

DISTRIBUTION OF NEUROTRANSMITTER SECRETION IN GROWING AXONS

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Abstract—Neurotransmitter secretion from the nerve terminal is mediated by the fusion of synaptic vesicles with the plasma membrane. It is generally believed that neurotransmitter release in mature synapses is localized to the presynaptic nerve terminals. To probe the topology of neurotransmitter secretion along developing axons in culture, we recorded membrane currents from myocytes manipulated into contact with axons. At the early stages of growth, exocytotic events were detected along the axon as well as at the growth cone. At the later stages of growth, neurotransmitter secretion adopted the form of a smooth proximodistal gradient, with the highest level of activity at the growth cone region.

Our results reveal the existence of a previously undetected early stage of axonal growth and suggest developmental regulation in the pattern of neurotransmitter secretion along the growing nerve processes. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: axonal differentiation, synaptic vesicles, neurotransmitter secretion, axonal growth.

Neurons secrete neurotransmitters via a highly regulated process involving the fusion of synaptic vesicles with the plasma membrane.^{4,33} Following exocytosis, the molecular components of synaptic vesicles are retrieved by endocytosis, and synaptic vesicles are recycled locally within the nerve terminal.¹⁷ This exo-endocytotic recycling pathway is orchestrated by a number of synaptic vesicle-specific proteins.¹³ In mature synapses, synaptic vesicles are clustered at the sites specialized for neurotransmitter secretion (“active zones”). The clustering of synaptic vesicles at the active zones is accompanied by accumulation of specific proteins at both the presynaptic nerve terminal and the postsynaptic membrane.^{10,57}

Synaptic vesicle exocytosis is tightly regulated by Ca^{2+} entry during excitation of the presynaptic cell,⁴² whereas fusion of synaptic vesicles in the resting state is inhibited. However, synaptic vesicle exocytosis and neurotransmitter secretion can also occur spontaneously.^{36,66} The spontaneous neurotransmitter release may be important in the development of initial synaptic contacts into mature and fully functional synapses.^{3,61}

It is generally believed that, in developing nerve processes free of contact with postsynaptic targets, spontaneous neurotransmitter secretion is localized primarily to the growth cone region.³⁶ In the majority of cases, insertion of newly synthesized plasma

membrane and cytoskeletal components into the growing nerve processes also occurs primarily at the growth cone area.^{16,18,63} Therefore, in addition to its function in neuronal pathfinding and navigation, the growth cone serves as the major site for the assembly of new axonal structure, exo-endocytotic activity and neurotransmitter secretion. In this model of axonal development, the function of the axonal shaft is limited primarily to the delivery of cell body-synthesized material to the growth cone region. However, experimental evidence suggests that some newly synthesized membrane components may be inserted along the axon.^{26,34,51} Moreover, constitutive endocytotic recycling of the plasma membrane along the axon has been reported in developing hippocampal^{27,44} and *Xenopus* spinal cord¹⁹ neurons.

EXPERIMENTAL PROCEDURES

Cell culture

Cultured *Xenopus* (purchased from Xenopus Express, Homosassa, FL) spinal cord neurons were prepared according to previously reported methods.^{1,59} The cells were plated on acid-washed cover glasses and grown in culture medium consisting of (v/v): 50% Leibovitz L-15 medium (Gibco), 49% Ringer solution (115 mM NaCl, 2 mM CaCl_2 , 2.5 mM KCl, 10 mM HEPES, pH 7.6) and 1% fetal bovine serum (Gibco). The cultures were used for experiments after one to four days incubation at 20°C. *Xenopus* myocytes were plated separately on Petri dishes, grown in a culture medium supplemented with 3% fetal bovine serum, and used for experiments after 24–48 h incubation at 20°C. In some experiments, the neurotrophic factor neurotrophin-3 (NT-3; Regeneron) was added to the culture medium during cell culture preparation at the final concentration of 50 ng/ml.

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Abbreviations: ACh, acetylcholine; HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid; MEPC, miniature endplate current; NT-3, neurotrophin-3; PBS, phosphate-buffered saline.

Micromanipulation

Manipulation of *Xenopus* myocytes followed the procedures described previously.^{30,46} Briefly, myocytes were gently detached from the surface of the Petri dish by heat-polished micropipettes attached to a hydraulic micromanipulator (Newport). The cells were transferred into the vicinity of the axon, allowed to reattach to the glass surface and manipulated into the contact axon. The micropipette used for myocyte manipulation was withdrawn. In the majority of patch-clamp recordings, the myocyte was firmly attached to the surface of the coverslip and was in tight contact with the axon. We found that myocytes plated on the plastic surface were easier to detach from the substrate than those plated on glass coverslips. Therefore, Petri dishes were routinely used for myocyte culture preparation.

