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MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

Filopodial protrusions induced by glycoprotein M6a exhibit high motility and aids synapse formation

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Abstract

M6a is a neuronal membrane glycoprotein whose expression diminishes during chronic stress. M6a overexpression in rat primary hippocampal neurons induces the formation of filopodial protrusions that could be spine precursors. As the filopodium and spine motility has been associated with synaptogenesis, we analysed the motility of M6a-induced protrusions by time-lapse imaging. Our data demonstrate that the motile protrusions formed by the neurons overexpressing M6a were more abundant and moved faster than those formed in control cells. When different putative M6a phosphorylation sites were mutated, the neurons transfected with a mutant lacking intracellular phosphorylation sites bore filopodia, but these protrusions did not move as fast as those formed by cells overexpressing wild-type M6a. This suggests a role for M6a phosphorylation state in filopodium motility. Furthermore, we show that M6a-induced protrusions could be stabilized upon contact with presynaptic region. The motility of filopodia contacting or not neurites overexpressing synaptophysin was analysed. We show that the protrusions that apparently contacted synaptophysin-labeled cells exhibited less motility. The behavior of filopodia from M6a-overexpressing cells and control cells was alike. Thus, M6a-induced protrusions may be spine precursors that move to reach presynaptic membrane. We suggest that M6a is a key molecule for spine formation during development.

Introduction

Neuronal remodeling is a fundamental process by which the brain responds to environmental influences, e.g. during stress (de Kloet *et al.*, 2005; McEwen, 2005; Krishnan & Nestler, 2008). In the hippocampus, chronic stress causes dendrite retraction in CA3 pyramidal neurons (Fuchs & Flugge, 1998). Dendritic spines are small protrusions from the main dendritic stalk that form the postsynaptic component of most excitatory synapses and have an important role in higher brain functions, such as learning and memory (Hering & Sheng, 2001). During development, dendritic filopodia – which are thought to be spine precursors – show high motility and their number correlate inversely with the onset of more stable spines and synapses (Dailey & Smith, 1996; Ziv & Smith, 1996; Fiala *et al.*, 1998; Dunaevsky *et al.*, 1999; Okabe *et al.*, 2001). These observations led to the hypothesis that filopodia may initiate synaptogenesis by extending themselves towards axons and, subsequently, stabilizing the resulting connections into mature synapses (Goda & Davis, 2003). Dendritic filopodia transiently extend and retract, probably, to maximize the chance encounter between a developing axon and a target dendrite. In general, spine density seems to be maintained by an 'optimal' level of synaptic activity. Thus, spine density increases when synaptic activity is insufficient and diminishes with excessive stimulus (Hering & Sheng, 2001; Calabrese & Halpain, 2005).

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We have recently identified *M6a* as a stress-responsive gene in the hippocampal formation. This gene is downregulated in the hippocampus of both socially and physically stressed animals, and this effect can be reversed by antidepressant treatment (Alfonso *et al.*, 2004a, 2006). M6a is a membrane glycoprotein abundantly expressed in neurons of the CNS, especially in the hippocampus (Yan *et al.*, 1996). M6a overexpression in rat hippocampal primary cultures induces neurite formation and a higher filopodium/spine density, while M6a-decreased expression reduces protrusion number (Alfonso *et al.*, 2005b).

M6a gene codes for a 278-amino acid transmembrane glycoprotein with four transmembrane domains and the N- and C- termini located in the cytoplasm. Transmembrane domains form one intracellular and two extracellular loops. There are several putative phosphorylation sites for casein-kinase 2 (CK2) in both loops, and for protein kinase C (PKC) in the N- and C- termini. In accordance, treatment of PC12 cells with PKC inhibitors blocks the M6a effect in Ca^{2+} increment induced by nerve growth factor (Mukobata *et al.*, 2002). Calcium modulates the activity of many proteins implicated in neurite, filopodium and spine formation (Cullen & Lockyer, 2002; Jourdain *et al.*, 2003; Colbran & Brown, 2004); therefore, putative intracellular phosphorylation sites in M6a could be involved in this pathway.

As mentioned, M6a is involved in filopodium formation; however, whether M6a plays a role in synaptogenesis remains to be tested. Thus, the aim of this work was to analyse the motility of M6a-induced protrusions by time-lapse microscopy and to examine the role of M6a

phosphorylation state in filopodium motility. Furthermore, in an effort to elucidate whether M6a-induced protrusions may be spine precursors and facilitate synaptogenesis, the stabilization of the filopodial protrusions upon contact with presynaptic region was explored.

Materials and methods

Animals

Animals used were Sprague–Dawley rats maintained at Facultad de Ciencias Veterinarias (Buenos Aires, Argentina). All animal procedures carried out in this study were in accordance with the guidelines laid down by the NIH regarding the care and use of animals for research.

