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REGULAR ARTICLE

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Differential appearance of dynamin in constitutive and regulated exo-endocytosis: a single-cell multiplex RT-PCR study

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Abstract Neurons in the central nervous system establish, via their axons and dendrites, an extended network that allows synaptic transmission. During developmental maturation and process outgrowth, membrane turnover is necessary for the enlargement and subsequent growth of axons and dendrites from the perikarya to the target cell (constitutive exocytosis/endocytosis). After targeting and synapse formation, small synaptic vesicles are needed for the quantal release of neurotransmitters from the presynaptic terminal with subsequent recycling by regulated exocytosis/endocytosis. An investigation of the onset of the appearance of mRNA and protein in dissociated cultures of neurons from mouse hippocampus or from chick retina has shown an early abundance of proteins involved in exocytosis, such as syntaxin 1, SNAP-25, and synaptotagmin 1, whereas dynamin 1, a protein necessary for clathrin-mediated endocytosis, can be detected only after neurons have established contacts with neighboring cells. The results reveal that constitutive membrane incorporation and regulated synaptic transmitter release is mediated by the same neuronal proteins. Moreover, the data exclude that dynamin 1 takes part in constitutive recycling before synapse formation, but dynamin 2 is present at this stage. Thus, dynamin 2 may be the constitutive counterpart of dynamin 1 in growing neurons. Synapse establishment is linked to an upregulation of dynamin 1 and thereby represents the beginning of the regulated recycling of membranes back into the presynaptic terminal.

Keywords Neuronal development · Exocytosis · Endocytosis · Presynaptic proteins · Dynamin · Chick (White Leghorn) · Mouse

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Introduction

In mature neurons, synaptic vesicles continuously recycle within the presynaptic nerve terminal. This cycle includes the fusion of synaptic vesicles with the plasma membrane and transmitter release by exocytosis followed by endocytic membrane internalization. The regulated exo-/endocytosis is thought to be mediated by presynaptic proteins. These proteins include the members of SNARE proteins involved in exocytic events (for a review, see Bruns and Jahn 2002) and a group of proteins including dynamin, amphiphysin, and endophilin involved in endocytic events (Herskovits et al. 1994; McPherson et al. 1996; Higgins and McMahon 2002; Reutens and Begley 2002).

However, membrane expansion and retrieval have to occur during neuronal development along the processes, at the tip of growing processes, and during the establishment of synapses. These mechanisms underlying diverse alterations within neuronal growth cones have been the subject of intense investigation. Many of these investigations have centered on the cytoskeleton, which displays significant changes during growth cone elongation (Letourneau 1996). Other studies have focused on the involvement of receptor proteins in ion channels (Contestabile 2000), adhesion molecules (Walsh and Doherty 1997), guidance molecules (Cook et al. 1998), or neurotrophins (Huang and Reichardt 2001). Additionally, the removal, translocation, and addition of membrane (and their proteins) must be in the transformation of the growth cone into a neuronal process and, later on, into a mature synaptic terminal. Although key proteins for both pathways have been named (Jahn et al. 2003), the exact mechanisms of membrane extension (exocytosis) and retrieval (endocytosis) in living neurons are still poorly understood in developing neurons.

In-situ and in-vitro studies have been shown that presynaptic proteins involved in exocytosis are expressed during neuronal development and undergo extensive redistribution prior to and during synapse formation (e.g., Bergmann et al. 1991; Catsicas et al. 1994; Grabs et al. 1994; Grosse et al. 1998) and are transported together as distinct packets (Ahmari et al. 2000). Several proteins have

been shown to mediate membrane expansion during outgrowth (Steiner et al. 2002). The cleavage of other proteins by clostridial neurotoxins inhibits axonal (Osen-Sand et al. 1993; Igarashi et al. 1996) and dendritic (Grosse et al. 1999) growth. Moreover, constitutive membrane recycling is reported not to be restricted to the nerve terminal but to occur in cycles of exo-/endocytosis over the whole axonal surface (Matteoli et al. 1992; Kraszewski et al. 1995). However, conflicting data from neuroendocrine pheochromocytoma cell lines (PC12) indicate that neurite extension can occur, although known members of the exocytic machinery are absent from specialized strains of these cells (Leoni et al. 1999; Grundschober et al. 2002). Additionally, data from null-mutant mice suggest that, in the absence of either VAMP2 (synaptobrevin 2; Schoch et al. 2001) or SNAP-25 (Washbourne et al. 2002), synaptic transmission is decreased, although normal axonal outgrowth and synaptic targeting still occurs.