Data acquisition and analysis

Gigaohm-seal whole-cell recording methods followed those described previously.³² Patch pipettes were fabricated from glass micropipettes (VWR) and pulled with a two-step puller (Narishigi). After heat polishing, the pipette tip diameter was 1.5–2 μm and the resistance was 2–5 M Ω . The intrapipette solution for the whole-cell recording from myocytes contained 140 mM KCl, 1 mM NaCl, 1 mM MgCl₂ and 10 mM HEPES (pH 7.4). All recordings were done at room temperature. The membrane currents were monitored by a patch-clamp amplifier (Warner PC501-A). The data were digitized and stored on a videotape recorder for later playback onto a storage oscilloscope (Tektronix 5113) or a chart recorder (Gould RS3200). The data were analysed with the SCAN program, kindly provided by Dr J. Dempster, Strathclyde University, U.K. The threshold for detection of current events was typically set at the level of 20–25 pA. For quantitative analysis of the shape of the current events recorded from myocytes, the following parameters of the individual membrane currents were calculated: (i) the peak amplitude; (ii) the rise time, defined as the time interval between 10% and 90% of the peak amplitude on the upstroke of the current; (iii) the half decay time, defined as the time interval between the peak and 50% of the peak amplitude on the decaying phase of the current.

To estimate the rate of decay of secretory activity along the axons (Fig. 5C, D), for each experiment the frequency of secretion events recorded at a particular location was normalized to that at the growth cone. For each data point, the ratio $\log_2(\text{normalized frequency})/(\text{distance from the growth cone})$ was calculated. Statistically significant difference between the two data sets (one-day-old neurons vs neurons grown for two days in the presence of NT-3) was evaluated using the Mann–Whitney test.

Immunocytochemistry

The cultures were grown for two days in the presence of NT-3 (50 ng/ml), fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, washed three times with PBS and permeabilized with blocking buffer (PBS containing 1% newborn calf serum, 100 mM glycine and 0.3% saponin) for 5 min. The cells were incubated with primary antibodies (anti-syntaxin monoclonal HPC-1 at 1:500, provided by Gregory Enikolopov, and anti-SNAP-25 polyclonal MC21 at 1:100, provided by Pietro De Camilli) in blocking buffer for 1 h, and with secondary antibodies (goat rhodamine-conjugated anti-mouse or anti-rabbit immunoglobulin G at 1:200; Jackson Immuno-Research, West Grove, PA) in blocking buffer for 1 h. Cells were mounted in Vectashield mounting medium (Vector Labs, Burlingame, CA) to resist bleaching. Cells were observed using an Olympus IX 50 inverted microscope equipped with differential interference contrast optics, a $\times 60$ Fluorite objective (NA 1.2) and a 100-W mercury

lamp. Images were acquired with a charge-coupled device camera (Sensys, Photometrics LTD, Tucson, AZ) driven by IPLab (Signal Analytics Corp., Vienna, VA) imaging software and processed with Photoshop (Adobe Systems, Mountain View, CA).

RESULTS

Spontaneous acetylcholine secretion along growing *Xenopus* axons

Nerve–muscle cultures from *Xenopus* embryos were plated at low density and isolated neurons were chosen for experiments. The first neurites could be observed about 4–6 h after plating. We will refer to all processes produced by *Xenopus* embryonic neurons in culture as “axons”.^{9,48,51,54} Experiments were performed one to three days after cell culture preparation on axons which were about 400–600 μm in length and had a shape close to cylindrical as judged by phase microscopy. An isolated cultured *Xenopus* myocyte was detached from the substrate using a micropipette and manipulated into contact either with the growth cone region of the axon, or the middle of the axonal shaft (Fig. 1A, B). The acetylcholine (ACh) receptors are distributed throughout the myocyte surface,^{40,50} and therefore myocytes may be used as detectors of ACh release. The micropipette used for manipulation was withdrawn and membrane currents in the myocyte were recorded using a conventional patch-clamp technique.³² Whole-cell voltage-clamp recordings from the myocyte revealed fast inward currents similar in shape to the miniature endplate currents (MEPCs) found at *Xenopus* neuromuscular synapses³⁰ (Fig. 1C, D). The individual current events were due to the release of packets of ACh molecules from the axons and most likely reflected spontaneous fusion of ACh-containing synaptic vesicles with the plasma membrane. The number of ACh molecules in each packet (N) can be estimated from the size of the membrane current elicited in the myocyte. For a typical MEPC (current amplitude ≈ 100 pA), N is of the order of 10^3 .^{47,56} To avoid potential accumulation of ACh receptors at the contact site,^{20,22} the recording was limited to the first 5–10 min after contact with the axon was formed. The exocytotic events could be detected immediately after establishment of whole-cell configuration in the myocyte, and their frequency did not change noticeably during the period of recording. Therefore, myocytes appear to serve as passive detectors rather than inducers of exocytotic events. The technique allows detection of ACh release from the axonal site in immediate contact with a myocyte, as well as within 1–2 μm from the contact site.^{30,47} The secretion of ACh that we observed is consistent with previously reported constitutive recycling of synaptic vesicles along *Xenopus* axons in culture as detected with FM1-43 fluorescent dye.¹⁹

