Regional Calcium Regulation within Cultured *Drosophila* Neurons: Effects of Altered cAMP Metabolism by the Learning Mutations *dunce* and *rutabaga*

Brett Berke¹ and Chun-Fang Wu^{1,2}

¹Interdisciplinary Program in Neuroscience and ²Department of Biological Sciences, University of Iowa, Iowa City, Iowa 52242

The *dunce* (*dnc*) and *rutabaga* (*rut*) mutations of *Drosophila* affect a cAMP-dependent phosphodiesterase and a Ca²⁺/CaM-regulated adenylyl cyclase, respectively. These mutations cause deficiencies in several learning paradigms and alter synaptic transmission, growth cone motility, and action potential generation. The cellular phenotypes either are Ca²⁺ dependent (neurotransmission and motility) or mediate a Ca²⁺ rise (action potential generation). However, interrelations among these defects have not been addressed. We have established conditions for fura-2 imaging of Ca²⁺ dynamics in the “giant” neuron culture system of *Drosophila*. Using high K⁺ depolarization of isolated neurons, we observed a larger, faster, and more dynamic response from the growth cone than the cell body. This Ca²⁺ increase depended on an influx through Ca²⁺ channels

and was suppressed by the Na⁺ channel blocker TTX. Altered cAMP metabolism by the *dnc* and *rut* mutations reduced response amplitude in the growth cone while prolonging the response within the soma. The enhanced spatial resolution of these larger cells allowed us to analyze Ca²⁺ regulation within distinct domains of mutant growth cones. Modulation by a previous conditioning stimulus was altered in terms of response amplitude and waveform complexity. Furthermore, *rut* disrupted the distinction in Ca²⁺ responses observed between the periphery and central domain of growth cones with motile filopodia.

Key words: learning and memory; *dnc*; *rut*; fura-2; Ca²⁺ dynamics; neuronal culture; growth cone; cAMP

Second messenger pathways involving cAMP-dependent protein kinase (PKA) and Ca²⁺/CaM-dependent protein kinase (CaMKII) have been implicated in the cellular basis of learning and memory (Cline, 1991; Carew, 1996; Chen and Tonegawa, 1997; Bailey et al., 2000; Dickson et al., 2001). Ca²⁺/CaM stimulation of cAMP production (Cooper et al., 1995) and activation of CaMKII (Soderling, 2000) may link neuronal activity to its regulation of use-dependent morphological and physiological plasticity.

The earliest identified mutations to disrupt learning in *Drosophila* included *dunce* (*dnc*) and *rutabaga* (*rut*), which were later found to affect a cAMP-specific phosphodiesterase and a Ca²⁺/CaM-activated adenylyl cyclase (Dudai et al., 1976; Byers et al., 1981; Aceves-Pina et al., 1983; Livingstone et al., 1984; Tully and Quinn, 1985). Consequentially, their defects in developmental and activity-dependent neuronal plasticity have been studied extensively. The *dnc* and *rut* mutations increase and reduce, respectively, the rate of habituation of a jump-and-flight escape response (Engel and Wu, 1996). Abnormal mechanosensory neuron development and adaptation have also been reported in adult *rut* flies (Corfas and Dudai, 1990, 1991). Both mutations inhibit the activity-dependent facilitation and potentiation of neurotransmitter release at the larval neuromuscular junction (Zhong and Wu,

1991) while causing opposite changes in the extent of presynaptic arborization (Zhong et al., 1992). During development in culture, *dnc* and *rut* suppress growth cone motility (Kim and Wu, 1996) and alter the kinetics and activity-dependence of neurotransmitter release from the growth cone (Yao et al., 2000) and presynaptic terminal (Lee and O'Dowd, 2000). Furthermore, K⁺ channel regulation and action potential patterning are also disrupted in larval muscle and cultured neurons from these mutants (Zhong and Wu, 1993a; Zhao et al., 1995; Zhao and Wu, 1997), which may contribute to defects in neurotransmitter release and growth cone motility.

In several species, neural activity and Ca²⁺ regulate growth cone behaviors during development. The frequency of Ca²⁺ transients determines the rate of neurite outgrowth in *Xenopus* embryos (Gomez and Spitzer, 2000), whereas the navigation by guidepost cells involves Ca²⁺ signaling in the grasshopper embryo (Bentley et al., 1991). In culture, different levels of evoked Ca²⁺ can lead to the enhancement or suppression of motility and neurite outgrowth (for review, see Kater et al., 1988; Kater and Mills, 1991; Fields et al., 1993; Rehder et al., 1996). For example, a spatially restricted Ca²⁺ increase in filopodia and the leading edge of the lamellipodia interact with cAMP to direct growth cone turning (Ming et al., 1997; Zheng, 2000).

In *Drosophila*, the “giant” neuron culture system (Wu et al., 1989) has facilitated investigations of neuronal development that may be hampered by the small size of central neurons and their processes. We have established conditions for imaging Ca²⁺ dynamics in cultured giant neurons and exploited the increased spatial resolution to differentiate the region-specific and use-dependent Ca²⁺ increase evoked by high K⁺ depolarization. Our initial exploration with the *dnc* and *rut* mutations identifies Ca²⁺

Received June 29, 2001; revised Nov. 13, 2001; accepted Dec. 7, 2001.

We thank Dr. Wei-Dong Yao for his generous help during the initial exploration of this work. We also thank Shetarra Walker and Daniel Choi for their assistance, Peter Taft for technical suggestions, and Drs. Atsushi Ueda, Mei-Ling Joiner, and Sudipta Saraswati for comments on this manuscript.

Correspondence should be addressed to Dr. Chun-Fang Wu, Department of Biological Sciences, University of Iowa, Iowa City, IA 52242. E-mail: cfwu@blue.weeg.uiowa.edu.

Copyright © 2002 Society for Neuroscience 0270-6474/02/224437-11\$15.00/0

regulatory defects caused by abnormal cAMP metabolism. The results also demonstrate that Ca^{2+} imaging in the giant neuron culture system may facilitate genetic dissection of Ca^{2+} -dependent neuronal development and plasticity.

MATERIALS AND METHODS

Animal stocks. All fly stocks were raised on standard *Drosophila* medium at room temperature. Two *dnc* alleles, *dnc*¹ (Dudai et al., 1976) and *dnc*² (Bellen and Kiger, 1988), and two *rut* alleles, *rut*¹ (Aceves-Pina et al., 1983; Livingstone et al., 1984) and *rut*² (Bellen et al., 1987), were used in this study. The wild-type strain Canton-Special was used as the control. Results from the different mutant alleles within each locus (*dnc* or *rut*) have been pooled because they did not differ significantly.

Embryonic cell culture. Individual cultures were prepared from either single embryos (Seecof et al., 1971; Salvaterra et al., 1987; Wu et al., 1989) or homogenized embryos as described previously (Zhao and Wu, 1997; Yao and Wu, 1999; Yao et al., 2000). Cultures were plated on glass coverslips in modified Schneider's medium (Invitrogen, Gaithersburg, MD) containing 200 ng/ml insulin (Sigma, St. Louis, MO), 20% fetal bovine serum (Summit Biotechnologies, Ft. Collins, CO), 50 $\mu\text{g}/\text{ml}$ streptomycin, and 50 U/ml penicillin (all from Invitrogen). The production of giant neurons (Saito and Wu, 1991; Zhao and Wu, 1997) involved the inhibition of neuroblast cytokinesis. This was achieved by adding 1–2 $\mu\text{g}/\text{ml}$ of cytochalasin B (Sigma), which blocks actin polymerization and cell division, to the culture media. All cells were grown in a humidified chamber at room temperature (21–24°C), and the cytochalasin B-containing media was replaced with fresh culture media within 24 hr after plating.