Whereas the main focus of the previous investigations has been on the exocytic machinery, little is known about the involvement of soluble presynaptic proteins used in endocytosis, such as dynamin, amphiphysin, and endophilin, in dendrite and axon outgrowth and synapse formation. Although dynamin and amphiphysin are established textbook members of clathrin-mediated endocytosis, endophilin (Ringstad et al. 1997) is thought to be necessary for lipid modification at the plasma membrane during endocytosis (Huttner and Schmidt 2000), and the blockage of endophilin has been shown to interfere with clathrin "uncoating" (Gad et al. 2000) but not with neurotransmission (Verstreken et al. 2002). However, multiple isoforms of dynamin and endophilin are differentially expressed in the central nervous system (Cook et al. 1994; Nakata et al. 1993; Ringstad et al. 2001). In-situ expression data for dynamin and amphiphysin in chick retinotectal neurons have suggested a delay between the upregulation of these proteins compared with presynaptic proteins involved in exocytosis (Bergmann et al. 1999; Grabs et al. 2000). Interestingly, a delayed appearance of dynamin has also been found during motor neuron development and neuromuscular synaptogenesis (Noakes et al. 1999). The growth cones of neurons treated with amphiphysin antisense-probes collapse (Mundigl et al. 1998). Nevertheless, the specific functions of these endocytic proteins in immature neurons and during synapse formation remains to be determined.

Materials and methods

Animals

All experiments were carried out in accordance with the guidelines published by the Swiss Academy of Medical Sciences) and the Swiss Academy of Natural Sciences regarding the use of animals for experimental procedures.

White Leghorn chicks (embryonic day 10, ED10) and mouse embryos (ED17) were used. Chicks were taken from termed eggs, whereas mother mice were anesthetized with Vetanarcol (Pentobarbital 100 mg/kg body weight), and their embryos were removed by Caesarian section.

Cell culture

Primary cultures were established from the hippocampi of ED17 mice or from retinae of ED10 White Leghorn chicks. Single-cell suspensions were prepared by mechanical and enzymatic dissociation in Neurobasal medium supplemented with B27 solution (Invitrogen, Basel, Switzerland). The cells were suspended at a density between 100,000 and 500,000 cells/cm^2 in poly-D-lysine-coated plastic dishes (Nunc, Wiesbaden, Germany) and kept for up to 21 days in culture.

Single-cell reverse transcription/polymerase chain reaction

Cultivated cells were chosen under visual control under a Leica DM-IRBE microscope and picked up with a 15-µm glass capillary (WPI, Aston, UK). Single cells were transferred into a proteinase/SDS/RNase inhibitor solution and heated for 5 min at 95°C for lysis and enzyme inactivation. Reverse transcription (RT) and polymerase chain reaction (PCR) were performed in single tubes by using the One-Step RT-PCR system (Qiagen, Basel, Switzerland). Thereafter, specific first-round primers for all tested protein sequences were added to the solution. A second run of PCR with nested primers used only one pair of primers for a specific protein in each tube.

First-round primers for the mouse hippocampus were as given below.

Protein	Clone	Forward primer	Reverse primer
Beta-actin	NM_007393	CCCTGAAGTACCCCATTGAACATG	CCTCAGGGCATCGGAACCGCT
Amphiphysin 1	NM_175007	GTATGGACGGGAAGATGTAAAGAT	CTGGGCAGGGGAGAAGG
Dynamin 1	NM_010065	TGCCGCAGATCGCCGTGGT	TCAGGTCCAACTTGGTGA TGAC
Alpha-adaptin	X14971	TCCGGCTCATCAACAACGCTATCA	GCCGCCCGCTGCCTCAC
Endophilin 2	U58885	GGGGCCGAAGGGACCAAACT	GCCGTGCAGCATCCCCTC ATAC
Syntaxin 1a	D45208	ACGGCCAAGGACAGCGATGAC	TGGTACTTGACGGCCTTCTT GGTG
Synaptotagmin 1	NM_009306	GCTGAACTGCCCGCCCTGGAC	GCTCTGCGCCGGTGCTGTT GTAG

Nested primers for the mouse hippocampus were as follows.

Protein	Clone	Forward primer	Nested primer
Beta-actin	NM_007393	GATCTGGCACCACACCTTCTAC	GCACAGCTTCTCTTTGATG
Amphiphysin 1	NM_175007	CTAAGCGCAGCAGGAAG	TGACGTAAAAGCCAACTCGA
Dynamin 1	NM_010065	GGCGTCCCCTGGTCCTG	GGTGCGCTGACCCTGGG
Alpha-adaptin	X14971	GTGCCGCCCTATGCCTACTG	ACACTCCACTAACCGCCCTT TCAC
Endophilin 2	U58885	CATGGCAAGGAACTAGGTGGAGA	GGGGCCGGGGCTTGAACT
Syntaxin 1a	D45208	TCCCCGAACCCCGATGAGA	AGAGGCAAAGATGGCAGGG TTCC
Synaptotagmin 1	NM_009306	CCACCGGAAAACCCTCAATC	AGCCACCCACATCCATCTTCT

First-round primers for the chick retina were as below.