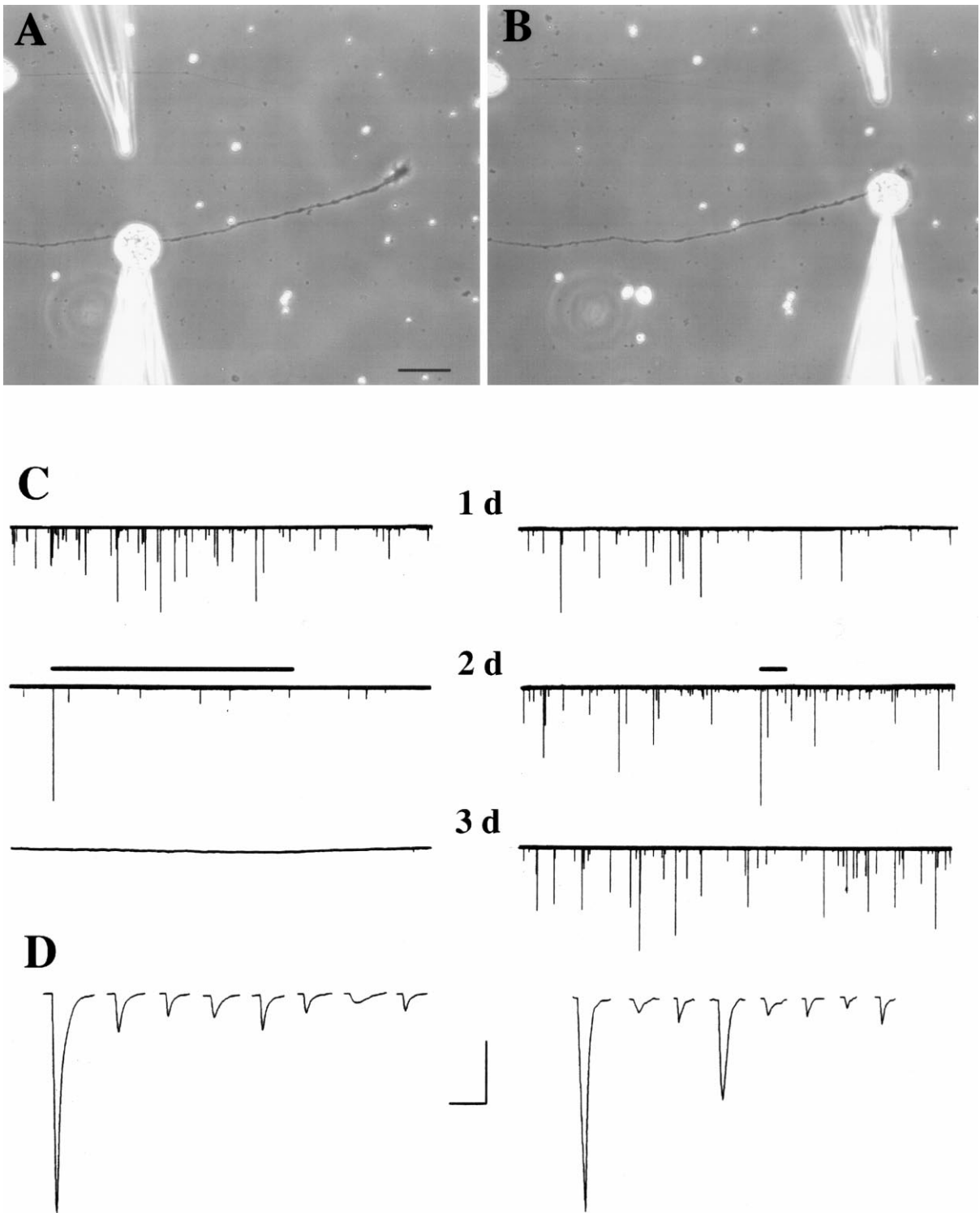


Fig. 1. Spontaneous ACh secretion along growing *Xenopus* axons. (A, B) Phase contrast images of a cultured *Xenopus* myocyte manipulated into contact with the middle (A) or the growth cone (B) region of a growing *Xenopus* axon. The top pipette was used for myocyte manipulation and the bottom one for whole-cell patch-clamp recordings from the myocyte. Scale bar = 40 μm . (C) Examples of MEPC-like currents recorded from myocytes in contact with the middle axonal segment (left traces) and growth cone area (right traces) one, two and three days after preparation of neuronal cultures. ACh secretion events, as shown by pulsatile inward currents (downward deflections), were monitored by whole-cell voltage-clamp recording from *Xenopus* myocytes at resting potential (-70 mV; filtered at 500 Hz). Note a decrease in the frequency of secretion events in recordings from the middle axonal region with increase in cell culture age. (D) Samples of current events recorded from myocytes in contact with *Xenopus* axons two days after cell culture preparation. The current events detected during the time intervals marked by lines in C are shown at a higher time resolution (filtered at 3 kHz). Bars: 1 nA, 60 s (C); 0.5 nA, 50 ms (D).