Imaging. Media containing 4–6 μM fura-2 AM and 10–15 $\mu\text{l}/\text{ml}$ pluronic acid was used to load 2- to 6-d-old cells in the dark at room temperature for 1.5–2 hr. Fura-2-loaded cells were perfused continuously in a chamber allowing for fast laminar flow of solution. The dead volume was <0.5 ml, and the time from valve opening to perfusion across the coverslip was on average 6 sec (up to 16 sec in early experiments). All of the salines used in this study were buffered at pH 7.1–7.2 with 5 mM HEPES (adjusted with NaOH). Normal bath saline contained (in mM): 128 NaCl, 2 KCl, 4 MgCl_2 , 1.8 CaCl_2 , and 35.5 sucrose. For high K^+ depolarizing salines, the following modifications to normal saline were made: (1) 60 K saline, 60 mM KCl, and 70 mM NaCl; (2) 42 K saline, 42 mM KCl, and 88 mM NaCl; (3) 30 K saline, 30 mM KCl, and 90 mM NaCl; (4) 0 Ca saline, normal saline with 0 mM CaCl_2 , 5.8 mM MgCl_2 , and 5 mM EGTA; (5) 30 K 0 Ca and 60 K 0 Ca saline, 30 or 60 K saline with the above changes. The duration of stimulation varied between 5 and 30 sec, with the latter being more common because of the lack of responsiveness from mutant neurons. In Figures 7B, 8, and 9, stimuli were used to achieve a signal-to-noise ratio of at least 5 and most often >10.

Giant neurons were imaged using an inverted Diaphot microscope equipped for epifluorescence (Nikon, Tokyo, Japan). Video images were obtained with an intensified CCD camera (IonOptix, Milton, MA) and a shutter/filter wheel (IonOptix) controlled by the IonWizard software (IonOptix). Images were collected with 40 $\times$ [1.3 numerical aperture (NA)] or 100 $\times$ (1.3 NA) oil-immersion objectives at 1 Hz or higher sampling rate, and single or averaged frames were saved on-line in a pseudocolor format. The camera gain was adjusted such that most cellular responses for the numerator (340 nm) and denominator (380 nm) were within a proper working range of the camera output (they approached neither the upper nor lower limits). This ensured a more linear measure and less disturbance by noise. Data collected with the 40 $\times$ and 100 $\times$ objectives were combined where applicable, because changes in magnification did not consistently alter the Ca^{2+} response. No autofluorescence caused by the media or microscope optics was observed, and compensation was made for spatial variations in the camera dark current, the camera gain, and the intensity of the excitation source. A full-frame (cell-free) background image collected before and a background zone used during the experiment helped compensate for the above systematic errors. Dye bleaching was not observed at the acquisition rates used in this study. The soma and processes differed in the intensity of basal fluorescence, so the camera gain and black level were adjusted to maintain the camera output within the optimal dynamic range, and these regions were imaged during different recording epochs.

The fura-2 binding constant and the coefficients for use in the Grynkiewicz formula (Grynkiewicz et al., 1985), $K_D(F_0/F_S)(R - R_{\min})/(R_{\max} - R) = [\text{Ca}^{2+}]$, were determined using a cell-free calibration (Williams et al., 1985). R is the ratio of fluorescence at 340 nm to the fluorescence at

380 nm, $R_{\min}$ and $R_{\max}$ are the 340 nm/380 nm ratios in 0 and 10 mM Ca^{2+} , K_D is the effective dissociation constant (147 nM), and F_0 and F_S are the 380 nm excitation efficiencies in 0 mM Ca^{2+} and at saturating Ca^{2+} (10 mM). The Ca^{2+} level was quantified by applying the Grynkiewicz formula to fura-2 fluorescence recorded from square zones that were 16 μm^2 in area. Running averages of $[\text{Ca}^{2+}]$ taken from selected neuronal compartments were performed before analysis using a window of five points.

Electrophysiology. Whole-cell patch-clamp recording from *Drosophila* giant neurons has been described previously (Saito and Wu, 1991; Zhao and Wu, 1997). Patch electrodes were pulled from glass capillaries (75 ml micropipettes; VWR, Chicago, IL) and had a tip resistance of 2–5 M Ω when measured in normal saline. Whole-cell recordings were obtained with an EPC-7 patch-clamp amplifier (Medical Systems Corp., Greenvale, NY), and electrodes were filled with (in mM): 144 KCl, 1 MgCl_2 , 0.5 CaCl_2 , 5 EGTA, 10 HEPES, and adjusted with KOH to a pH of 7.1.

RESULTS

Regional heterogeneity

Giant neuron cultures grown from cytokinesis-arrested embryonic neuroblasts were loaded with fura-2 AM and subsequently imaged during perfusion with depolarizing high K^+ saline. Comparable with the proportion previously found in cultures of non-cytokinesis-arrested larval neurons (Kim and Wu, 1991, 1996), ~30% of these giant embryonic neurons contained neurites ending with large growth cones. After depolarization, a robust Ca^{2+} increase was imaged throughout single, isolated neurons. Thirty second depolarizations of the wild type by an increase from 2 to 60 mM $[\text{K}^+]$ (60 K saline) caused a Ca^{2+} increase to appear first in the growth cone and neurites (Fig. 1). Later, an increase was observed throughout the soma, beginning in regions nearest the neurite (not shown in the image sequence). We quantified the intracellular Ca^{2+} transient within the growth cone to study how its regulation is distinct from the soma in terms of amplitude, kinetics, and Ca^{2+} source. Perfusion with high K^+ saline caused a larger Ca^{2+} increase in the growth cone that peaked earlier and decayed more rapidly than in the cell body (Fig. 1B, inset).

Involvement of Ca^{2+} and Na^+ channels in the Ca^{2+} increase

Drosophila cultured neurons express various ion channels, leading to several categories of electrical excitability when investigated with patch-clamp techniques applied to the soma (Zhao and Wu, 1997; Yao and Wu, 1999; Yao et al., 2000). However, the electrophysiological study of ion channels in the neurite and growth cone has been hampered by the small size of these structures [but see Saito and Wu (1991)]. A pharmacological approach was taken to selectively block or isolate the involvement of specific ion channel types because their differential expression may underlie the temporal and spatial characteristics of the Ca^{2+} signal. Depolarization in 0 Ca^{2+} saline (Fig. 2A) or in the presence of a Ca^{2+} channel blocker, cobalt (Fig. 2B), reversibly eliminated the response. Furthermore, a Na^+ channel blockade by TTX decreased the amplitude of the Ca^{2+} increase (Fig. 2C). From these results, we conclude that an influx through Ca^{2+} channels is required and Na^+ channels are involved in the high K^+ depolarization-induced response. It is possible that fura-2 loading alters aspects of ionic currents and spike activity that may affect Ca^{2+} influx and thus cytosolic Ca^{2+} concentration. To examine these potential effects, we compared Na^+ -dependent action potential generation (Wu et al., 1989; Saito and Wu, 1993) and whole-cell outward currents between neurons with and without fura-2 loading. We verified that fura-2 does not impair action potential generation (Fig. 3A). In giant neuron cultures, the cell population can be classified (types 1–4) according to the propor-

Figure 1. The high K^+ depolarization-induced Ca^{2+} increase is distinct between the soma and growth cone. *A*, Phase-contrast and pseudocolor images of a wild-type cell are shown during and after perfusion of 60 mM K^+ (60 K) from 0 to 30 sec. Depolarization caused a Ca^{2+} increase within discrete locations along the proximal neurite and at the growth cone, later appearing in the cell body (see color scale bar in *B* for Ca^{2+} levels). In the phase-contrast and 25 sec pseudocolor images, boxed regions indicate where fura-2 fluorescence was quantified. *B*, Quantification of intracellular Ca^{2+} over time accentuates the differences in response amplitude between the soma (*S*) and growth cone (*GC*). Accompanying the larger amplitude, the growth cone responds with faster kinetics (inset, amplitude normalization). Amplitude normalization was performed by subtracting the minimum value from every point and then dividing by the peak value. This caused the normalized value of each point of the trace to vary between 0 and 1. Scale bar (shown in *A*): 10 μ m. Black bars in *B* indicate the perfusion of 60 K saline.

tion between the peak and sustained components and the decay time constants of the total outward current (Fig. 3*B*) (I.-F. Peng and C.-F. Wu, unpublished results). A similar classification has been used to describe outward currents in non-cytochalasin B-treated larval neurons in culture (Delgado et al., 1998). We did not observe a substantial change in the population distribution among the different classes (fura vs non-fura, type 1–1/24 vs 2/30, type 2–7/24 vs 6/30, type 3–8/24 vs 7/30, type 4–8/24 vs 18/33). Our data therefore imply that fura-2 does not strongly affect ionic currents underlying neuronal spike activity.