Cell culture

Hippocampal primary cultures were established from 19-day-old fetal Sprague–Dawley rat hippocampi as described previously (Brocco *et al.*, 2003). Rats were killed with CO_2 followed by cervical dislocation. Immediately, fetuses were removed by cesarean section and decapitated. Hippocampi from all fetuses dissected were pooled to obtain a single-cell suspension. Tissue was treated with 0.25% trypsin (Gibco®; Invitrogen Life Science, Carlsbad, CA, USA) in Hank's solution for 15 min at 37°C. Then, the cell suspension was prepared by dissociation with a narrow polished Pasteur pipette in 10% horse serum-supplemented Neurobasal medium (Gibco®; Invitrogen Life Science) with 4.5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were seeded at a density of 20 000 cells/cm^2 on 35-mm glass-bottom Petri dishes with a 10-mm micro well (MatTek, Ashland, MA, USA) coated with 0.1 mg/mL poly-L-lysine hydrobromide (Sigma, St Louis, MO, USA) and 20 μg/μL laminin (Gibco®; Invitrogen Life Science). After 2 h, medium was changed to Neurobasal medium supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 g/L ovalbumin and B27 serum-free supplements from Gibco®, Invitrogen Life Science.

Cell transfections

Neurons were transfected 4, 7 or 8 days after plating. Transfections were carried out with Lipofectamine 2000 (Gibco®; Invitrogen Life Science), as described elsewhere. Briefly, 10 μg of Qiagen-purified DNA (Qiagen GmbH, Germany) was complexed with 5 μL of Lipofectamine per 35-mm dish, for 20 min in OptiMEM, and then cells were incubated with transfection mix for 4 h at 37°C. Finally, the media was changed and replaced by minimal Eagle's medium without phenol-red supplemented with 4.5 g/L glucose, 2 mM glutamine, 10 μM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, 25 mM HEPES, 1 g/L ovalbumin and B27 serum-free supplements from Gibco®, Invitrogen Life Science. Cells were transfected with the following constructions: enhanced green fluorescent protein (pEGFP-C1; from Clontech, Mountain View, CA, USA), M6a coding sequence fused to GFP C-terminus (GFP-M6a; Alfonso *et al.*, 2005b), non-phosphorylatable M6a fused to GFP C-terminus (GFP-M6a-no-P-EC and GFP-M6a-no-P-IC), proteolipid protein fused to GFP C-terminus (GFP-PLP) and synaptophysin coding sequence fused to red fluorescent protein C-terminus (RFP-syn; pDsRed-Monomer-C1, Clontech).

Imaging and image analysis

Imaging was performed using a Nikon TE2000U inverted microscope enclosed within an incubator system set at 37°C (Solent Scientific, Segensworth, UK). A 1.4 NA 60× Plan Apo objective from Nikon was used for all experiments. Rapid switching of fluorescent wavelengths was accomplished by excitation and emission filter wheels (EGFP, excitation 470/20, emission 525/40; DsRed2, excitation 565/25, emission 620/60) used in combination with the 86007bs multiband dichroic mirror (Chroma Technology, Brattleboro, VT, USA). Epifluorescence Smart shutter and filter wheels were controlled by the Lambda 10-B controller (Sutter Instrument, Novato, CA, USA). Images were acquired with an Orca-AG cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) using 1×1 binning. Under our experimental conditions, no significant photobleaching was detected. All peripherals were controlled with METAMORPH 6.3r0 software (Molecular Devices Downingtown, PA, USA). Exposure time was 0.5–1.0 s per frame. Images were captured at intervals of 30 s, resulting in 21 frames per stack. The stacks were mounted into videos using METAMORPH software.

During imaging, few protrusions could move out of the focus due to the drift in *z*-dimension. These protrusions were not analysed. We made a stack for each neuron and 15–20 protrusions per neuron were analysed. Images were analysed for changes in tip position and length of each protrusion. To measure protrusion motility, we used the Track Points command from METAMORPH. The different protrusion tip (dashed circles in inserts from Fig. 1B) positions were tracked with respect to a defined origin, i.e. the point in the neurite stalk from where each protrusion emerges (arrowheads in inserts from Fig. 1B). To do so, we added a red cross over the protrusion tip in each frame. Figure 1B shows crosses from each image of the stack overlaid to denote protrusion motility. For a non-motile filopodium, the crosses clustered in a small area around the protrusion tip. By contrast, a typical motile protrusion shows a scattered distribution of the crosses. Protrusions clearly emerging from the dendritic shaft, irrespective of apparent shape, were measured.

For motility analysis, we measured the protrusion length in each time point and we then calculated two types of measurements: motility and length motility index (LMI). Motility estimated movement magnitude and was calculated as the accumulated length change divided by duration of video ($M = \sum|x_n - x_i|/10$; where x_n is the length for protrusion x in frame n, and x_i is the length for the same protrusion in the first frame). LMI specifically measured the extension retraction motility (Konur & Yuste, 2004). For calculating the LMI, the smallest length was subtracted from the maximum length and divided by the average length of the protrusion during the video. For comparisons, LMI values were related to GFP (GFP LMI = 1).

Statistics

Experiments were done in triplicate or duplicate. Each replica was a completely independent experiment. Thus, for each replica hippocampi dissected from all fetuses from one or two pregnant rats were disaggregated to obtain a single-cell suspension. This cell suspension was used to prepare one plate per plasmid. Then, five-seven transfected cells per plasmid were analysed by time-lapse. Finally, 15–20 protrusions per cell were recorded by time-lapse imaging. Comparison of the number of motile filopodia, LMI and motility for protrusions induced by the different proteins overexpressed was performed using non-directional paired Student's *t*-tests (GFP vs. M6a) or one-way repeated-measures ANOVA followed by *post hoc* analysis with a Dunnet test when comparing M6a mutants. Significance was accepted at $P \leq 0.05$. Values are expressed as mean ± standard error. All statistics calculations were done with the software Analyse-it® for Microsoft Excel (Analyse-it Software, England, UK).

