,[2] Benjamin P. Berman,[2] Todd R. Laverty,[2] Reed A. George,[1] Lisa Ciesiolka,[1] Mohammed Naeemuddin,[1] Andrew D. Arenson,[3] James Durbin,[3] Robert G. David,[3] Paul E. Tabor,[3] Michael R. Bailey,[3] Denise R. DeShazo,[3] Joseph Catanese,[4]†‡ Aaron Mammoser,[4] Kazutoyo Osoegawa,[4]† Pieter J. de Jong,[4]†‡ Susan E. Celniker,[1] Richard A. Gibbs,[3] Gerald M. Rubin,[2] Steven E. Scherer[3]

We constructed a bacterial artificial chromosome (BAC)–based physical map of chromosomes *2* and *3* of *Drosophila melanogaster*, which constitute 81% of the genome. Sequence tagged site (STS) content, restriction fingerprinting, and polytene chromosome in situ hybridization approaches were integrated to produce a map spanning the euchromatin. Three of five remaining gaps are in repeat-rich regions near the centromeres. A tiling path of clones spanning this map and STS maps of chromosomes *X* and *4* was sequenced to low coverage; the maps and tiling path sequence were used to support and verify the whole-genome sequence assembly, and tiling path BACs were used as templates in sequence finishing.

The fruit fly *Drosophila melanogaster* is a principal model organism in metazoan genetics and molecular biology. Here, we describe a BAC-based physical map of chromosomes *2* and *3* constructed as part of the effort to determine the *D. melanogaster* genome sequence (*1*). There are five chromosomes (*X*, *2*, *3*, *4*, and *Y*), and the second and third together account for ~97 Mb of the ~120-Mb euchromatic portion of the genome. Several clone-based physical maps have been described previously. Low-resolution yeast artificial chromosome maps of the genome have been produced by polytene chromosome in situ hybridization (*2*), and cosmid maps of regions of the *X* chromosome have been made by STS content and fingerprint mapping (*3*). The most complete previous map is the P1-based map by Kimmerly *et al.* (*4*) [also see (*5*)], constructed by polymerase chain reaction–based STS content mapping and polytene chromosome in situ hybridization. On chromosomes *2* and *3*, it comprises 348 sets of contiguously overlapping clones (contigs), each with at least two STS markers.

The contiguity of the P1 map was limited by the shallow genome coverage of the library (about sixfold) and the relatively small insert size of the clones (80 kb). BAC vectors can accommodate larger inserts, so we created a BAC map using the P1 map as a starting point. We constructed a BAC library (RPCI-98) from an isogenic y^1; cn^1 bw^1 sp^1 strain (*6*). High-molecular-weight (HMW) DNA was prepared from adults (*7*), partially digested with Eco RI and Eco RI methylase, size fractionated, and cloned into the pBACe3.6 vector (*8*). The library consists of 17,540 recombinant clones with an average insert size of 163 kb and represents ~24-fold coverage of the euchromatic portion of the genome (*9*).

We hybridized radioactively labeled oligonucleotide probes made from STS markers selected from the P1 map to colony arrays representing the RPCI-98 library (*10*); 1226 markers from the P1 map are included in the BAC map, at an average spacing of 80 kb. Because these markers had been previously localized, the data for each of the four chromosome arms (2L, 2R, 3L, and 3R) could be assembled separately, and this reduced the complexity of the assembly process.

To join the initial contigs together, new markers were added to the map in multiple iterations of STS design, hybridization, and data assembly. The new markers included 690 designed from BAC end sequences (*1*), 5 designed from genomic sequences, and 2 designed from coding sequences of known genes. Potential markers with substantial sequence similarity to more than one location in the genome were rejected. These were identified by scanning databases of known repeats and scanning for instances of the sequence in multiple, nonoverlapping BAC and P1 clones. In the latter stages of the project, restriction fingerprints (see description below) were used in STS design to identify BACs that extended farthest into the map gaps. The map presented here includes 1923 markers at an average spacing of 50 kb.

STS content data were assembled by chromosome arm in the program SEGMAP v3.49 (*11*) and manually edited. Cytological data associated with markers from the P1 map were used to identify false joins in the BAC map. These were due to markers that hybridized to multiple sites in the genome and were resolved by removing the markers from the map. Markers that had been mapped to the wrong chromosome arm in the P1 map were identified by

[1]Berkeley *Drosophila* Genome Project, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA. [2]Berkeley *Drosophila* Genome Project, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA. [3]Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX 77030, USA. [4]Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, NY 14263, USA.

*To whom correspondence should be addressed. E-mail: hoskins@bdgp.lbl.gov
†Present address: Children's Hospital Oakland Research Institute, Oakland, CA 94609, USA.
‡Present address: Parke Davis Laboratory for Molecular Genetics, Alameda, CA 94502, USA.