Variation in the amplitude and kinetic properties of the Ca^{2+} response

Individual neurons displayed considerable variation in the high K^+ -induced Ca^{2+} increase. We analyzed a large number of cells for the regional differences in the evoked response (Fig. 4). Compared with the soma, a larger number of growth cones

responded to 30 K saline, some with a Ca^{2+} increase above 0.6 μ M (growth cone, GC, 0.31 ± 0.14 vs soma, S, 0.05 ± 0.02 ; mean $\pm$ SEM in micrometers; sample size given in figure legends). Response amplitude distributions displayed obvious differences between 30 and 60 K stimulations (Fig. 4*A*) (S: 30 K, 0.05 ± 0.02 , 60 K, 0.31 ± 0.07 ; GC: 30 K, 0.31 ± 0.14 , 60 K, 0.78 ± 0.23). Using the stronger stimulus, we measured response latency from the beginning of the stimulus to 10% of the response amplitude, rise-time from 10 to 90% of the response, and duration from 50% of the rise to 50% of the decay. There was a clear trend of faster response kinetics in the growth cone compared with the soma (Figs. 1*B*, 4*B*) (see below).

Figure 2. TTX-sensitive influx through voltage-gated Ca^{2+} channels. *A*, Depolarization in 0 Ca^{2+} saline (represented as a black bar within a white bar) eliminated the increase by 30 mM K^+ saline (30 K). Images of peak Ca^{2+} are shown before, during, and after exchange of the bath saline with one lacking Ca^{2+} . *B*, Application of 6 mM $CoCl_2$ during 60 K stimulation had a similar affect. *C*, The addition of 1 μ M TTX only partially eliminated the Ca^{2+} increase during 30 sec of 60 K stimulation.

Figure 3. Action potential generation and whole-cell outward currents from fura-2-loaded giant neurons. *A*, Action potential generation was not impaired in fura-2-loaded neurons during whole-cell patch-clamp experiments. Representative action potential activities in response to current injection of two tonically firing neurons are shown, one loaded with fura-2 (+) and the other not (–). *B*, Whole-cell outward currents, composed mostly of various types of K^+ currents, were measured from loaded and nonloaded cultures. Four categories (types 1–4) are shown based on the proportion between the peak and sustained components and the decay time-constants of the total outward current. The proportion of cells with a response that belonged to each category of current kinetics was unaffected by fura-2 (see Results).

The variation among neurons may stem from intrinsic differences between cell types, suggesting that a restricted cell population would reduce response variability. We investigated this possibility using the GAL4-UAS system (Brand and Perrimon, 1993) of *Drosophila* to express green fluorescent protein (GFP; in a UAS-GFP construct) in a subset of giant neurons derived from mushroom body neuroblasts with the lineage-specific driver 201Y-GAL4 (Yang et al., 1995). We found that normal Ca^{2+} measurements could be acquired despite GFP expression (Supplemental Fig. 1). However, such a restricted cell population did not reduce variability in response amplitude or change the characteristic differences between the soma and growth cone responses (Supplemental Fig. 2). Even within the GFP-labeled population, responses from the growth cone and soma of single neurons tend to covary from the population mean, suggesting that measurements from one area (growth cone or neuritic terminal) can serve as an indicator for responsiveness in other cellular regions.

***dunce* and *rutabaga* mutations disrupt the high K^+ response**

After the depolarization of *dnc* and *rut* neurons, several hallmarks were immediately noticeable. We observed a suppression of response amplitude (Fig. 5*A,B*) and a change in response kinetics (Figs. 5*C*, 6). However, the magnitude of these changes differed between the soma and growth cone. Representative soma and growth cone Ca^{2+} traces from single neurons are shown (Fig. 5*A*) (collected using 30 sec depolarizations with 60 K saline), indicating the effects of altered cAMP metabolism. As a population, *dnc* and *rut* growth cones showed a weaker response to 30 K

stimulation (Fig. 5*B*, 30 K) (*WT*, 0.31 ± 0.14 ; *dnc*, 0.02 ± 0.01 ; *rut*, 0.06 ± 0.02). With 60 K saline, mutant growth cone responses approached wild-type amplitudes (Fig. 5*B*, 60 K) (*WT*, 0.78 ± 0.21 ; *dnc*, 0.87 ± 0.40 ; *rut*, 0.49 ± 0.12). In contrast, only *rut* suppressed the high K^+ response within the soma (*WT*, 0.31 ± 0.06 ; *dnc*, 0.38 ± 0.08 ; *rut*, 0.11 ± 0.02).

Similar to wild-type neurons, the amplitude and kinetics of responses from the soma and growth cone of mutant cells also covaried from the population mean. The preferential suppression

Figure 4. Regional differences in dose dependence and response kinetics. *A*, Frequency distributions of response amplitudes during 30 sec depolarization with the 30 and 60 K salines. Scattered responses above $0.6 \mu M$ (600 nM) are represented by a single class. Neurons in this culture system varied in the extent of the Ca^{2+} increase, with a subpopulation containing highly sensitive growth cones and cell bodies. In general, however, the soma required 60 K stimulation to elicit a distribution comparable to that produced by 30 K in the growth cone [growth cone (GC) at 30 K 0.31 ± 0.14 vs soma (S) at 60 K 0.31 ± 0.07 ; mean $\pm$ SEM in micrometers for all figures except Figures 5 and 8]. *B*, Although the latency, rise-time, and duration vary among cells, the mean latency and rise-time are smaller in the growth cone [Latency (seconds): S 23.06 ± 1.14 vs GC 13.24 ± 0.99 ; Student's *t* test; $p < 0.005$; Rise-Time (seconds): S 16.31 ± 0.76 vs GC 11.00 ± 0.93 ; $p < 0.005$; Duration (seconds): S 31.03 ± 2.37 vs GC 27.24 ± 2.52 ; $p < 0.5$]. Latency was measured from the beginning of the stimulus to 10% of the response amplitude, rise-time from 10 to 90% of the response, and duration from 50% of the rise to 50% of the decay. The number of zones, number of neurons, and number of cultures used for data presented in this and all figures are represented as zones, neurons, and cultures. For *A* and *B*, S 30 K: 30, 21, 13, 60 K: 101, 57, 32; GC 30 K: 37, 21, 16, 60 K: 37, 26, 18.

Figure 5. The *dnc* and *rut* mutations lower the sensitivity to high K^+ depolarization more strongly in the growth cone than soma. **A**, Example traces from the growth cone and soma of *dnc* and *rut* neurons during 30 sec perfusion of 60 K saline (indicated by black bars). **B**, Response amplitudes were suppressed among *dnc* and *rut* growth cones with 30 K and *rut* growth cones with 60 K (30 K: WT, 0.31 ± 0.14 , *dnc*, 0.02 ± 0.01 , *rut*, 0.06 ± 0.01 ; 60 K: WT, 0.78 ± 0.21 , *dnc*, 0.87 ± 0.40 , *rut*, 0.49 ± 0.12). For the soma, suppression was indicated only in *rut* (WT, 0.31 ± 0.07 ; *dnc*, 0.40 ± 0.08 ; *rut*, 0.13 ± 0.05). **C**, Cultured neurons could be categorized by regional differences in response latency (time from beginning of stimulation to 10% of the peak amplitude), with the growth cone response latency being either earlier or later than that of the soma. Samples of normalized growth cone and soma responses from the same cell of the two categories are shown. The *dnc* and *rut* mutations reversed the relationship found in the wild type, causing a larger percentage of soma responses to rise before the growth cone (quantified for 15, 15, and 10 WT, *dnc*, and *rut* neurons). For **B**, 60 K (S: WT, 101, 57, 32, *dnc*, 89, 56, 28, *rut*, 91, 46, 20; GC: WT, 37, 26, 18, *dnc*, 51, 26, 21, *rut*, 68, 26, 16); 30 K (GC: WT, 37, 21, 16, *dnc*, 24, 11, 8, *rut*, 56, 16, 7).

during low depolarizations (30 K saline) implies that the threshold for induction of the Ca^{2+} increase may be altered by *dnc* and *rut*, and the soma and growth cone may be differentially affected as well (Fig. 5B). As shown previously in wild-type neurons, response latency, defined as the time from beginning of the stimulus to 10% of the peak, was shorter in the growth cone than soma (Figs. 1, 4B, 5A). However, mutant growth cones often had a longer response latency than the soma (Figs. 5C). Together with

stronger amplitude suppression in the growth cone (Fig. 5B), these results suggest that alterations in cAMP metabolism affect the reaction speed as well as the sensitivity to depolarization.