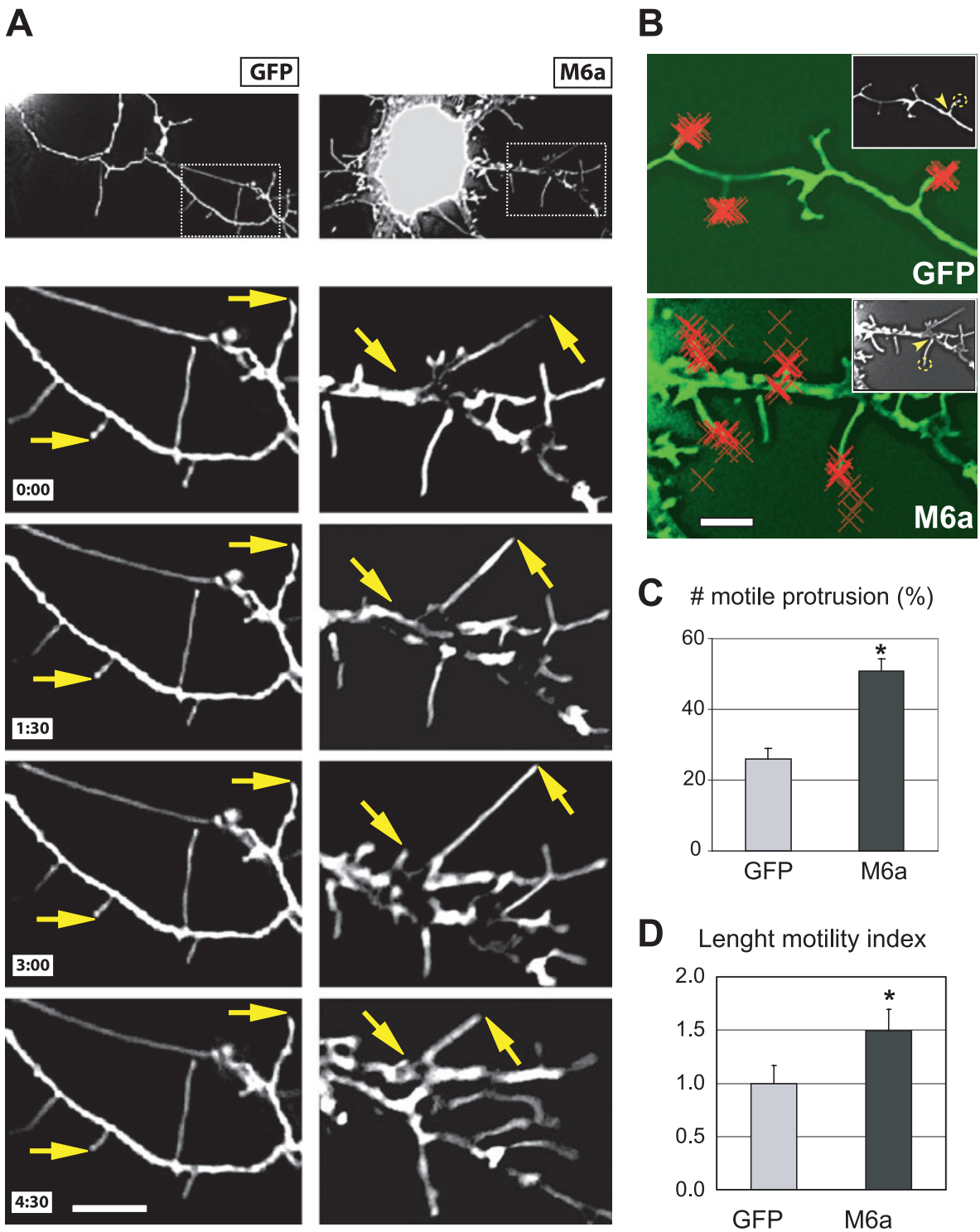

FIG. 1. Membrane glycoprotein 6a (M6a)-transfected cells exhibited more motile filopodia. (A) Images of 4 days-*in vitro* (DIV) neurons transfected at 3 DIV with green fluorescent protein (GFP)-M6a and GFP are shown. Boxed regions from upper pictures are shown in the lower panel at higher magnification. Time-lapse images are shown as a time sequence of representative frames. Arrows track different filopodia. Note protrusions in the M6a-expressing cell appearing disappearing and extending retracting within the lapse shown (4 min 30 s), in contrast to almost 'stable' filopodia in the GFP-transfected cell. Pictures were taken every 90 s with a 60× lens. (B) Tracking filopodium movement between planes in an image stack. Neurites from neurons transfected with GFP or GFP-M6a are shown. Dashed circles indicate protrusion tip and arrowheads show defined origins. Protrusion tip position in each image was marked with a red cross. Crosses from each image of the stack were overlaid to denote protrusion motility. Note that in the GFP-transfected cell, crosses clustered in a small area around the protrusion tips. By contrast, motile protrusions from the GFP-M6a transfected neuron show a scattered distribution of the crosses. (C) Number of motile filopodia in 4-days-primary cultures transfected with GFP-M6a and GFP is presented as percentage of total filopodia per cell (*M6a vs. GFP, $t_{35} = 4.97$, $P = 0.0077$). (D) LMI was calculated by subtracting the smallest protrusion length from the longest and divided by the average length of the protrusion during the video (*M6a vs. GFP, $t_{192} = 3.69$, $P = 0.0003$). Mean ± standard error are shown. Scale bars: 20 μm (A) and 10 μm (B).