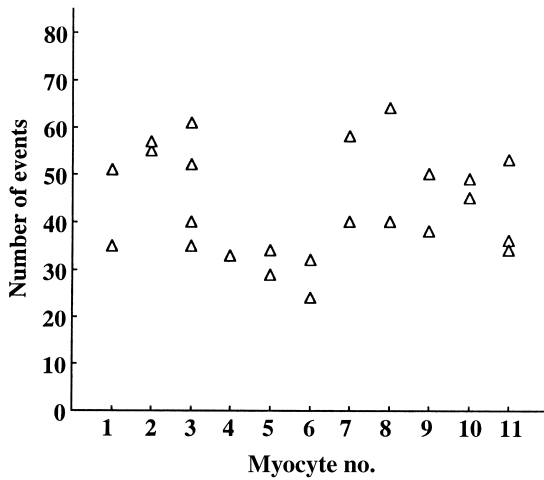


Fig. 2. Control measurements of exocytotic activity at the same axonal site. Eleven different myocytes were consecutively manipulated into contact with the same axonal segment of a neuron. The segment was located at a distance of about 320 μm from the soma. Data points represent the number of MEPCs detected during a 5-min period of recording. After the end of recording, most of the myocytes (except myocyte no. 4) were removed and repeatedly manipulated into contact with the same axonal site. All 24 recordings were performed during a 6-h period. The axon was not damaged by myocyte manipulations as evidenced by continuous growth (data not shown). The number of secretion events observed during a 5-min period of recording (N) was 43.5 ± 11.1 (average of all 24 experiments \pm S.D.). Assuming that the observed variability in N was due entirely to the stochastic nature of exocytotic events, one would expect $\text{S.D.} = N^{1/2} \approx 6.6$ events. The actual S.D. of the data set was 11.1, indicating a significant contribution of other factors. The main factor is likely to be the area of contact between axon and myocyte. Indeed, MEPC frequency usually increased when the myocyte was moved into tighter contact with the axon using the recording patch-clamp pipette (data not shown).

One day after cell culture preparation, ACh secretion events could be observed at both the growth cone region and the middle axonal segment. However, with increase in cell culture age, secretory activity along the axon was inhibited (Fig. 1C). To semiquantitatively characterize the topology of ACh secretion in axons, we systematically measured parameters of current events in recordings from myocytes at the spontaneously formed synapses ("preformed synapses"), and from myocytes manipulated into contact with the middle axonal segment or the growth cone region. For these measurements, we made an effort to perform myocyte manipulation and patch-clamp recordings in a reproducible fashion. Control experiments using repeated manipulation of different myocytes to the same axonal segment demonstrated that MEPC frequency recorded at the same site may differ by as much as 2.6-fold (Fig. 2). The stochastic nature of the secretory events, as well as variations in the area of contact between the axon and the myocyte and in the density of ACh receptors in different myocytes, are all likely to contribute to the observed differences in the MEPC frequency. Therefore, we performed at least 14 recordings from the middle

and distal axonal regions to detect statistically significant differences in the MEPC frequency between these regions. In one-day-old neuronal cultures, the frequency of secretion events was 10.0 ± 1.7 (mean \pm S.E.M., $n = 17$) and 9.4 ± 2.1 events/min ($n = 17$) at the middle axonal segment and at the growth cone region, respectively (Fig. 3A). In experiments two and three days after cell culture preparation, the frequency of spontaneous secretion events at the middle segment of neurites declined dramatically to 1.9 ± 0.3 ($n = 18$) and 0.28 ± 0.2 events/min ($n = 15$), respectively. At the same time, MEPC frequency did not change significantly at the growth cone area and in preformed synapses (Fig. 3A). Exocytotic events were also observed in recordings from the myocytes manipulated into contact with the soma (data not shown). We have not attempted to analyse quantitatively secretory activity at the soma.

The average amplitude of MEPCs at the middle axonal segment was somewhat lower in comparison to that at the growth cone region or at the spontaneously formed synapses (Fig. 3A). However, there was substantial variability in the amplitude of MEPCs and this difference was not statistically significant. The amplitude distribution of the current events was skewed towards smaller sizes, both in recordings from the middle axonal segment and from the growth cone (Fig. 3E, F), similar to that found at developing *Xenopus* neuromuscular synapses.⁶⁴ The absence of well-defined quanta in these synapses is believed to reflect the immaturity of synaptic vesicles. The distribution of MEPCs becomes bell-shaped four to five days after cell culture preparation, and synaptic maturation can be accelerated by neurotrophic factors released from the postsynaptic myocytes.⁶⁴

The average rise times of the current events recorded from different axonal segments were identical (Fig. 3C), suggesting a similar (within the time resolution of the method) rate of neurotransmitter release at various axonal regions.^{30,62} The average half decay time, a measure of the average open time of ACh channels and an indirect indicator of the distance between the sites of ACh release and myocyte ACh receptors,^{30,47,56} was also similar (Fig. 3D). The quantitative similarity between parameters of MEPCs at various axonal regions supports the idea that quantal ACh release throughout the whole axonal surface is mediated by similar synaptic vesicles.^{19,27,44}

Secretory activity along the axon is inhibited by chronic exposure to neurotrophin-3

The rate of axonal growth under cell culture conditions used in this study slows down two days after cell culture preparation, which may reflect the general decline in the synthesis of molecular components necessary for nerve growth. To investigate