Region-specific effects were also revealed by comparisons of response kinetics (Fig. 6). The rise-time of the growth cone response was decreased more strongly by *rut* but that of the soma was increased equally by both mutations (Fig. 6, Rise-Time(s)) (S: WT, 16.31 ± 0.76 , *dnc*, 19.53 ± 0.73 , *rut*, 22.81 ± 1.86 ; GC: WT,

Figure 6. Kinetic alterations of the Ca^{2+} increase by *dnc* and *rut* with no change in basal Ca^{2+} levels. Similar to wild type, *dnc* and *rut* showed regional differences between the soma and growth cone in the rise-time and duration (Fig. 2, see legend for definition). However, the rise of Ca^{2+} within the growth cone was shortened by *rut* [Rise-Time(s): WT, 11.00 ± 0.93 , *dnc*, 12.58 ± 1.23 , *rut*, 7.81 ± 0.62], whereas both mutations more strongly increased the rise-time in the soma [Rise-Time(s) and Student's *t* test: WT, 16.31 ± 0.76 , *dnc*, 19.53 ± 0.73 ; $p < 0.005$; *rut*, 22.81 ± 1.84 ; $p < 0.005$]. *dnc* and *rut* increased response duration in the soma [Duration(s): WT, 31.03 ± 2.37 , *dnc*, 48.40 ± 3.65 ; $p < 0.0005$; *rut*, 47.88 ± 3.22 ; $p < 0.0005$], with little effect on the growth cone (WT, 27.24 ± 2.52 ; *dnc*, 22.85 ± 1.80 ; *rut*, 26.84 ± 2.15). In contrast to these dynamic measures, the basal Ca^{2+} level was not affected in the mutants (values in nM; S: WT, 22.00 ± 0.91 , *dnc*, 23.3 ± 0.81 , *rut*, 21.51 ± 0.82 ; GC: WT, 24.61 ± 0.92 , *dnc*, 22.73 ± 1.42 , *rut*, 25.57 ± 3.07). S: WT, 101, 57, 32, *dnc*, 89, 56, 28, *rut*, 91, 46, 20; GC: WT, 37, 26, 18, *dnc*, 51, 26, 21, *rut*, 68, 26, 16.

11.00 ± 0.93 , *dnc*, 12.58 ± 1.23 , *rut*, 7.81 ± 0.62]. Interestingly, neither mutation altered response duration in the growth cone, whereas the duration was increased within the soma (Fig. 6, Duration(s)) (S: WT, 31.03 ± 2.37 , *dnc*, 48.40 ± 3.65 , *rut*, 47.88 ± 3.22 ; GC: WT, 27.24 ± 2.52 , *dnc*, 22.85 ± 1.80 , *rut*, 26.84 ± 2.15), indicating that chronic changes in cAMP metabolism caused by these mutations disrupt the decay of the high K^+ response as well as the rise. In contrast to these dynamic measures of Ca^{2+} , *dnc* and *rut* did not alter the basal Ca^{2+} levels in either region (Fig. 6).

Release of Ca^{2+} from intracellular stores (Friel and Tsien, 1992; Mironov et al., 1993) or the complex electrical activity caused by ion channel interactions could lead to multiple peaks during the high K^+ response or to multiple time-constants during

its decay. We found that the prevalence of a shoulder or second peak could further differentiate the soma and growth cone of *Drosophila* neurons. The above two response categories were distinguished by their local amplitudes with respect to the peak Ca^{2+} during 30 sec stimulation with a high K^+ saline (Fig. 7, see legend). In all genotypes, the complex waveforms were more common in the growth cone than soma (Fig. 7A). However, the shoulder or second peak appeared to be suppressed more strongly in *dnc* than *rut* (Fig. 7A). The shape of the Ca^{2+} response was not related to the amplitude suppression described above, because the different waveform categories did not correlate with the peak Ca^{2+} level in any genotype (data not shown). Our results indicate that the Ca^{2+} response is not a simple linear process and that alterations in cAMP metabolism may affect the individual processes differently in the growth cone and soma.

Use-dependent defects in *dnc* and *rut*

The *dnc* and *rut* mutations alter activity-dependent regulation of action potential firing (Zhao and Wu, 1997) and neurotransmitter release from the synapse (Zhong and Wu, 1991; Lee and O'Dowd, 2000). In addition, *dnc* disrupts activity-dependent modulation of neurotransmitter release from the developing growth cone (Yao et al., 2000). We therefore investigated use-dependent Ca^{2+} regulation in the growth cone with a twin-pulse protocol (Fig. 7B). A pair of depolarizations (S1 and S2), given at two different interstimulus intervals (ISIs) (30 and 160–185 sec), revealed that previous depolarization altered the complexity of response kinetics in several ways. An increase (or decrease) of response complexity, as indicated by the emergence (or disappearance) of a shoulder or second peak, was observed in all genotypes. Noticeably, the response kinetics did not change in *rut* at the longer ISI in contrast with wild type and *dnc* (Fig. 7B).

A previous depolarization also altered the amplitude of the Ca^{2+} increase (Fig. 8A). A facilitation/suppression indicator, *R*, represents the log of the ratio of the second to the first response. (For example, $R = 0$ indicates no change, $R = 1$ indicates a 10-fold increase, and $R = -1$ indicates a 10-fold decrease.) Frequency distributions of *R* for the three genotypes are shown for both the short (30 sec) and long (160–185 sec) ISI (Fig. 8B). Among the population of cells, wild-type growth cones showed facilitation at both ISIs. We found that the *dnc* mutation caused less facilitation at the shorter ISI (WT, 0.16 ± 0.03 ; *dnc*, 0.09 ± 0.04), whereas *rut* suppressed the response at the longer one (WT, 0.20 ± 0.09 ; *rut*, -0.07 ± 0.06). Our results indicate that altered cAMP metabolism by *dnc* and *rut* affects at least two aspects of Ca^{2+} regulation after a previous depolarization. The likelihood of generating more complex waveforms is suppressed (Fig. 7B), and the facilitation of response amplitude ($R > 1$) seems to be decreased or in some cases converted to depression ($R < 1$) (Fig. 8B).