Results

Previous studies from our laboratory showed an increase of 100% in filopodium/spine density in cells overexpressing GFP-M6a (Alfonso *et al.*, 2005b). Because those analyses were done with fixed cells it was not possible to evaluate protrusion motility. In this work, we analysed M6a-induced filopodium motility in living transfected cells. Primary hippocampal neurons were transfected after 3 days in culture and imaged 24 h later. Time-lapse recordings showed higher filopodium motility in neurons transfected with GFP-M6a than in GFP-expressing cells. Videos of these protrusions can be found in the Supporting information (Videos S1–S4). Figure 1A shows image series for neurons transfected with M6a-GFP or GFP as control. Arrows indicate filopodia. In control cells, most protrusions did not change their position within the period shown. In contrast, many protrusions from M6a-overexpressing cells moved, disappeared and appeared, extended and/or retracted during the same time. To quantify these results, we first analysed the number of motile filopodia in M6a- or GFP-overexpressing cells. Filopodia whose tip changed its position at least once during the time-lapse recorded were classified as motile. About 25% of filopodia from GFP-transfected cells changed their position along 10 min imaging. In the case of the M6a-transfected neurons, more than 50% of the filopodia moved at least once during the same period ($t_{35} = 4.97$, $P = 0.0077$; Fig. 1C). Then, to study motility, filopodium movement was tracked and an example is shown with red crosses in Fig. 1B. To analyse the filopodium motility degree (for details, see Materials and methods), we did an analysis called 'LMI'. This index indicates the degree of filopodium extension and retraction. Results showed that filopodia formed by M6a overexpression extend and retract more than those formed in GFP-control neurons (1.50 ± 0.20 vs. 1.00 ± 0.17; $t_{192} = 3.69$, $P = 0.0003$; Fig. 1D). Altogether, these data indicate that M6a not only increases filopodium density but that most filopodia are mobile as compared with protrusions in control cells. Moreover, M6a-induced protrusions exhibited higher motility than filopodia formed in control cells. An additional control experiment was carried out to show that changes in cell morphology were due to M6a protein itself and not to the use of fusion proteins. We analysed the effect of the proteolipid protein (PLP), which belongs to the family of proteolipid proteins as well as M6a. PLP was also fused to GFP. We first examined the overall morphology in cells overexpressing the fusion protein. PLP-transfected neurons exhibit a similar morphology to GFP-transfected ones and greatly differ to those transfected with M6a (Fernandez *et al.*, 2009; supporting Fig. S1A). Next, we studied the motility of filopodia protruding from PLP-overexpressing neurons. Supporting Fig. S1B, and supporting Video S5 show that protrusion motility in PLP-transfected cells is comparable to the motility of protrusions from GFP-transfected neurons.

M6a downstream signaling is not known. Therefore, we studied if phosphorylation could regulate filopodium motility. Comparison of the modeled structure and the primary sequence of M6a with that of members of the tetraspanin protein family allowed the identification of many putative sites for different posttranslational modifications. Among them, there are several putative intra- and extracytoplasmic motifs for PKC and CK2 phosphorylation. Given the possibility that M6a may transmit its signal through changes in its phosphorylation state, we analysed mutated M6a lacking the putative intra- or extracellular phosphorylation sites. Using site-directed mutagenesis we constructed two M6a mutants in which threonine and serine residues at indicated positions were replaced by alanine to create non-phosphorylatable forms of M6a. Mutant GFP-M6a-no-P-EC has point mutations at the key amino acid residues for putative CK2 motifs from major extracellular loop (i.e. T166, T184, T193 and S195). To obtain mutant GFP-M6a-no-P-IC, we mutated the two putative intracellular PKC sites and the CK2 site (i.e. e. T10, S256, S267 and T268). Expression of both mutants in primary hippocampal neurons did not affect M6a localization in the plasma membrane nor its ability to promote filopodium formation (Fig. 2A and B). However, they differentially affected protrusion motility. Filopodia induced by GFP-M6a-no-P-EC mutant moved with similar velocity to those induced by wild-type M6a (Fig. 2C), suggesting that CK2 sites from major extracellular loop may not be involved in protrusion motility. On the other hand, protrusions formed by GFP-M6a-no-P-IC mutant did not move as much as those induced by the wild-type M6a protein (16.3 ± 2.3 vs. 9.1 ± 1.5 μm/min, $F_{3,270} = 7.56$, $P = 0.0001$; Fig. 2C). These results suggest that intracellular phosphorylatable amino acid residues could be relevant for M6a function in filopodium motility.