Protein	Clone	Forward primer	Reverse primer
Beta-actin	NM_205518	CCCTGAAGTACCCCATTGAACACG	CCTCGGGGCACCTGAACCTCT
Amphiphysin 1	X60422	GTCCAGAAACGCCTCAACC	TAATCCTGCCTTCATCTTTT CTTT
Dynamin 1	XM_415501	TGCCGCAGATCGCCGTGGT	TAAGATCCAGCTTAGTGA TGAC
Synaptotagmin 1	S64957	ATGGTGAGTGAAAGTCAT	GTAAATTGCTCATTGAAA

Nested primers for the chick retina were as follows.

Protein	Clone	Forward primer	Nested primer
Beta-actin	NM_205518	GATCTGGCACCACACTTTCTAC	GCACAGCTTCTCCTTGATG
Amphiphysin 1	X60422	ACATGAAGACGGGCATC	CATCACATTTCTCACCAA
Dynamin 1	XM_415501	GGCGTCCGCTGGTCCTG	GGTGCGTTGCCCTTGAG
Synaptotagmin 1	S64957	GGAGGAGGAGGAAAAGAAGAT	GAATAATTCCAACCAGAAGC

To compare dynamin 1 and dynamin 2 in the mouse hippocampus different primers were used. First-round primers were as given below.

Protein	Clone	Forward primer	Reverse primer
Dynamin 1	NM_010065	CCCGGCGTCCCCTGGTCCTG	ACGTCCCGCGCATCTGTG
Dynamin 2	NM_007871	TACCCCGAGAAGGACCAGGCAGAG	TGGCGCAGAGAAGGGGTCGT TGTT

Nested primers for the mouse hippocampus were as follows.

Protein	Clone	Forward primer	Nested primer
Dynamin 1	NM_010065	CACCGGCACCAACAAGGGCATTTC	CCGAGTTGGCAGGGGACACAGC
Dynamin 2	NM_007871	CAGGCTCAGCGGCGGGACGAC	GGCGCTGTGGAGTGGGGCTGTG

Immunostaining of primary cultures

For immunocytochemical detection, cell cultures were fixed for 30 min with 4% paraformaldehyde in 0.1 M phosphate buffer saline (pH 7,4). Fixed cultures were permeated with 0.1% Triton X-100 for 30 min. For immunofluorescence, mouse monoclonal or rabbit polyclonal antibodies recognizing dynamin 1 (Transduction Laboratories), dynamin 1 (*DG1*, kindly provided by P. DeCamilli; see Butler et al. 1997), synaptotagmin 1 (*clone 41.1*, kindly provided by R. Jahn; see Brose et al. 1992), and SNAP-25 (*SMI-81*, Sternberger Monoclonals) were diluted 1:100 in goat serum dilution buffer (20 mM $NaPO_4$ pH 7.4, 15% normal goat serum, 450 mM NaCl) and incubated overnight at 4°C. Antigen/antibody complexes were visualized by using Alexa 488- or Alexa 546-conjugated goat anti-rabbit or goat anti-mouse IgGs 1:100 for 2 h (for further details, see Grabs et al. 1994; Grosse et al. 1998, 1999). The cultures were examined and photographed by using a Leitz DM-IRBE microscope equipped with epifluorescence.

For ultrastructural analysis, cultures were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 30 min. Cultures were processed for pre-embedding staining by the avidin-biotin-complex technique (for further details, see Grosse et al. 1998). Sections were postfixed with 1% OsO_4, counterstained with 4% uranyl acetate and 0.2% lead citrate, and examined in a Zeiss EM10 electron microscope.

Results

Low density cultures were established either from embryonic mouse hippocampus (ED17) or from embryonic chick retina (ED10). For investigation of the developmental appearance of the mRNA of synaptic proteins, single cells were kept under controlled in-vitro conditions. Two different stages of neuronal development were compared:

1. neurons grown in isolation with no connections to neighboring neurons (Fig. 1a) and
2. neurons with established connections to one or more adjacent neurons (Fig. 1b).