Spatial alterations of subcellular Ca^{2+} regulation

In many species, the lamellipodia, filopodial protrusions, and central domain of the growth cone vary their structure and movement depending on elevations in Ca^{2+} (for review, see Kater and Mills, 1991). Local Ca^{2+} increases may induce the growth of filopodia (Rehder and Kater, 1992; Lau et al., 1999) and determine the direction of growth cone turning (Zheng, 2000). In *Drosophila*, the *dnc* and *rut* mutations suppress the expansion and contraction of growth cone lamellipodia in dissociated larval CNS cultures (Kim and Wu, 1996) and influence the

Figure 7. The Ca²⁺ waveform and its activity-dependent modulation in *dnc* and *rut*. *A*, High K⁺-induced Ca²⁺ responses were categorized into three groups: simple responses of a single peak (black) and more complex responses with a shoulder during decay (gray) or with two or more distinct peaks (white, deviation from the simple decay with >25% of the peak amplitude). Data from 30 sec, high K⁺ stimulation indicates that the growth cone exhibits more complex waveforms than the soma of wild-type neurons. *Dnc* and *rut* appeared to suppress responses with a complex waveform, especially in the soma. *B*, A twin-pulse paradigm revealed the influence of previous activity on the Ca²⁺ increase. A pair of 30 sec stimulations (*S1* and *S2*) were given at two interstimulus intervals (*ISI*) (30 and 160–185 sec), and Ca²⁺ dynamics

were recorded in the growth cone. The Ca²⁺ waveform acquired a shoulder or second peak (white), did not change (gray), or lost its shoulder or second peak (black). Notably, at the 160–185 sec ISI, the *rut* mutation blocked the modulation of response kinetics. The 30, 42, and 60 K salines were used according to the responsiveness of individual cells, and mutant growth cones were in general less responsive, so the results presented for *dnc* and *rut* contain a larger proportion of 60 K data. The kinetic categories did not correlate with amplitude (data not shown). Response categories are as follows: *S*: WT, 131, 70, 43, *dnc*, 92, 51, 29, *rut*, 88, 40, 22; *GC*: WT, 87, 38, 20, *dnc*, 46, 26, 15, *rut*, 74, 26, 16. Activity-dependent changes are as follows: 30 sec ISI: WT, 38, 20, 15, *dnc*, 39, 13, 12, *rut*, 60, 20, 15; 160–185 sec ISI: WT, 20, 12, 9, *dnc*, 28, 11, 9, *rut*, 30, 15, 14.

terminal arborization of motor axons at the larval neuromuscular junction (Zhong et al., 1992).

We therefore collected higher magnification images (with 100× objectives) to distinguish how *dnc* and *rut* alter Ca²⁺ regulation within structurally and functionally distinct regions of the growth cone (Fig. 9). Growth cones of giant neurons had structures similar to those of larval neurons (Kim and Wu, 1991, 1996), but when viewed with phase-contrast optics, they were larger and appeared less motile in terms of the percentage change in lamellipodial area over time. The motility that we most often observed consisted of filopodial movements or lamellipodial expansion or retraction during 1 min recording epochs (Fig. 9A). This is similar to the motility of non-fura-loaded giant neurons (data not shown) and contrasts with more active lamellipodial ruffling and filopodial motility observed in larval cultures (Kim and Wu, 1991, 1996). However, in both embryonic giant neuron and larval CNS cultures, nonmotile growth cones often appeared phase-dark and had a bulging central domain, whereas motile growth cones appeared phase-light, contained more filopodia, and had a larger, flattened lamellipodia. We found that regional Ca²⁺ regulation could be correlated with motility (Fig. 9A,B). Motile growth cones exhibited Ca²⁺ levels in the peripheral lamellipodia that were greater than in the central domain (Figs. 9B). This spatial variation was less apparent in nonmotile than motile growth cones (Fig. 9C). A response amplitude ratio, edge/center, was used to facilitate the comparison with normalized values. In *dnc* and *rut* cells, some growth cones showed an anomalous relationship between the edge/center response ratio and motility. In *dnc*, peripheral Ca²⁺ levels were well beyond those found in motile growth cones of the wild type, whereas extreme ratios were seen in nonmotile *rut* growth cones (Fig. 9C). This is in stark contrast to the ability to predict motility based on regional Ca²⁺ signaling in the wild-type growth cone. These data suggest that altered cAMP metabolism may enhance peripheral Ca²⁺ signaling as compared with the central domain and disrupt the relationship between Ca²⁺ levels and motility.

DISCUSSION

Regional differences within cultured neurons

Here, we describe the first use of fura-2 imaging for intracellular Ca²⁺ in a dissociated culture system of *Drosophila*. Unlike *in vivo* preparations such as the neuromuscular junction (Karunanithi et al., 1997) or the adult CNS (Rosay et al., 2001; Wang et al., 2001), optical imaging in culture can relate subcellular Ca²⁺ dynamics throughout different regions of single neurons (Fig. 1). The spatial resolution offered by optical imaging complements electrophysiological studies of *Drosophila* giant neurons in culture (Saito and Wu, 1991, 1993; Zhao and Wu, 1997; Yao and Wu, 1999) and may indicate the functional significance of membrane excitability differences in subneuronal regions. The two approaches in combination will greatly enhance the neurogenetic study of Ca²⁺-dependent processes involved in neuronal development and physiology.

Our initial characterization of regional differences in high K⁺-induced Ca²⁺ regulation in the soma and growth cone (Figs. 1, 4) indicates that both Ca²⁺ and Na⁺ channels are involved (Fig. 2). *Drosophila* central neurons in culture contain two types of Ca²⁺ currents (L and T type) distinguished by their activation voltage, decay kinetics, voltage dependence, and underlying single-channel activities (Byerly and Leung, 1988; Leung and Byerly, 1991; Saito and Wu, 1991, 1993). In other species, electrical recordings from the growth cone (for review, see Gottman and Lux, 1995) indicate that similar L- and T-type Ca²⁺ channels are expressed throughout the neuron, but that channel density may be higher and the channels more clustered in the growth cone. Such variation may relate to our findings that the growth cone and soma differed in sensitivity and response kinetics during depolarization (Figs. 1, 4–7). In future investigations, it will be interesting to use identified mutations in Na⁺ and Ca²⁺ channel genes to dissect their involvement in Ca²⁺ regulation throughout the neuron.

Local Ca²⁺ levels regulate filopodial formation (Rehder and Kater, 1992; Lau et al., 1999) and play an important role in

Figure 8. Activity-dependent modulation of response amplitude in the growth cone. *A*, Example traces from the growth cones of two cells show facilitation and suppression of the high K^+ response amplitude during the twin-pulse protocol. A facilitation/suppression indicator, R , was calculated from the ratio of the two amplitudes [$R = \log(\text{Amp}_1/\text{Amp}_2)$, $R = -1$ represents a 10-fold suppression in amplitude, whereas $R = 1$ represents a 10-fold facilitation]. *B*, Frequency histograms of the facilitation/suppression indicator R showing the modulation of response amplitude in the growth cone. Wild-type cells showed slight facilitation of response amplitude regardless of the long (160–185 sec) or short (30 sec) ISI. In the case of *dnc*, a tendency for less facilitation was observed, whereas for *rut*, suppression was observed at the long ISI. Data from different stimulus durations (2, 5, 10, and 30 sec) and magnitudes (30, 42, and 60 K) were combined because the mutants required stronger stimulation and because no correlation between the stimulus and facilitation/suppression was observed. 30 sec ISI: WT, 38, 20, 15; *dnc*, 39, 13, 12; *rut*, 60, 20, 15; 160–185 sec ISI: WT, 20, 12, 9; *dnc*, 28, 11, 9; *rut*, 30, 15, 14.

directing growth cone turning (Zheng, 2000) in cultured neurons from other species. Our data show that the magnitude of the high K^+ response was larger in the periphery of motile, as opposed to nonmotile, growth cones. Distinct regions within the growth cone exhibit differences in the localization of cytoskeletal elements and cytoplasmic organelles (Forscher et al., 1987; Davenport et al., 1993; Azhderian et al., 1994). It may be questioned whether the cytokinesis inhibition technique used to generate giant neurons affects the distribution of Ca^{2+} channels in the soma and growth cone because of actin cytoskeletal disruption. However, previous

electrophysiological studies did not detect differences in action potential and ion current properties with and without the removal of cytochalasin B (Saito and Wu, 1991). This result is consistent with our preliminary study indicating that differences in response characteristics between the soma and growth cone were still evident in embryonic cultures made in the absence of CCB (data not shown). In future studies, untreated larval neurons in culture can be used to examine Ca^{2+} signaling in the same *dnc* and *rut* growth cones that display retarded motility (Kim and Wu, 1996).