Finally, we investigated M6a-induced filopodia as putative postsynaptic partners for presynaptic synaptophysin-overexpressing processes. Therefore, we asked if motile M6a-induced protrusions could be stabilized upon contact and mature into synaptic spines. To study this, 8 days-*in vitro* cultures were transfected with plasmids expressing synaptophysin fused to red fluorescent protein (RFP-syn) and, 24 h later, the same culture was transfected with plasmids expressing control GFP or GFP-M6a fusion protein. Transfections with the different constructs were done on different days to avoid co-transfection, i.e. so that cells were transfected with RFP-syn or GFP plasmids, but not with both of them. We analysed the LMI of protrusions (green structures) that were (arrowheads, Fig. 3A and A') or were not (arrows) in apparent contact in the plane of focus with synaptophysin-overexpressing cells (red processes). Experimental results are also shown in the supporting Videos S6 and S7. The motility of GFP-induced protrusions that contacted synaptophysin-expressing cells was lower than the motility of those protrusions not contacting them (1.0 ± 0.17 vs. 0.44 ± 0.06, $t_{40} = 2.72$, $P = 0.09$; Fig. 3B, gray bars). Interestingly, M6a-induced filopodia exhibited a similar behavior (0.90 ± 0.27 vs. 0.40 ± 0.12, $t_{35} = 2.72$, $P = 0.04$; Fig. 3B dark bars) to those naturally formed in control cells (M6a vs. GFP, $F_{1,98} = 0.37$, $P = 0.5448$). Reduced motility in M6a-induced protrusions that touched synaptophysin-overexpressing processes suggests that protrusions induced by M6a could be stabilized upon contact with the presynaptic zone.

Discussion

M6a forms highly motile protrusions

We have recently identified *M6a* as a stress-responsive gene. This gene is downregulated in its expression in the hippocampus of both socially and physically stressed animals, and this effect can be reversed by antidepressant treatment (Alfonso *et al.*, 2004a,b, 2006). Moreover, we showed that M6a is a key modulator for neurite outgrowth and filopodium/spine formation (Alfonso *et al.*, 2005b). In order to study if protrusions induced by M6a could induce synapse formation, in this work we analysed filopodial motility by time-lapse imaging. The obtained results show that protrusions formed by M6a could be the site for future synapses during development. Reduction in motility, when M6a cannot be phosphorylated, suggests that filopodium motility might be regulated by phosphorylation or dephosphorylation events.

M6a expression in the adult brain has been observed in axonal fibers (Cooper *et al.*, 2008). However, in fetal rat hippocampal primary culture, endogenous M6a as well as overexpressed M6a is