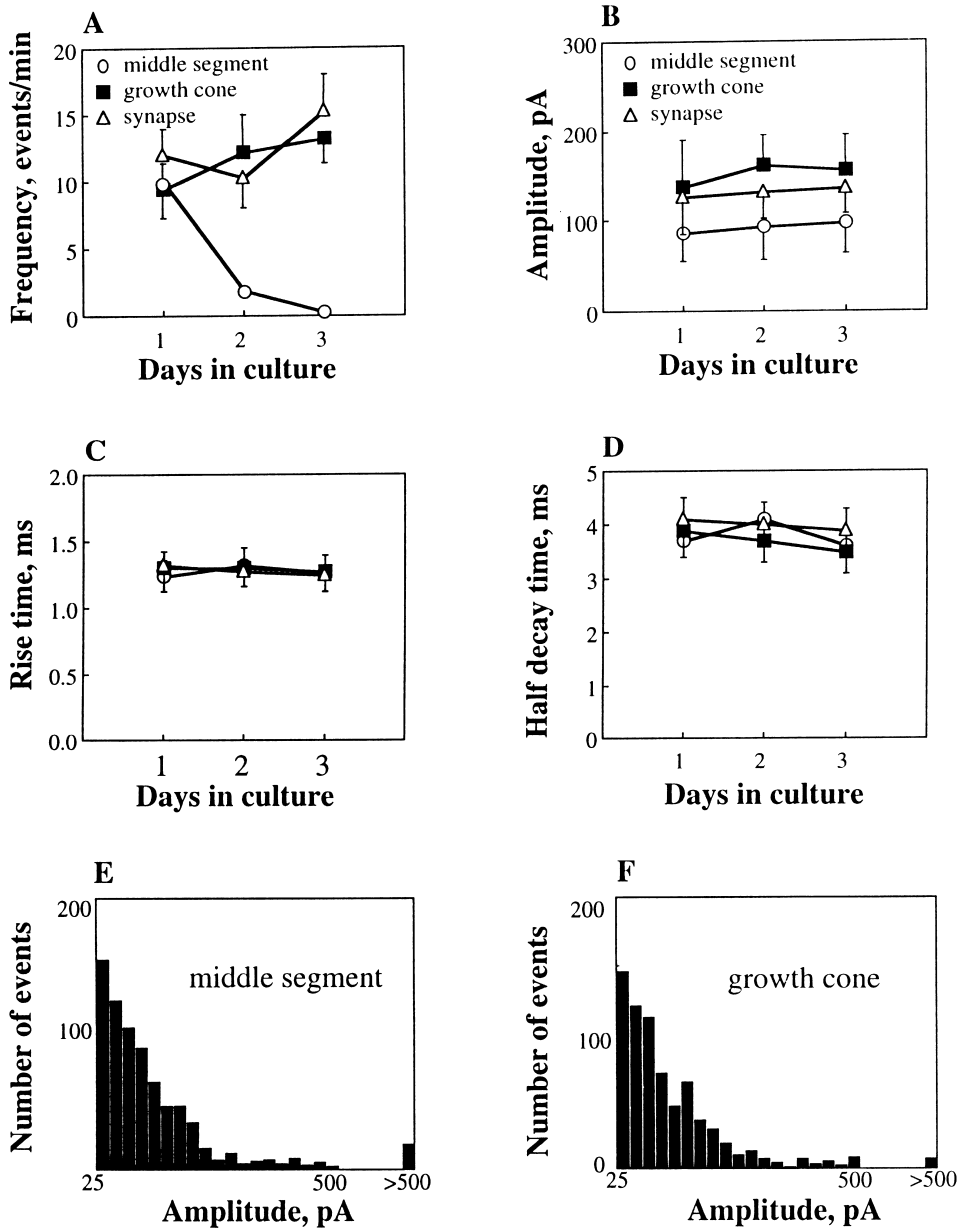


Fig. 3. Characterization of the exocytotic activity at the growth cone and middle axonal regions. (A–D) Parameters of the individual MEPCs recorded from the myocytes in spontaneously formed neuromuscular synapses (open triangles) and from the myocytes manipulated into contact with the middle (filled squares) or the growth cone (open circles) axonal regions one to three days after neuronal culture preparation. Myocytes were manipulated into contact with the middle axonal segment or the growth cone area by a micropipette (Fig. 1). Typically, recordings started 1–2 min after myocyte manipulation and the data were collected for a period of 5 min. For each individual recording, the average frequency (A), amplitude (B), rise time (C) and half decay time (D) of MEPCs were determined. Each point in A–D represents the mean \pm S.E.M. of 14–21 different experiments. The total number of experiments was 169. No secretion events were recorded in eight different experiments at the middle axonal segment four days after cell culture preparation (data not shown). (E, F) Representative histograms of MEPC amplitude distribution in recordings from the myocytes manipulated into contact with the middle axonal segment (E) and growth cone region (F). The data were collected for a period of 45 min.

whether the drop in the frequency of secretion events at the middle axonal segment was directly related to the decrease in the efficiency of biosynthetic apparatus and/or to axonal degeneration, we measured

exocytotic activity along the axons in the presence of the neurotrophic factor NT-3 in the culture medium. Chronic exposure to NT-3 is known to promote axonal growth, survival and differentiation