The *dnc* and *rut* Ca^{2+} regulatory phenotypes

Our analysis of these well studied mutants by fura-2 imaging revealed previously unknown mutant phenotypes and suggests the usefulness of Ca^{2+} imaging for a wide range of other mutations. We observed that *dnc* and *rut* decreased sensitivity to high K^+ stimulation for a cytosolic Ca^{2+} increase (Figs. 5, 6) while affecting both the activity dependence (Figs. 7, 8) and spatial distribution of $[Ca^{2+}]$ within motile and nonmotile growth cones (Fig. 9). Chronic changes in cAMP metabolism imposed by the *dnc* and *rut* mutations decreased sensitivity most strongly in the growth cone while prolonging the Ca^{2+} increase only in the soma (Figs. 5, 6).

It is known that *dnc* and *rut* alter the modulation of K^+ currents gated by the *Sh* and *eag* channel subunits (Zhong and Wu, 1993b; Zhao and Wu, 1997). Moreover, enhanced spike activity has been detected with patch-clamp recordings from the soma of *dnc* and *rut* giant neurons (Zhao and Wu, 1997). Such altered excitability may explain the prolonged soma response that we observed (Fig. 6) and should be further examined during patch-clamp experiments with high K^+ depolarization. Electrophysiological studies of other currents in *dnc* and *rut* neurons have been lacking, but a Ba^{2+} current flowing through wild-type Ca^{2+} channels increased during the application of cAMP analogs (Alshuaib and Byerly, 1996). Further support for Ca^{2+} channel defects stems from findings that *dnc* and *rut* increase and decrease an L-type (dihydropyridine-sensitive) Ca^{2+} current in larval muscle, phenotypes that were mimicked by short-term pharmacological manipulations on wild-type muscles (Bhattacharya et al., 1999). However, some *dnc* and *rut* physiological phenotypes at the larval neuromuscular junction cannot be mimicked by acute pharmacological treatments and are attributed to long-term, developmental effects of the mutations (Renger et al., 2000). Different aspects of the Ca^{2+} signaling phenotypes may be caused by either acute or chronic effects. Therefore, the combination of genetic and pharmacological analyses will offer a more comprehensive picture of how the cAMP pathway regulates neuronal Ca^{2+} .

The effects of *dnc* and *rut* on Ca^{2+} dynamics may be mediated by misregulation of multiple mechanisms, including channel distribution, cytoskeletal organization, and intracellular Ca^{2+} stores. Region-specific differences in the cAMP pathway may lead to a higher incidence of responses containing a shoulder or second peak in the growth cone than soma (Fig. 7*A*) and affect waveform modulation by previous activity (Fig. 7*B*). In other culture systems, the prolonged decay of intracellular Ca^{2+} triggered by high K^+ depolarization has been used to implicate Ca^{2+} uptake into and release from intracellular stores (Friel and Tsien, 1992; Mironov et al., 1993; Babcock et al., 1997; Pivovarov et al., 1999). In this respect, Ca^{2+} imaging of the large collection of *Drosophila* learning and memory mutants (Davis, 1996; Dubnau and Tully, 1998) may complement studies in other organisms designed to assay the role of intracellular Ca^{2+} stores in neuronal

Figure 9. The spatial distribution of Ca^{2+} within distinct regions of the growth cone. *A*, Phase images of a giant neuron and its growth cone (captured with 40 and 100 $\times$ objectives, respectively) showing filopodial and lamellipodial movement (arrows). For this experiment, motility was indicated by changes in filopodial or lamellipodial shape over the course of 1 min. *B*, Pseudocolor images (*B*₁) showing Ca^{2+} within this growth cone after a 5 sec stimulation with 60 K. Ca^{2+} levels in the peripheral lamellipodium (zones 1 and 2) reached a higher peak concentration than within the central domain (zone 3), as quantified in *B*₂. *C*, The ratio of peak Ca^{2+} at the leading edge to levels in the central domain of the wild type were larger in motile growth cones (●) than nonmotile growth cones (○) (e.g., the ratio of an average of 2–6 zones in the periphery to an average of 1–2 zones in the central domain). In *dnc*, ratios of peripheral-to-central peak Ca^{2+} levels in motile growth cones were well beyond those of wild-type neurons, whereas extreme ratios were seen in nonmotile *rut* growth cones. Upward-directed arrows indicate ratios that were outside the range of the y axis. Scale bars in *A* are 10 μ m (left) and 7 μ m (right). For *C*, WT (cells, cultures) 16, 13; *dnc*, 14, 11; *rut*, 19, 13.

plasticity (Cormier et al., 2000; Nishiyama et al., 2000; Emptage et al., 2001).

Expansion and retraction of the growth cone have been implicated in synapse formation at the larval neuromuscular junction of *Drosophila* (Halpern et al., 1991; Keshishian et al., 1993; Chiba and Keshishian 1996; Yoshihara et al., 1997). Defects in lamellipodial expansion and retraction caused by *dnc* and *rut* in cultured neurons (Kim and Wu, 1996) suggest that this altered growth cone behavior may disrupt arborization of the motor axon terminal as was described for these mutants (Zhong et al., 1992). Here we characterized defects in the spatial distribution of Ca^{2+} signaling in *dnc* and *rut* growth cones (Fig. 9), suggesting that the interplay between Ca^{2+} and the cAMP pathway may be important for growth cone behaviors. In *Xenopus* cultured neurons, an interaction between the cAMP pathway and Ca^{2+} influences the growth cone response to an external guidance cue (Ming et al., 1997). As suggested by data shown in Figure 9, *dnc* and *rut* altered the regulation of Ca^{2+} with reference to growth cone motility. *Drosophila* culture systems and available mutations can be used to link cAMP to several Ca^{2+} -dependent aspects of growth cone behavior described in other preparations (Kater et al., 1988; Kater and Mills, 1991; Rehder and Kater, 1992; Lau et al., 1999; Zheng, 2000).

Drosophila culture systems to study the genetics of Ca^{2+} regulation

Highest expression of the *dnc* and *rut* products is found in the mushroom bodies (Crittenden et al., 1998), whereas lower levels were observed elsewhere. However, using the GAL4-UAS system (Brand and Perrimon, 1993) to label a subset of mushroom body neurons with GFP, we showed that this restricted cell population displayed similar variability and response levels (Fig. 5B, compare with Supplemental Fig. 2). Previous electrophysiological data on a similar population of mushroom body neurons in larval CNS cultures (Wright and Zhong, 1995) also exhibit considerable variation in the amplitude and kinetics of voltage-gated K^{+} currents. Significant mutant phenotypes may not be restricted to cells with high expression levels. In fact, physiological defects have been observed in neurons with lower expression levels such as the larval neuromuscular junction (Zhong and Wu, 1991). *dnc* and *rut* defects in neuronal firing patterns (Zhao and Wu, 1997) and growth cone motility (Kim and Wu, 1996) have been documented in the general population of neurons in culture (with and without CCB treatment). Therefore, the reported *dnc* and *rut* phenotypes in our study may reflect defects common to different types of neurons.

Given the multiple downstream targets of the cAMP pathway that may vary in spatial or temporal distribution, the phenotypes of the *dnc* and *rut* mutations are necessarily complex. Mutant phenotypes within subcellular compartments may indicate localized actions of these proteins. For example, the abundance of PKA subunits varies throughout the *Drosophila* CNS (Kalderon and Rubin 1988; Park et al., 2000), and the cellular expression of PKA function is controlled through binding to membrane-associated attachment proteins (for review, see Gray et al., 1998). Available mutations in *Drosophila* offer the unique opportunity to study how different isoforms of regulatory and catalytic subunits [encoded by *PKA-RI* and *PKA-RII* (Park et al., 2000) and *DCO* (Kalderon and Rubin 1988)] PKA subunits as well as anchoring proteins (AKAP200) (Li et al., 1999; Rossi et al., 1999) contribute to subcellular Ca^{2+} regulation. Together with pharmacological manipulations of cAMP levels and PKA activity, these studies should yield a more complete picture of how PKA and other cAMP-related mechanisms produce the phenotypes that we have described.