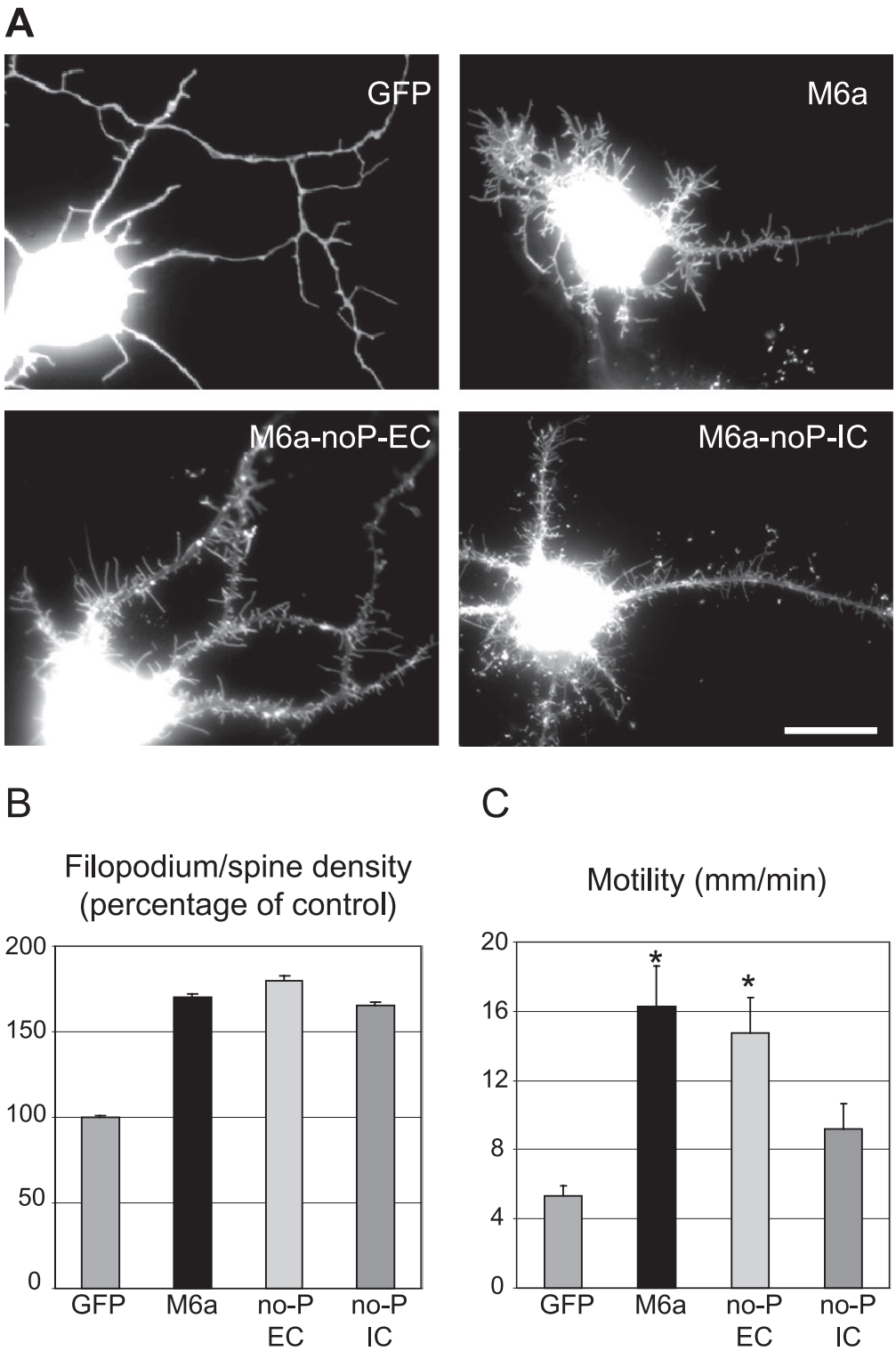

FIG. 2. Intracellular membrane glycoprotein 6a (M6a) phosphorylation sites are required for protrusion motility. Hippocampal primary cultures were transfected with green fluorescent protein (GFP), wild-type M6a, M6a-no-P-EC or M6a-no-P-IC (mutants M6a lacking putative extra- or intracytoplasmic phosphorylation sites, respectively). (A) Like wild-type M6a, M6a-no-P-EC and M6a-no-P-IC induce filopodium formation in neurons. (B) Protrusion density (number of protrusions per 20 μm neurite length) was quantified. Values are expressed as a percentage of control, and the mean ± SEM of 40–60 neurites per group from a representative experiment is shown. None of the mutations changed M6a ability to form protrusions. (C) Filopodium motility was measured and means ± standard errors are shown. Protrusions formed in neurons transfected with M6a-no-P-EC moved similarly to those induced by wild-type M6a (*M6a and M6a-no-P-EC vs. GFP, $F_{3,270} = 7.56$, $P = 0.0001$). Significant differences were determined using one-way ANOVA followed by Dunnet's *post hoc* test. M6a-no-P-IC induces the formation of filopodia that do not move as those induced by wild-type M6a. Scale bar: 20 μm.