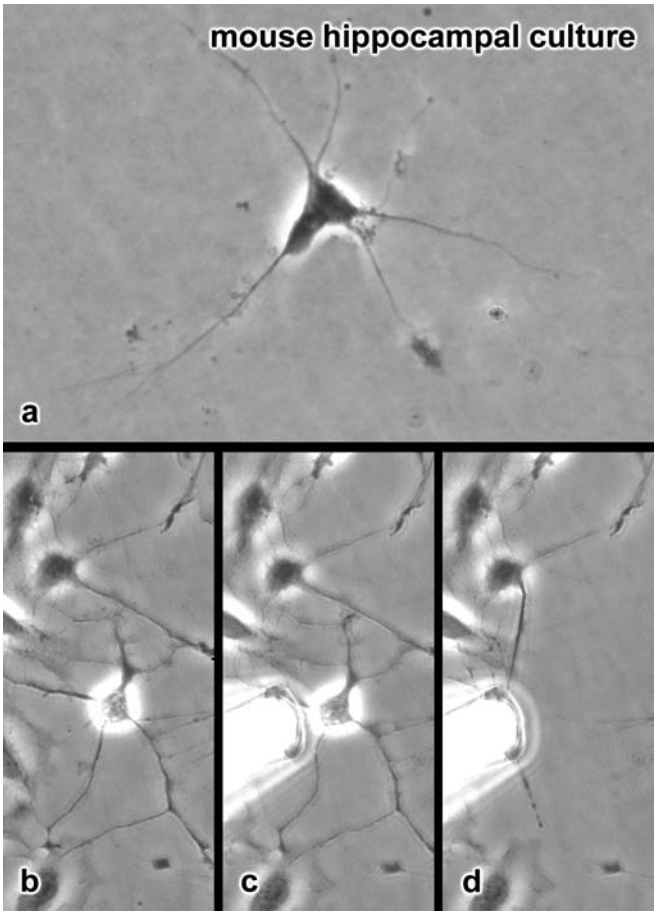

Fig. 1 Selection of single cells. Single neurons either grown in isolation (**a**) or integrated in a neuronal network (**b**) were chosen as described. By means of micropipettes with a 15-μm tip containing phosphate-buffered saline (PBS), target cells were localized (**c**), picked up by negative pressure in the pipette (**d**), and transferred into Eppendorf tubes filled with RNase protection medium

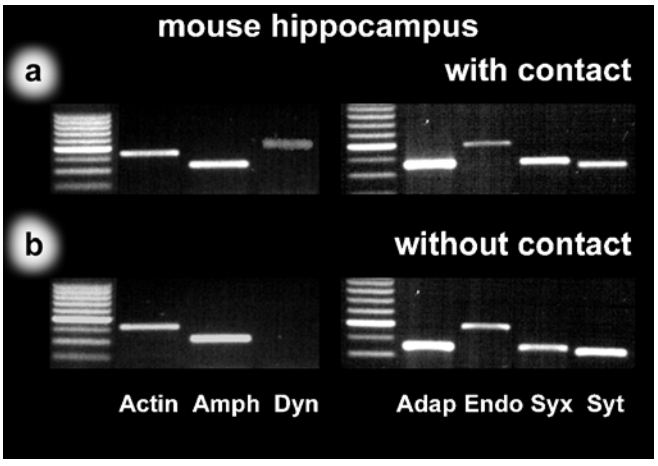

Fig. 2 Single-cell RT-PCR from mouse hippocampus (*lanes 1*, *5* size markers). Identified pyramidal cells from mouse hippocampal cultures were used in order to detect differences in the mRNA content between neurons with and without established contacts to neighboring neurons. Although transcripts were found for beta-actin (*Actin*), amphiphysin 1 (*Amph*), alpha-adaptin (*Adap*), endophilin 2 (*Endo*), syntaxin 1 (*Syx*), and synaptotagmin 1 (*Syt*) in cells with contacts (**a**) and in cells without contacts (**b**), dynamin 1 (*Dyn*) transcripts were detected in cells with neuronal contacts (**a**, *lane 4*) but were missing in cells without contacts (**b**, *lane 4*)

All cells were taken between day-in-vitro 5 (DIV5) and 16 (DIV16) from the culture system. In order to withdraw single cells out of the culture, fine capillaries were drawn out to give tips with a diameter of 15 µm. Capillaries were placed under microscopical control beside the chosen neurons (Fig. 1c). Single cell bodies were picked up by negative pressure by using an UltraMicroPump II (WPI) and immediately transferred into RNase protection medium (Fig. 1d).

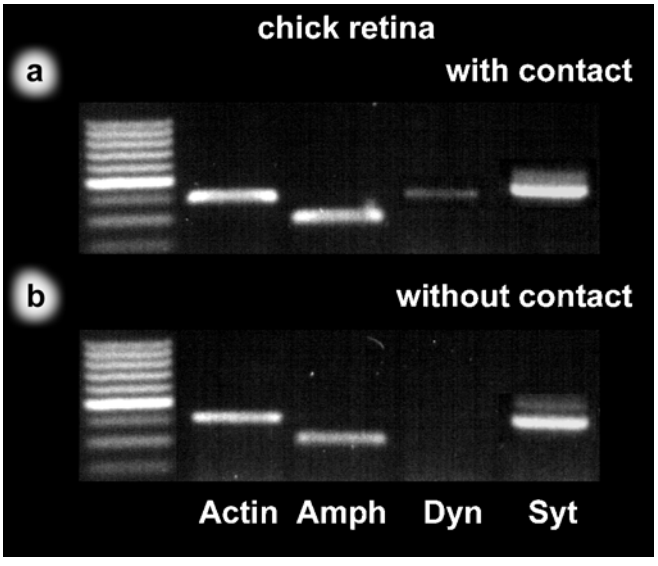

Fig. 3 Single-cell RT-PCR from chick retina (*lane 1* size marker). As found in the hippocampus, we could detect beta-actin (*Actin*), amphiphysin 1 (*Amph*), and synaptotagmin 1 (*Syt*) in cells with contacts (**a**) and in cells without contacts (**b**). Dynamin 1 (*Dyn*) could be found in neurons connected to neighboring neurons (**a**, lane 4) but was missing in cells without contacts (**b**, lane 4)