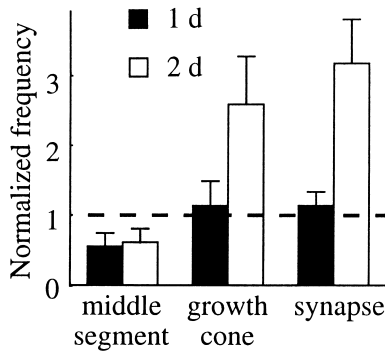


Fig. 4. Effect of NT-3 on the exocytotic activity along the axon. NT-3 (50 ng/ml; Regeneron) was added to the culture medium during cell culture preparation and was present throughout the experiment. The frequency of secretion events at the middle axonal region, growth cone area and spontaneously formed synapses (mean \pm S.E.M. of six to 11 experiments) was normalized to those recorded from control cultures, not treated with NT-3 (data from Fig. 3).

of *Xenopus* embryonic neurons.^{14,45} In agreement with previously published data,⁶⁴ in recordings from both the preformed synapses and the growth cones of isolated axons two days after cell culture preparation, the frequency of secretion events was significantly higher in comparison with control neurons untreated with NT-3 (Fig. 4). However, in one- and two-day-old cultures, chronic exposure to NT-3 resulted in the inhibition of secretory activity along the axon (Fig. 4). These results argue against the idea that the inhibition of exocytotic activity along the axon is related to axonal degeneration. The data are consistent with a scenario in which NT-3 treatment facilitates the shift of exocytotic fusion events from the proximal to distal axon during axonal differentiation.

Gradient of exocytotic activity along the axon

To construct a more accurate map of the exocytotic activity along the length of neurites, we repeatedly manipulated myocytes into contact with various regions of the same axon and measured the frequency of MEPCs in myocytes. Since the level of secretory activity along the axon three days after cell culture preparation was low (see Figs 1C and 3A), we have not attempted to perform these measurements at the later stages of axonal growth. Instead, we concentrated our efforts on neurons growing for one day in the regular culture medium and on neurons growing for two days in the culture medium supplemented with NT-3. Figure 5A and B depicts representative data on the frequency of secretion events along two different axons recorded at different distances from the growth cone. As expected, the secretion events could be observed all over the length of the neurite one day after cell culture preparation. When fitted with a single exponential, the distance from the growth cone, where the frequency of secretion events decreased two-fold

in comparison with that at the growth cone region ($\lambda_{1/2}$), was 459 μm (Fig. 5A). For an axon growing for two days in the presence of NT-3 (Fig. 5B), the corresponding $\lambda_{1/2}$ was 162 μm . Figure 5C and D summarizes the results of 128 measurements performed on six axons one day after cell culture preparation (Fig. 5C) and on six axons grown for two days in the presence of NT-3 (Fig. 5D). The average axonal lengths were 398 and 740 μm for one- and two-day old neuronal cultures, respectively. In each experiment, the frequency of secretion events recorded at a particular location was normalized to that at the growth cone. When interpolated with an exponential function, for one-day-old neuronal cultures $\lambda_{1/2}$ was 334 μm , indicating rather uniform exocytotic activity along the axon (Fig. 5C). For neurites grown for two days in the presence of NT-3, the corresponding $\lambda_{1/2}$ was 190 μm , reflecting significantly higher levels of secretory activity at the distal axonal regions in comparison with that at the proximal segments. The difference in $\lambda_{1/2}$ between the two series of experiments was statistically significant ($P < 0.01$; see Experimental Procedures). Exocytotic events were not precisely localized to the growth cone in any of the 12 axons. Instead, secretory activity could be observed at axonal segments proximal to the growth cone region, even in neurons grown for two days in the presence of NT-3.

Localization of syntaxin and SNAP-25

Syntaxin and SNAP-25 are putative SNAREs,⁵⁵ which are believed to be required for the docking and/or fusion of synaptic vesicles. Previously, these proteins have generally been localized to synapses. However, in isolated hippocampal neurons or in PC12 cells, which are free of contact with other cells, both proteins are distributed throughout the neuronal surface.^{27,28,58} To determine the distribution of syntaxin and SNAP-25 along growing *Xenopus* neurites, we stained the neuronal cultures with antibodies to these proteins. Immunoreactivity was largely restricted to the neurons. In individual neurons, immunoreactivity for both SNAP-25 and syntaxin was detected at the soma, the growth cone and along the axon (Fig. 6).

DISCUSSION

We have used an electrophysiological technique for the detection of individual ACh secretion events to characterize ACh secretion along growing *Xenopus* axons in culture. The main finding of the present study is that, at the early stage of axonal growth, ACh release from the neuron takes place along the axon, as well as at the growth cone. At the later stages of growth, the exocytotic activity is preferentially localized to the distal axonal region. To our knowledge, this is the first semiquantitative