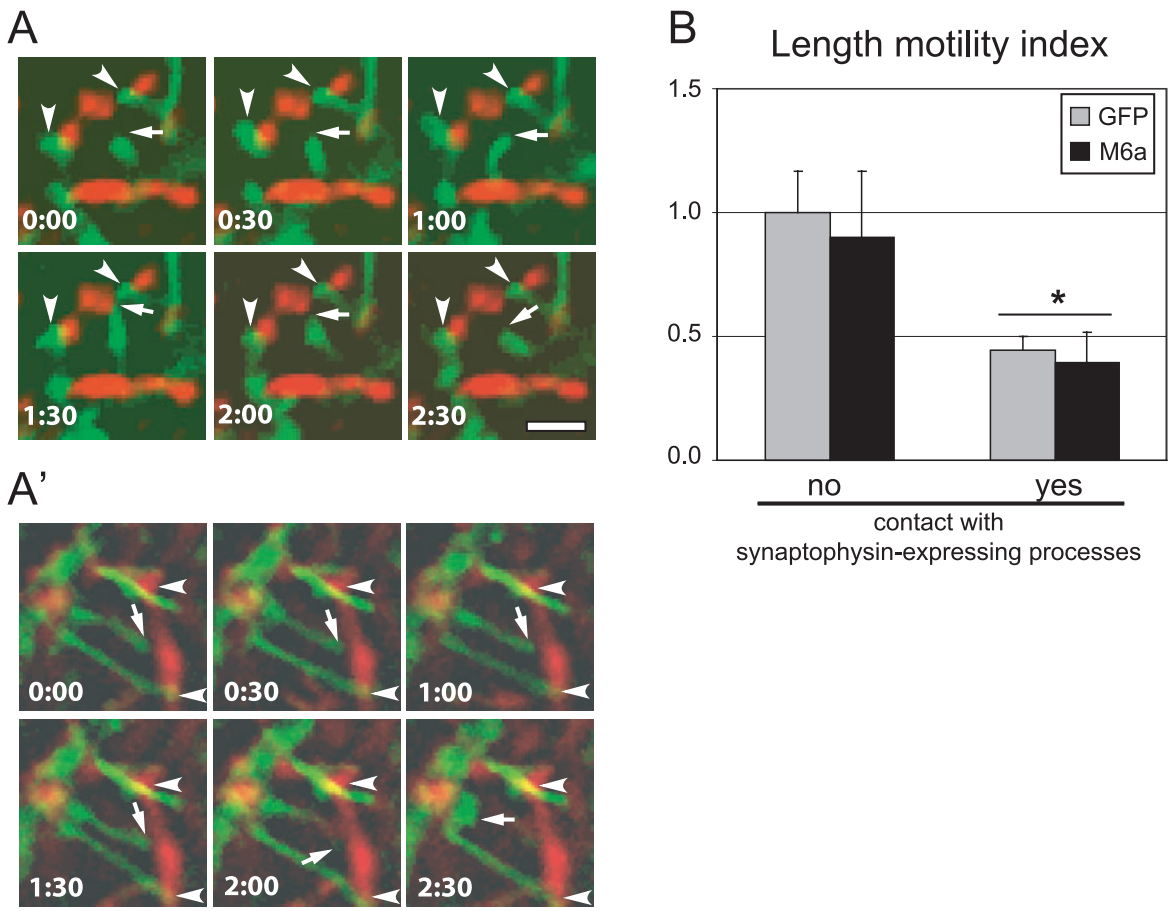

FIG. 3. Protrusions contacting with presynaptic membrane display less motility. (A and A') Filopodium motility in cells expressing green fluorescent protein (GFP)-membrane glycoprotein 6 (M6a; green) was analysed for protrusions that were next to processes expressing RFP-synaptophysin (red). Two representative fields are shown. Arrows indicate a motile protrusion and arrowheads point to stable spines. (B) Motility quantification for GFP- and M6a-induced protrusions in hippocampus neurons from 9 DIV is shown. LMI for spines contacting or not synaptophysin-overexpressing processes was compared (gray bars for GFP, $t_{40} = 2.72$, $P = 0.09$; dark bars for M6a, $t_{35} = 1.85$, $P = 0.05$). Protrusions that were in apparent contact with presynaptic membranes exhibited a reduced motility. Interestingly, M6a-induced filopodia exhibited a similar behavior to those naturally formed in control cells (*M6a vs. GFP, $F_{1,75} = 0.37$, $P = 0.5448$). Mean ± standard error is shown. Scale bar: 10 μm.

expressed in all neuronal extensions (Alfonso *et al.*, 2005b). Moreover, M6a-induced filopodia develop from neurites expressing MAP2, a dendritic marker (Alfonso *et al.*, 2005b), suggesting that, during development, M6a may be involved in dendritic filopodium formation. (Alfonso *et al.*, 2005b). Here, we analysed M6a overexpression effects in living neurons. Time-lapse microscopy revealed that M6a not only augmented filopodium number but it also increased about 100% the percentage of motile filopodia as compared with control cells. Because filopodium motility subserves for transient interactions with presynaptic membrane (Konur & Yuste, 2004), it has been associated to synaptogenesis. Thus, during embryonic development, M6a could be one of the several molecules involved in spine and synapse formation. Alternatively, as M6a promotes neuronal differentiation from undifferentiated embryonic stem cells (Michibata *et al.*, 2008), M6a might also participate in neuronal differentiation during neurogenesis. Throughout adulthood, environmental as well as endocrine factors regulate adult neurogenesis in the hippocampus (van Praag *et al.*, 2002), and M6a expression in newly generated neurons could contribute to spine formation in these neurons.

M6a induced not only an increase in the number of motile filopodia, but also an increase in protrusion motility. LMI, which reflects extension retraction movement, was higher for M6a-induced protrusions compared with control cells. Because spine 'health' is linked to synaptic function, altered spines in psychiatric and neurological diseases may have functional effects (Calabrese *et al.*, 2006). Because chronic stress induces spine lost (Stewart *et al.*, 2005), and given that M6a levels diminish during chronic stress and can be reverted by antidepressant treatment, the increased motility observed for M6a-induced protrusions could be relevant to reestablish spine morphology.

M6a phosphorylation state affects M6a-induced protrusion motility

M6a downstream signaling is largely undefined. M6a has several putative phosphorylation sites in the extra- and intracellular domains. Two recent phosphoproteomic studies in brain tissue (DeGiorgis *et al.*, 2005; Xia *et al.*, 2008) indicate that M6a is phosphorylated in two of those sites (S256 and S267). Treatment of PC12 cells with a PKC inhibitor eliminates the ability of M6a to promote nerve growth factor-primed neurite extension (Mukobata *et al.*, 2002). Mutation of extracellular phosphorylation sites seems not to affect filopodium formation or their motility. On the contrary, phosphorylation of the M6a intracellular sites seems to regulate motility. We analysed protrusion movement in cells transfected with a mutant M6a lacking

the three putative intracellular phosphorylation sites, two for PKC and one for CK2. Although this mutant M6a induced filopodium formation similarly to wild-type M6a, mutation to non-phosphorylatable alanine residues reduced filopodium motility, suggesting that movement is regulated by phosphorylation. Similarly, non-phosphorylatable MARCKS (myristoylated, alanine-rich C-kinase substrate), a major PKC substrate, affects spine motility (Calabrese & Halpain, 2005). Besides MARCKS, there are many other proteins involved in spine morphology regulated by phosphorylation, e.g. WAVE1 [Wiskott-Aldrich syndrome protein (WASP)-family verprolin homologous protein 1; Kim *et al.*, 2006], TrkB (Huang & Reichardt, 2003) and others that recruit intermediates in intracellular signaling cascades. In general, these proteins regulate the number and shape of spines modifying the actin cytoskeleton (Hering & Sheng, 2001; Ehlers, 2002). One of these proteins is the kinase CK2 (Canton & Litchfield, 2006). Hence, the rapid actin rearrangements involved in filopodium motility may be inhibited when M6a lacks the CK2 site in the non-phosphorylatable mutant. On the other hand, protein phosphatase-1 (PP1) that also regulates hippocampal plasticity (Hu *et al.*, 2006) may dephosphorylate M6a, thus reducing filopodium motility to allow pre- and postsynaptic components assembly.