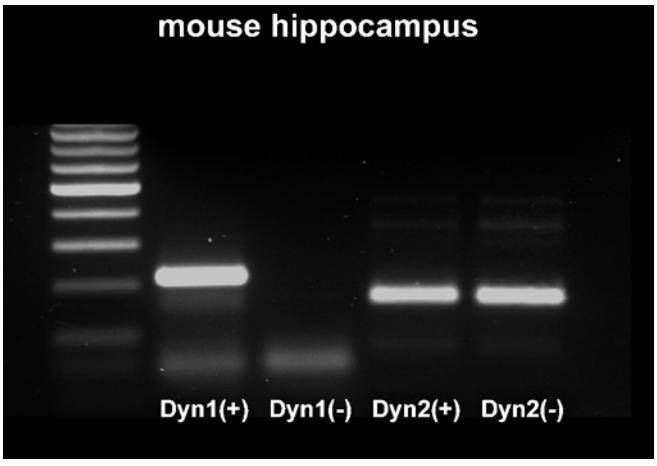

Fig. 4 Differential appearance of dynamin isoforms in mouse hippocampus (*lane 1* size marker). Mouse hippocampal cultures were used to detect differences in mRNA expression between dynamin isoforms 1 (*Dyn1*) and 2 (*Dyn2*). Neurons with established contacts to neighboring neurons (+) expressed both mRNAs (*lanes 2*, *4*). Cells without contacts (-) exhibited no transcripts for dynamin 1 (*Dyn1-*, *lane 3*), whereas transcripts for dynamin 2 (*Dyn2-*) were detectable (*lane 5*)

mRNA was extracted from these single cells and reverse-transcribed. cDNA was then amplified by two rounds of multiplex RT-PCR in the same cell for beta-actin (control), for proteins involved in endocytosis (amphiphysin 1, dynamin 1, alpha-adaptin, endophilin 2), and for proteins involved in exocytosis (syntaxin 1, synaptotagmin 1). In the chick retina, we tested for beta-actin, amphiphysin 1, dynamin 1, and synaptotagmin 1.

In the mouse hippocampus, all cells with established contacts exhibited mRNAs for all tested endo- and exocytic proteins (Fig. 2a). The results for cells grown in isolation were similar for most of the protein mRNAs, which were abundant in these cells. In contrast, in isolated neurons without a neuronal network, we were never able to detect a signal for dynamin 1 (Fig. 2b, lane 4).

The results from cultures of chick retina cells were identical to those from the mouse hippocampus. Cells with established contacts revealed signals for all tested mRNAs (Fig. 3a), whereas cells lacking contacts to neighboring cells were devoid only of dynamin 1 mRNA (Fig. 3b, lane 4).

In order to determine the differential expression of dynamin isoforms 1 and 2, we investigated their presence in the mouse hippocampal system. Interestingly, we found that dynamin 2 was expressed in neurons with and without synaptic contact (Fig. 4, lanes 3, 4), whereas the data for dynamin 1 was identical to that described above (Fig. 4, lanes 1, 2).

To verify the data obtained by RT-PCR at the protein level, we investigated the expression of proteins by using the immunocytochemical double-fluorescence technique. The analysis of the distribution for synaptotagmin 1 and dynamin 1 in the mouse hippocampus revealed that the pattern of immunostaining was strictly stage-dependent. Early developmental stages (DIV5) revealed an immunosignal for synaptotagmin 1 in the cell body and neuronal