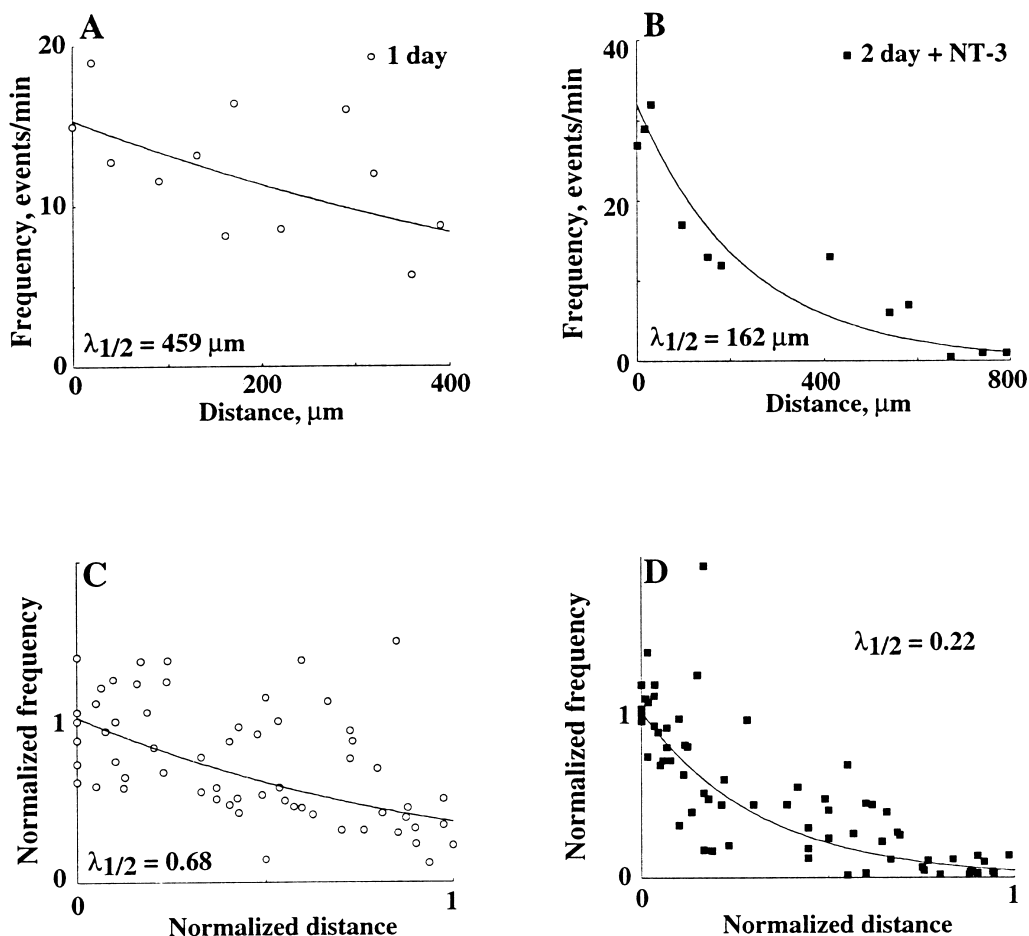


Fig. 5. Distribution of secretion events along growing axons. (A, B) Representative plots for one-day-old (A) and two-day-old neuronal cultures grown in the presence of NT-3 (B). Myocytes were manipulated into contact with various axonal regions as described in Fig. 1. The average frequency of MEPCs recorded from different myocytes is plotted as a function of the distance between the growth cone and the myocyte. The axons were 420 μm (A) and 820 μm (B) long. The data were least-squares fitted with an exponential (continuous line). The half lengths of the exponential decay $\lambda_{1/2}$ were 459 μm (A) and 162 μm (B). (C, D) Summary of all experiments. For each recording, the frequency of MEPCs at different distances from the growth cone was normalized to that at the growth cone region. In total, 60 measurements were performed on six different axons one day after cell culture preparation (average axonal length 398 μm). For two-day-old neuronal cultures growing in the presence of NT-3, 68 measurements were made on six different axons (average axonal length 740 μm). The data were least-squares fitted with the exponential (continuous lines). The normalized frequency of secretion events declined with half decay lengths $\lambda_{1/2}$ of 334 μm (C) and 190 μm (D).

measurement of the topology of neurotransmitter release along developing axons.

Constitutive acetylcholine secretion along the axons

Neurotransmitters appear early in the developing embryo and may play a role in morphogenesis, neuronal signaling and axonal navigation.^{11,35,41,67} In addition, neurotransmitter secretion from growing axons³⁶ is likely to contribute to development of neuronal networks. Therefore, it is important to determine the distribution of neurotransmitter secretion in growing axons, which are free of contact with postsynaptic cells. Towards this aim, we used whole-cell myocytes, manipulated into contact with growing *Xenopus* axons in culture, as detectors

of ACh release. It should be noted that this assay allows one to quantitatively characterize quantal, but not non-vesicular,⁵² release of ACh from the axon. The former results in the MEPC-like current events in the recordings from whole-cell myocytes; the latter leads to a steady-state depolarization of the myocyte membrane and to an increase in the baseline noise level.³⁸

Our results firmly establish the phenomenon of spontaneous quantal ACh release along growing *Xenopus* axons in culture. It is now accepted that, in an overwhelming majority of cases, release of neurotransmitter quanta in neurons is due to the fusion of ACh-containing vesicles with the plasma-lemma (a possible exception to this model may include the electric organ of *Torpedo*, where ACh