Preliminary results from our group postulate M6a as a lipid raft-associated protein (C. Scorticati and A.C. Frasch, unpublished observations). Lipid rafts are plasma membrane platforms with a characteristic lipid composition as well as links to the cytoskeleton that mediate various cellular events such as signal transduction (Allen *et al.*, 2007). Thus, M6a phosphorylation or dephosphorylation might transmit signals within lipid rafts to activate different signaling pathways involved in actin dynamics. The Rho family of GTPases regulates different aspects of actin organization (Nakayama *et al.*, 2000; Luo, 2002), including spine motility and stability (Tashiro & Yuste, 2004). In the case of M6a-induced filopodia, it seems that Rho GTPases do not affect M6a ability to form filopodia/spines (Alfonso *et al.*, 2005b); however, these proteins may be regulating M6a-induced protrusion motility. Further work is in progress to address these issues.

M6a-induced protrusions can be stabilized by presynaptic partners

Finally, we asked if protrusions formed in M6a-overexpressing neurons could provide a cellular substrate for synaptic plasticity. Filopodia motility has been observed in dendrites as well as in axons (Konur & Yuste, 2004), therefore our observations could fit well for both processes type. In the last experiment (see Fig. 3), we focused on putative dendritic filopodium motility, as we analysed those protrusions interacting with synaptophysin-expressing processes. Previous studies from our laboratory showed a reduction in synaptophysin puncta in cultures where M6a mRNA was interfered (Alfonso *et al.*, 2005b), suggesting a role in synaptogenesis for M6a. Filopodia, which are thin and headless protrusions, are thought to be precursors of dendritic spines. Once contact with the presynaptic membrane is made, filopodia undergo rapid changes in shape and pre- and postsynaptic components can be assembled (Kayser *et al.*, 2008). Thus, a synapse can be initiated and proceed through appropriate maturational steps (Fiala *et al.*, 1998). Hence, M6a-induced protrusions were exposed to neurites overexpressing synaptophysin – a protein localized in the synaptic vesicle membrane – to analyse if an interaction with the presynaptic region could reduce filopodium motility as it was proposed for naturally formed ones. Our data showed that protrusions that were in apparent contact with presynaptic membranes exhibited a reduced motility. Moreover, comparison with filopodia formed in control cells showed no differences between M6a-induced protrusions and control ones, suggesting that M6a-induced filopodia could initiate synapse as naturally formed filopodia do. This could represent the first step to localize synaptic machinery and commence synapse formation. Synaptogenesis involves various transmembrane signaling cascades; however, the molecular targets that transduce such signals into spine structure changes remain uncharacterized. Although there are currently no known M6a ligands, our results suggest that M6a is an attractive candidate as a transmembrane synaptogenic molecule.

Genetic and environmental factors, such as stress, are involved in the etiology of many psychiatric disorders (Alfonso *et al.*, 2005a). Several neurological and psychiatric diseases exhibit dendritic spine defects (Halpain *et al.*, 2005). Those alterations comprise abnormal density and morphology of dendritic spines, synapse loss, and aberrant synaptic signaling and plasticity (Blanpied & Ehlers, 2004). As M6a is involved in filopodium formation (Alfonso *et al.*, 2005b), filopodium motility and synaptogenesis (this work) and its expression is regulated by chronic stress (Alfonso *et al.*, 2004b, 2006), our findings provide a molecular link between this environmental factor and spine morphology.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Only membrane glycoprotein 6a (M6a) increases filopodium density and motility.

Video S1. Time-lapse video of protrusions in GFP-transfected neurons. Images were acquired every 30 s for 10 min in 4 DIV neurons.

Video S2. Time-lapse video corresponding to box shown in Video S1.

Video S3. Time-lapse video of protrusions in GFP-M6a-transfected neurons. Images were acquired every 30 s for 10 min in 4 DIV neurons transfected with GFP-M6a.

Video S4. Time-lapse video corresponding to box shown in Video S3.

Video S5. Time-lapse video of protrusions in PLP fused to GFP-transfected neurons. Images were acquired every 30 s for 10 min in 4 DIV neurons.

Video S6. Protrusions contacting with synaptophysin-expressing processes display less motility (Corresponding to Fig. 3A).

Video S7. Protrusions contacting with synaptophysin-expressing processes display less motility (Corresponding to Fig. 3A').

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Abbreviations

CK2, casein-kinase 2; GFP, green fluorescent protein; LMI, length motility index; M6a, membrane glycoprotein 6a; MARCKS, myristoylated, alanine-rich C-kinase substrate; PKC, protein kinase C; PLP, proteolipid protein.

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