REFERENCES

- Aceves-Pina EO, Booker R, Duerr JS, Livingstone MS, Quinn WG, Smith RF, Sziber PP, Tempel BL, Tully T (1983) Learning and memory in *Drosophila*, studied with mutants. Cold Spring Harb Symp Quant Biol 48:831–839.
- Alshuaib W, Byerly L (1996) Modulation of membrane currents by cAMP in cleavage-arrested *Drosophila* neurons. J Exp Biol 199:537–548.
- Azhderian EM, Hefner D, Lin CH, Kaczmarek LK, Forscher P (1994) Cyclic AMP modulates fast axonal transport in *Aplysia* bag cell neurons by increasing the probability of single organelle movement. Neuron 12:1223–1233.
- Babcock D, Herrington J, Goodwin P, Park Y, Hille B (1997) Mitochondrial participation in the intracellular Ca^{2+} network. J Cell Biol 136:833–844.
- Bailey CH, Giustetto M, Huang YY, Hawkins RD, Kandel ER (2000) Is heterosynaptic modulation essential for stabilizing Hebbian plasticity and memory? Nat Rev Neurosci 1:11–20.
- Bellen HJ, Kiger Jr JA (1988) Maternal effects of general and regional specificity on embryos of *Drosophila melanogaster* caused by *dunce* and *rutabaga* mutant combinations. Roux's Arch Dev Biol 197:144–166.
- Bellen HJ, Gregory BK, Olsson CL, Kiger JA (1987) Two *Drosophila* learning mutants, *dunce* and *rutabaga*, provide evidence for a maternal role for cAMP on embryogenesis. Dev Biol 121:432–444.
- Bentley D, Guthrie PB, Kater SB (1991) Ca^{2+} ion distribution in nascent pioneer axons and coupled preaxogenesis neurons *in situ*. J Neurosci 11:1300–1308.
- Bhattacharya A, Gu GG, Sing SGG (1999) Modulation of dihydropyridine-sensitive Ca^{2+} channels in *Drosophila* by a cAMP-mediated pathway. J Neurobiol 39:491–500.
- Brand AH, Perrimon N (1993) Target gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–415.
- Byerly L, Leung HT (1988) Ionic currents of *Drosophila* neurons in embryonic cultures. J Neurosci 8:4379–4393.
- Byers D, Davis RL, Kiger JA (1981) Defect in cAMP phosphodiesterase due to the *dunce* mutation of learning in *Drosophila melanogaster*. Nature 289:79–81.
- Carew TJ (1996) Molecular enhancement of memory formation. Neuron 16:5–8.
- Chen C, Tonegawa S (1997) Molecular genetic analysis of synaptic plasticity, activity-dependent neural development, learning, and memory in the mammalian brain. Annu Rev Neurosci 20:157–184.
- Chiba A, Keshishian H (1996) Neuronal pathfinding and recognition: roles of cell adhesion molecules. Dev Biol 180:424–432.
- Cline HT (1991) Activity-dependent plasticity in the visual systems of frogs and fish. Trends Neurosci 14:104–111.
- Cooper DMF, Mons N, Karpen JW (1995) Adenylyl cyclases and the interaction between Ca^{2+} and cAMP signaling. Nature 374:421–424.
- Corfas G, Dudai Y (1990) Adaptation and fatigue of a mechanosensory neuron in wild-type *Drosophila* and in memory mutants. J Neurosci 10:491–499.
- Corfas G, Dudai Y (1991) Morphology of a sensory neuron in *Drosophila* is abnormal in memory mutants and changes during aging. Proc Natl Acad Sci USA 88:7252–7256.
- Cormier RJ, Greenwood AC, Connor JA (2000) Bi-directional synaptic plasticity correlated with the magnitude of dendritic Ca^{2+} transients above a threshold. J Neurophysiol 85:399–406.
- Crittenden JR, Skoulakis EM, Han KA, Davis RL (1998) Tripartite mushroom body architecture revealed by antigenic markers. Learn Mem 5:38–51.
- Davenport RW, Dou P, Rehder V, Kater SB (1993) A sensory role for neuronal growth cone filopodia. Nature 361:721–724.
- Davis RL (1996) Physiology and biochemistry of *Drosophila* learning mutants. Physiol Rev 76:299–317.
- Delgado R, Davis R, Bono MR, Latorre R, Labarca P (1998) Outward currents in *Drosophila* larval neurons: *dunce* lacks a maintained outward current component downregulated by cAMP. J Neurosci 18:1399–1407.
- Dickson BJ, Cline H, Polleux F, Ghosh A (2001) Making connections. Meeting: axon guidance and neural plasticity. EMBO J 2:182–186.
- Dubnau J, Tully T (1998) Gene discovery in *Drosophila*: new insights for learning and memory. Annu Rev Neurosci 21:407–444.
- Dudai Y, Jan YN, Byers D, Quinn WG, Benzer S (1976) *dunce*, a mutant of *Drosophila* deficient in learning. Proc Natl Acad Sci USA 73:1684–1688.
- Emptage NJ, Reid CA, Fine A (2001) Ca^{2+} stores in hippocampal synaptic boutons mediate short-term plasticity, store-operated Ca^{2+} entry, and spontaneous transmitter release. Neuron 29:197–207.
- Engel J, Wu C-F (1996) Altered habituation of an identified escape circuit in *Drosophila* memory mutants. J Neurosci 16:3486–3499.
- Fields RD, Guthrie PB, Russell JT, Kater SB, Molhota BS, Nelson PG (1993) Accommodation of mouse DRG growth cones to electrically induced collapse: kinetic analysis of Ca^{2+} transients and set point theory. J Neurobiol 24:1080–1098.
- Forscher P, Kaczmarek LK, Buchanan JA, Smith SJ (1987) Cyclic AMP induces changes in distribution and transport of organelles within growth cones of *Aplysia* bag cell neurons. J Neurosci 7:3600–3611.
- Friel D, Tsien R (1992) A caffeine- and ryanodine-sensitive Ca^{2+} store in bullfrog sympathetic neurons modulates effects of Ca^{2+} entry on $[\text{Ca}^{2+}]$. J Physiol (Lond) 450:217–246.
- Gomez TM, Spitzer NC (2000) Regulation of growth cone behavior by Ca^{2+} : new dynamics to earlier perspectives. J Neurobiol 44:174–183.
- Gottman K, Lux HD (1995) Growth cone calcium ion channels: properties, clustering, and functional roles. Perspect Dev Biol 2:371–377.
- Gray PC, Scott JD, Catterall WA (1998) Regulation of ion channels by cAMP-dependent protein kinase and A-kinase anchoring proteins. Curr Opin Neurobiol 8:330–334.
- Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.
- Halpern ME, Chiba A, Johansen J, Keshishian H (1991) Growth cone behavior underlying the development of stereotypic synaptic connections in *Drosophila* embryos. J Neurosci 11:3227–3238.
- Kalderon D, Rubin GM (1988) Isolation and characterization of *Drosophila* cAMP-dependent protein kinase genes. Genes Dev 2:1539–1556.
- Karunanithi S, Georgiou J, Charlton MP, Atwood HL (1997) Imaging of Ca^{2+} in *Drosophila* larval motor nerve terminals. J Neurophysiol 78:3465–3467.
- Kater SB, Mills LR (1991) Regulation of growth cone behavior by Ca^{2+} . J Neurosci 11:891–899.
- Kater SB, Mattson MP, Cohan C, Connor J (1988) Ca^{2+} regulation of the neuronal growth cone. Trends Neurosci 11:315–321.
- Keshishian H, Chiba A, Chang TN, Halfon MS, Harkins EW, Jarecki J, Wang L, Anderson MD, Cash S, Halpern ME, Johansen J (1993) Cellular mechanisms governing synaptic development in *Drosophila melanogaster*. J Neurobiol 24:757–787.
- Kim YT, Wu C-F (1991) Distinctions in growth cone morphology and motility between monopolar and multipolar neurons in *Drosophila* CNS cultures. J Neurobiol 22:263–275.
- Kim YT, Wu C-F (1996) Reduced growth cone motility in cultured neurons from *Drosophila* memory mutants with a defective cAMP cascade. J Neurosci 16:5593–5602.
- Lau PM, Zucker RS, Bentley D (1999) Induction of filopodia by direct local elevation of intracellular Ca^{2+} ion concentration. J Cell Biol 145:1265–1275.
- Lee D, O'Dowd DK (2000) cAMP-dependent plasticity at excitatory cholinergic synapses in *Drosophila* neurons: alterations in the memory mutant *dunce*. J Neurosci 20:2104–2111.
- Leung HT, Byerly L (1991) Characterization of single Ca^{2+} channels in *Drosophila* embryonic nerve and muscle cells. J Neurosci 11:3047–3059.
- Li Z, Rossi EA, Hoheisel JD, Kalderon D, Rubin CS (1999) Generation of a novel A kinase anchor protein and a myristoylated alanine-rich C kinase substrate-like analog from a single gene. J Biol Chem 274:27191–27200.
- Livingstone MS, Sziber PP, Quinn WG (1984) Loss of Ca^{2+} /CaM responsiveness in adenylyl cyclase of *rutabaga*, a *Drosophila* learning mutant. Cell 37:205–215.
- Ming GL, Song HJ, Berninger B, Holt CE, Tessier-LeVigne M, Poo MM (1997) cAMP-dependent growth cone turning by netrin-1. Neuron 19:1225–1235.