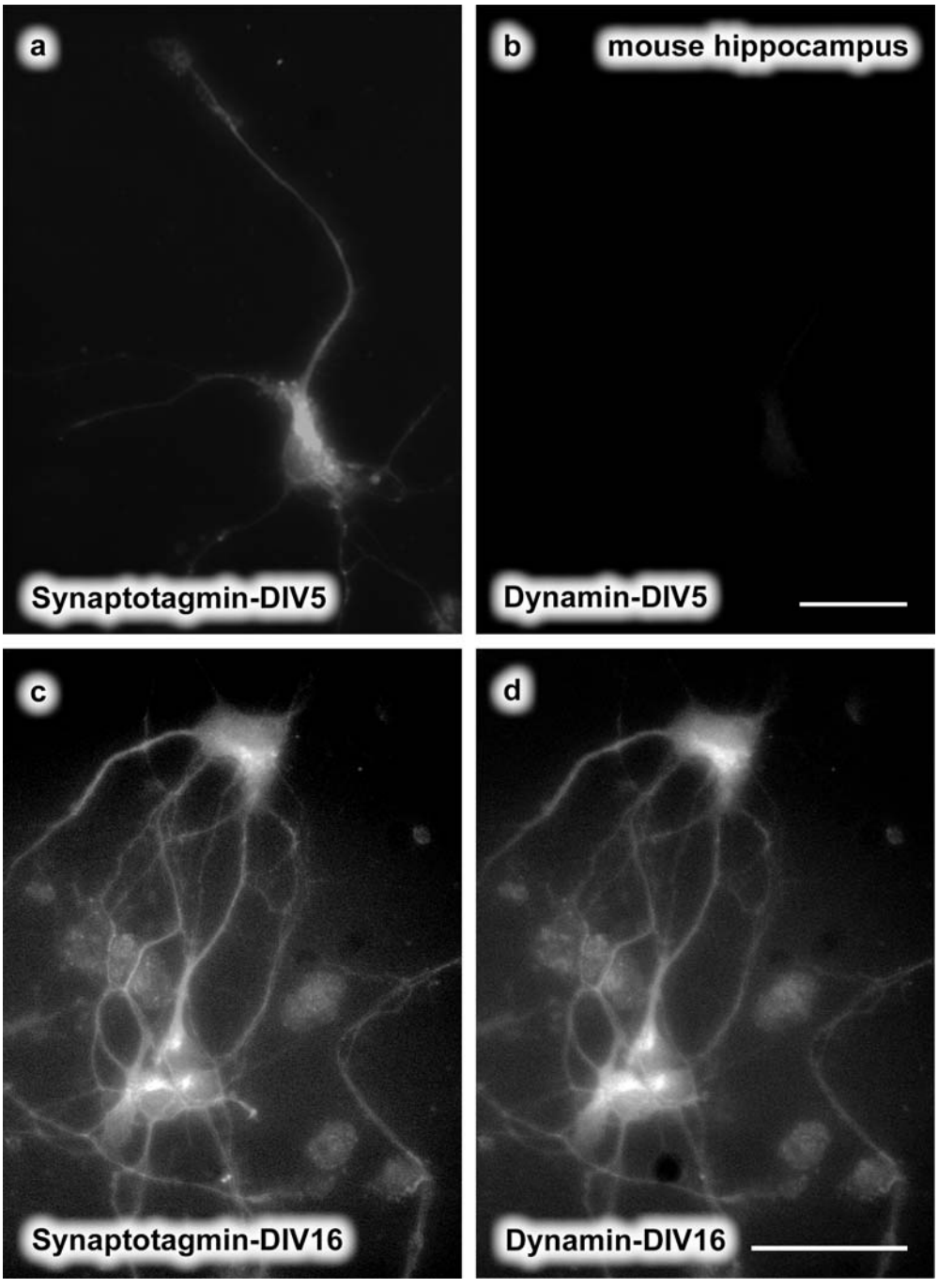

Fig. 5 Immunostaining of mouse hippocampal cultures. Mouse hippocampal cultures express synaptotagmin 1 (**a**) in the cell body and processes from early stages of development (*DIV5* day-in-vitro 5), but no dynamin 1 (**b**) can be detected at this stage. Later stages (*DIV16*) exhibit a similar immunostaining for synaptotagmin 1 (**c**) and for dynamin 1 (**d**). *Bars* 20 μm

processes, whereas dynamin 1 was not detectable (Fig. 5a, b). If cells were kept in culture for more then 2 weeks (DIV16) and developed a neuronal network, double-staining showed a similar expression pattern for synaptotagmin 1 and dynamin 1 in the cell bodies and neuronal processes (Fig. 5c, d). In agreement with the data from the mouse hippocampus, we found a stage-dependent expression pattern of SNAP-25 and dynamin 1 in the chick retina. Again, early developmental stages (DIV6) were immunostained for SNAP-25, a protein known as t-SNARE in the presynaptic terminal, and were devoid of dynamin 1 (Fig. 6a, b). Cells that were grown in culture for longer times (DIV15) and that had visible contacts to adjacent cells showed equivalent immunoreactivity for SNAP-25 and dynamin 1 in the same neurons (Fig. 6c, d). At the ultrastructural level, we found dynamin 1 localized throughout the presynaptic terminal in DIV 13 neurons with staining of synaptic vesicles and the plasma membrane (Fig. 7).