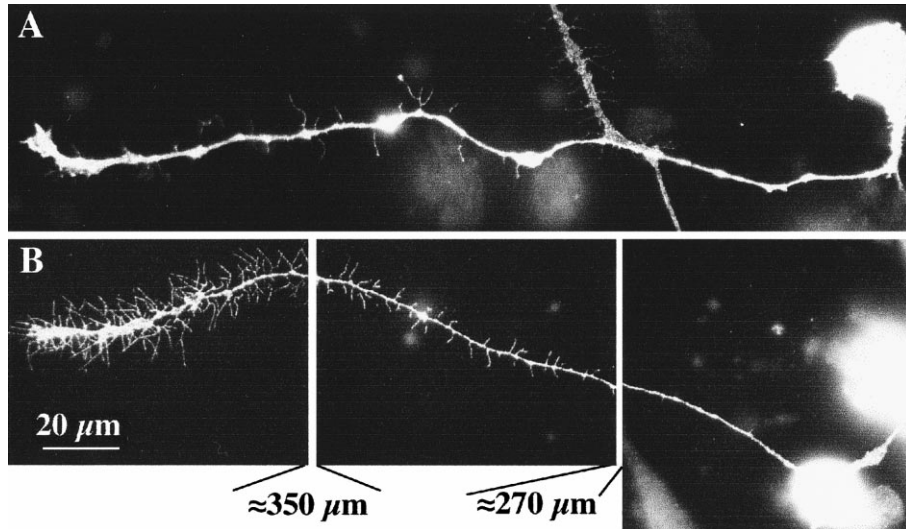


Fig. 6. Syntaxin and SNAP-25 are distributed throughout the neuronal surface. Immunofluorescence localization of syntaxin (A) and SNAP-25 (B) was performed as described in the Experimental Procedures. Immunoreactivity was detected at the soma, along the axon and at the growth cone region. In A, the whole axon (about 500 μm in length) is shown. The length of the axon shown in B was $\approx 1100 \mu\text{m}$; only three axonal segments (growth cone region, middle axon and soma) are shown.

translocation through the plasma membrane may be mediated by a Ca^{2+} -dependent plasma membrane protein²³). Vesicular exocytosis at various axonal regions is consistent with the presence of v-SNAREs⁵⁵ on these vesicles and of corresponding t-SNAREs along the entire axon.²⁸ Some of the ACh-containing vesicles appear to be docked at the plasma membrane along the axon, since their exocytosis can be triggered by an action potential (our unpublished data). Therefore, these vesicles are likely to contain molecular components of the docking machinery. Moreover, parameters of MEPCs recorded from different axonal segments are very similar (Fig. 3). Although the exact molecular composition of ACh-containing vesicles in various axonal regions remains to be firmly established, our results and previously reported data^{19,44} suggest that ACh-containing vesicles at the middle axonal region are similar, if not identical, to synaptic vesicles at the growth cone region.

Significant progress has recently been made in identifying the cellular mechanisms and molecular components involved in synaptic development and maturation.^{20,24,29,53,57} Activity dependence is a hallmark of many of these processes.^{8,12,25,53,61,65} ACh secretion throughout the whole axonal surface suggests that, in developing axons, there may be no clear-cut functional distinction between the growth cone and the axonal shaft; both regions have the potential to differentiate into a pre-synaptic nerve terminal. In the process of axonal development, ACh secretion adopts the form of a proximodistal gradient, with the highest level of activity at the distal axonal segment. This gradient is established in the absence of contact with other neuronal cells and is likely to be

governed by an endogenous program of axonal development.

Potential mechanisms for the generation of the gradient of exocytotic activity along growing axons

A large body of experimental evidence indicates that the distal and proximal axonal regions differ in molecular composition. Thus, a spatial gradient of second messengers,^{2,37} endocytotic organelle acidification⁴⁹ and microtubule-associated proteins^{7,15,39} may exist in growing axons. Post-translational modifications of tubulin,^{6,31} neurofilaments^{5,60} and microtubule-associated proteins^{7,43} also exhibit gradients along the axons. In addition, the turnover rate of axonal microtubules, as measured by the fluorescence recovery after photobleaching, is higher at the distal axon.^{14,21} The mechanisms of such gradient development have yet to be firmly established.

The main finding of the present study is the detection of a gradient of secretory activity along the axon, with the highest level at the growth cone region (proximodistal gradient). The steepness of the gradient apparently depends on the age of cell culture and on the presence of the neurotrophic factor NT-3 in the culture medium. The cellular mechanisms for the creation of such an exocytotic gradient remain unknown. The possible factors may include developmentally regulated redistribution of t-SNAREs to the growth cone region, modifications of the axonal cytoskeleton which favor delivery and/or accumulation of the synaptic vesicle precursors to the distal axon, or biophysical factors such as a gradient of mechanical tension along the axon. *Xenopus* embryo neurons in culture may be used

as a model system to address the molecular and cellular mechanisms of regulation of secretory activity along growing nerve processes. Our finding that, in growing axons, ACh is spontaneously secreted in the proximodistal gradient adds a new level of complexity to the problem of neurotransmitter-mediated signaling within the developing nervous system.

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