- Mironov SL, Usachev UM, Lux HD (1993) Spatial and temporal control of intracellular free Ca^{2+} in chick sensory neurons. *Pflügers Arch* 424:183–191.
- Nishiyama M, Hong K, Mikoshiba K, Poo MM, Kato K (2000) Ca^{2+} stores regulate the polarity and input specificity of synaptic modification. *Nature* 408:584–588.
- Park SK, Sedore SA, Cronmiller C, Hirsh J (2000) Type-II cAMP-dependent protein kinase-deficient *Drosophila* are viable but show developmental, circadian, and drug response phenotypes. *J Biol Chem* 275:20588–20596.
- Pivovarova NB, Hongpaisan J, Andrews SB, Friel DD (1999) Depolarization-induced mitochondrial Ca^{2+} accumulation in sympathetic neurons: spatial and temporal characteristics. *J Neurosci* 19:6372–6384.
- Rehder V, Kater SB (1992) Regulation of neuronal growth cone filopodia by intracellular Ca^{2+} . *J Neurosci* 12:3175–3186.
- Rehder V, Williams C, Kater SB (1996) Functional compartmentalization of the neuronal growth cone: determining Ca^{2+} 's place in signaling cascades. *Perspect Dev Biol* 4:215–226.
- Renger JJ, Ueda A, Atwood HL, Govind CK, Wu CF (2000) Role of cAMP cascade in synaptic stability and plasticity: ultrastructural and physiological analyses of individual boutons in *Drosophila* memory mutants. *J Neurosci* 20:3980–3992.
- Rosay P, Armstrong JD, Wang Z, Kaiser K (2001) Synchronized neural activity in the *Drosophila* memory centers and its modulation by *amnesiac*. *Neuron* 30:759–770.
- Rossi EA, Li Z, Feng H, Rubin CS (1999) Characterization of the targeting, binding, and phosphorylation site domains of an A kinase anchor protein and a myristoylated alanine-rich C kinase substrate-like analog that are encoded by a single gene. *J Biol Chem* 274:27201–27210.
- Saito M, Wu C-F (1991) Expression of ion channels and mutational effects in giant *Drosophila* neurons differentiated from cell division-arrested embryonic neuroblasts. *J Neurosci* 11:2135–2150.
- Saito M, Wu C-F (1993) Ionic channels in cultured *Drosophila* neurons. In: *Comparative molecular neurobiology* (Pichon Y, ed), pp 366–389. Basel: Birkhauser Verlag.
- Salvaterra PM, Bournias-Vardiabasis N, Nair T, Hou G, Lieu C (1987) *In vitro* neuronal differentiation of *Drosophila* embryo cells. *J Neurosci* 7:10–22.
- Seecof RL, Alleaume N, Teplitz RL, Gerson I (1971) Differentiation of neurons and myocytes in cell cultures made from *Drosophila* gastrulae. *Exp Cell Res* 69:161–173.
- Soderling TR (2000) CaM-kinases: modulation of synaptic plasticity. *Curr Opin Neurobiol* 10:375–380.
- Tully T, Quinn WG (1985) Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J Comp Physiol [A]* 157:263–277.
- Wang Y, Wright NJD, Guo HF, Xie Z, Svoboda K, Malinow R, Smith DP, Zhong Y (2001) Genetic manipulation of the odor-evoked distributed neural activity in the *Drosophila* mushroom body. *Neuron* 29:267–276.
- Williams DA, Fogarty KE, Tsien RY, Fay FS (1985) Ca^{2+} gradients in single smooth muscle cells revealed by the digital imaging microscope using fura-2. *Nature* 318:558–561.
- Wright NJD, Zhong Y (1995) Characterization of K^{+} currents and cAMP-dependent modulation in cultured *Drosophila* mushroom body neurons identified by *lacZ* expression. *J Neurosci* 15:1025–1034.
- Wu C-F, Sakai K, Saito M, Hotta Y (1989) Giant *Drosophila* neurons differentiated from cytokinesis-arrested embryonic neuroblasts. *J Neurobiol* 21:499–507.
- Yang MY, Armstrong JD, Vilinsky I, Strausfield NJ, Kaiser K (1995) Subdivision of the *Drosophila* mushroom bodies by enhancer-trap expression patterns. *Neuron* 15:55–61.
- Yao W-D, Wu C-F (1999) Auxiliary *Hyperkinetic B* subunit of K^{+} channels: regulation of firing properties and K^{+} currents in *Drosophila* neurons. *J Neurophysiol* 81:2472–2484.
- Yao WD, Rusch J, Poo MM, Wu C-F (2000) Spontaneous acetylcholine secretion from developing growth cones of *Drosophila* central neurons in culture: effects of cAMP pathway mutations. *J Neurosci* 20:2626–2637.
- Yoshihara M, Rheuban MB, Kidokoro Y (1997) Transition from growth cone to functional motor nerve terminal in *Drosophila* embryos. *J Neurosci* 17:8408–8426.
- Zhao ML, Wu C-F (1997) Alterations in frequency coding and activity dependence of excitability in cultured neurons of *Drosophila* memory mutants. *J Neurosci* 17:2187–2199.
- Zhao ML, Sable EO, Iverson LE, Wu C-F (1995) Functional expression of *Shaker K* $^{+}$ channels in cultured *Drosophila* “giant” neurons derived from *Sh* cDNA transformants: distinct properties, distribution, and turnover. *J Neurosci* 15:1406–1418.
- Zheng JQ (2000) Turning of nerve growth cones induced by localized increases in intracellular Ca^{2+} ions. *Nature* 403:89–93.
- Zhong Y, Wu C-F (1991) Altered synaptic plasticity in *Drosophila* memory mutants with a defective cAMP cascade. *Science* 251:198–201.
- Zhong Y, Wu C-F (1993a) Modulation of different K^{+} currents in *Drosophila*: a hypothetical role for the *eag* subunit in multimeric K^{+} channels. *J Neurosci* 13:4669–4679.
- Zhong Y, Wu C-F (1993b) Differential modulation of K^{+} currents by cAMP and its long-term and short-term effects: *dunce* and *rutabaga* mutants of *Drosophila*. *J Neurogenet* 9:15–27.
- Zhong Y, Budnik V, Wu C-F (1992) Synaptic plasticity in *Drosophila* memory and hyperexcitable mutants: role of the cAMP cascade. *J Neurosci* 12:644–651.