Taken together, our results reveal that exocytic protein mRNA is present in all investigated cells at all stages. Data from the chick retina and the mouse hippocampus show that mRNA transcripts are also present for endocytic proteins (chick: amphiphysin 1; mouse: amphiphysin 1, alpha-adaptin, and endophilin 2) in all preparations. Dynamin 1 has however only been detected in cells with established neuronal contacts, whereas dynamin 2 has been found in

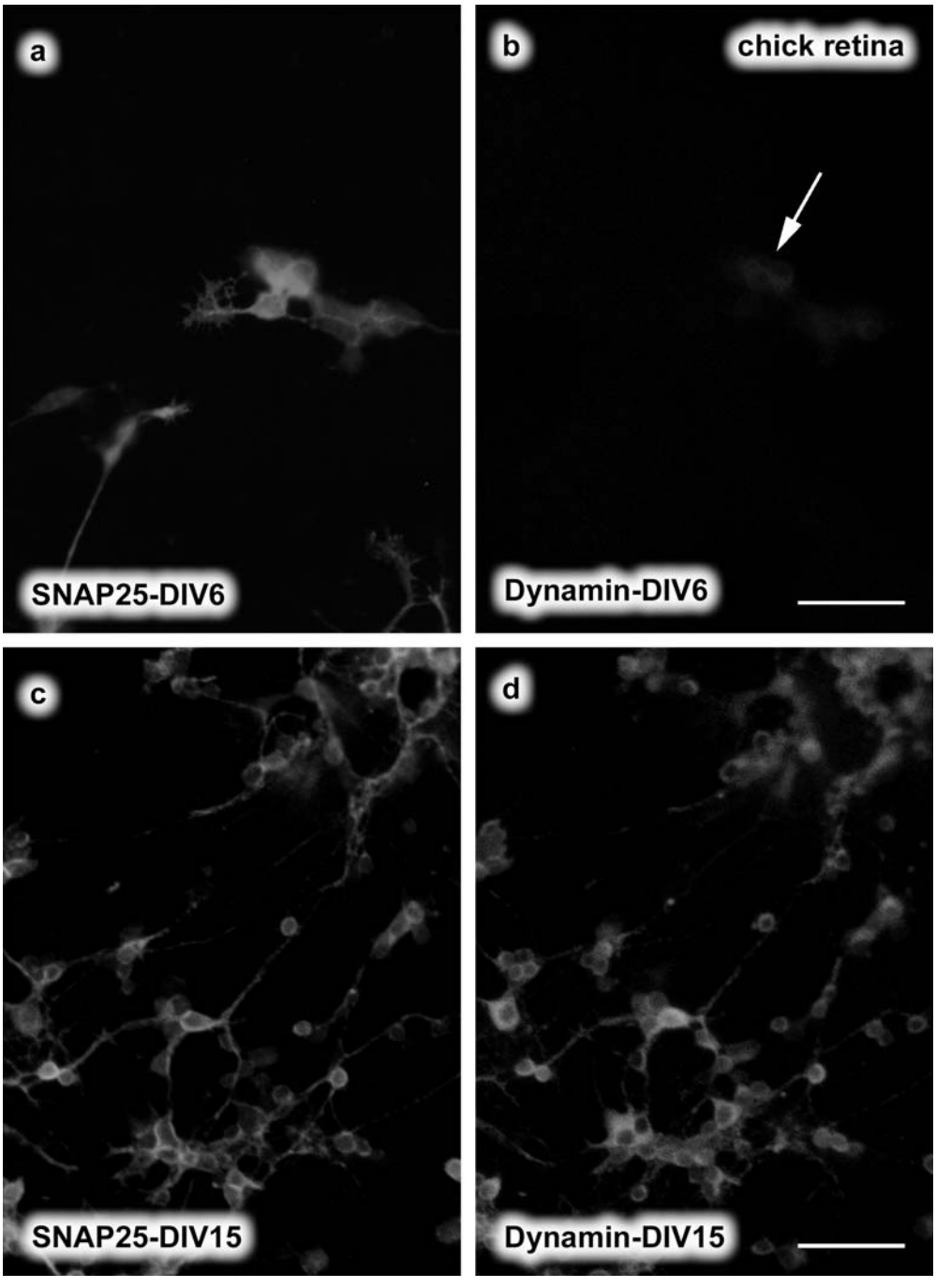

Fig. 6 Immunostaining of chick retinal cultures. Cultures taken from chick retina express SNAP-25 (**a**) in the cell body and processes from early stages of development (*DIV6*). Only faint staining of dynamin 1 (**b**) can be detected in cell clusters (*arrow*) but none in isolated neurons or growth cones at this stage. Later stages (*DIV15*) exhibit similar immunostaining for SNAP-25 (**c**) and for dynamin 1 (**d**). *Bars* 50 μm

all the investigated cells from our mouse hippocampal cultures.

Discussion

The data from this study reveal two main aspects of neuronal development. On the one hand, single-cell multiplex RT-PCR has detected that all tested mRNAs, with the exception of dynamin 1, are abundant even at early stages of neuronal development. On the other hand, dynamin 1 has only been detected once neurons have established connections to neighboring cells, whereas the ubiquitously expressed dynamin isoform 2 is detectable before synapse formation.

In addition to the active reconstruction of microfilaments (Letourneau 1996; Tseng and Wirtz 2004), early stages of neuronal maturation and process outgrowth are characterized by the incorporation of newly synthesized membrane (Ahmari et al. 2000; Zakharenko and Popov 2000), which is transported as so-called constitutive vesicles to the growth cone. It remains a matter of discussion whether vesicles for the enlargement of the membrane surface are identical to the synaptic vesicles that are found in mature synapses for the release of transmitters (Leoni et al. 1999; Grundschober et al. 2002). Our results are of relevance to

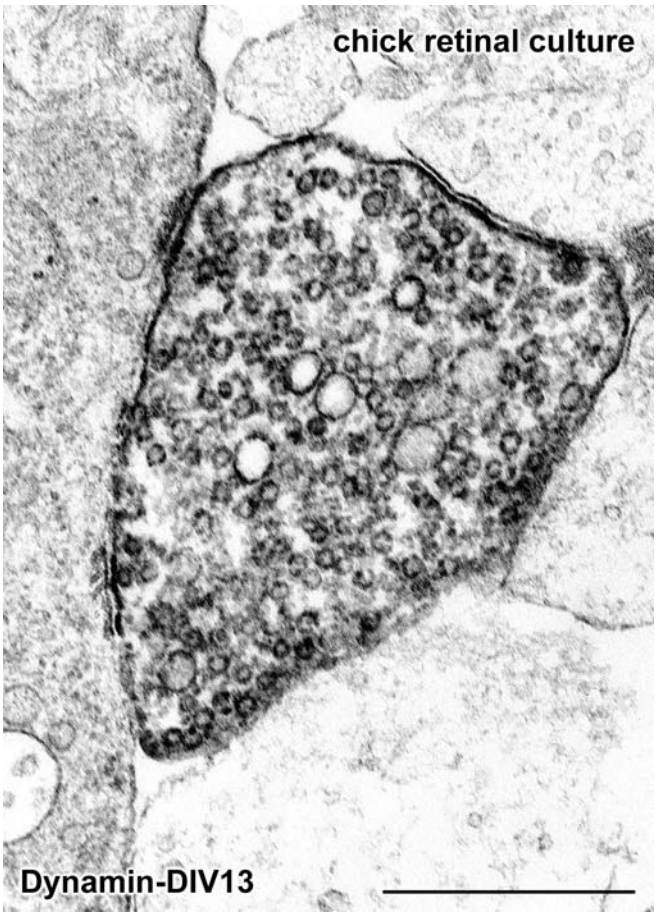

Fig. 7 Ultrastructural distribution of dynamin 1 in chick retinal cultures. Immunoelectron microscopy reveals that dynamin 1 is localized throughtout the presynaptic terminal in DIV13 neurons. Synaptic vesicles and the plasma membrane are heavily stained for dynamin 1. *Bar* 1 μm

this debate and to findings that have revealed the existence of spontaneous exocytosis during neurite extension along the processes (Gao and Van den Pol 2000). We have found that most of the tested proteins, including the plasma membrane anchors (t-SNAREs) syntaxin 1 and SNAP-25, are expressed at these early stages, and thus it is tempting to speculate that constitutive and synaptic vesicles share a similar set of membrane proteins.

We have also found that dynamin 1 mRNA and protein are not detectable in any of the tested cells from the two studied culture systems before the establishment of synaptic contacts. Interestingly, the results are strictly stage-related (contacts to other neurons being present or absent), depending on the density of neurons, and are not time-dependent. As we have shown previously in the retinotectal system of the chick in situ, a significant delay occurs between the onset of the expression of exocytic proteins and that of endocytic proteins (Bergmann et al. 1999, 2000; Grabs et al. 2000). Shortly after synapse formation, however, endocytic proteins are upregulated to fulfill their role in the recycling of membrane from the plasma membrane back to the presynaptic terminal (Südhof 2004). We have found, by studying single cells, that amphiphysin 1 mRNA is available in isolated neurons, but that dynamin 1 mRNA is first detectable after synaptogenesis with neighboring cells. Previous studies have shown that the blocking of dynamin by mutant isoforms or by peptides leads to an arrest in endocytosis (Shupliakov et al. 1997; Damke et al. 2001), but that membrane recycling occurs earlier (Matteoli et al. 1992; Kraszewski et al. 1995). Our data thus exclude that dynamin 1 is part of the machinery of membrane recycling during neurite growth. However, dynamin 2 is expressed before synapse formation.

The appearance of dynamin 1 mRNA and the presence of known components of endocytosis thus mark a time-point at which neurites stop growing and start recycling their plasma membrane. Our data have revealed that dynamin 1 is specific for the regulated recycling of synaptic vesicles after synapse formation, whereas dynamin 2 may act as the constitutive counterpart of dynamin 1.

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