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International Society for Neurochemistry

Camila Scorticati Ph.D

Address: Instituto de Investigaciones Biotecnológicas, CONICET-UNSAM, Av 25 de Mayo s/n y Francia. San Martin. Buenos Aires-Argentina Tel: +54 114006-1500 ext 2118
http://www.iib.unsam.edu.ar
Email address: cscorticati@iib.unsam.edu.ar

Current Position: Assistant Professor/Researcher

Do the M6a´s phosphorylation motifs influence neuronal plasticity?

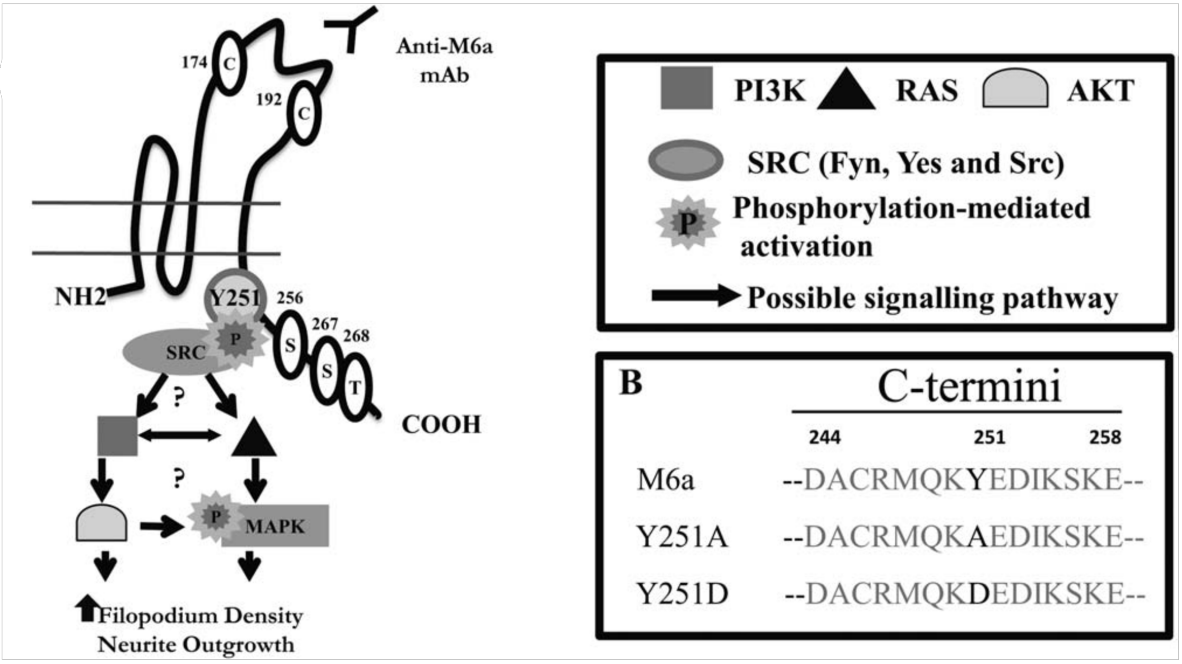
Background:

The neuronal membrane glycoprotein M6a is a member of the proteolipid protein family and is distributed mainly in neurons of the central nervous system. M6a is composed of 278 amino acids that form four transmembrane domains, two external loops, and the N- and C-terminal regions facing the cell cytoplasm. Sato and coworkers (2011) and workers in our laboratory have found that M6a induces neurite/axon outgrowth, increases filopodium/spine density, and participates in synaptogenesis (Alfonso et al., 2005a). In humans, polymorphisms in the human M6a gene GPM6A sequence have been associated with pathological situations such as schizophrenia (Boks et al., 2008), bipolar disorders (Greenwood et al., 2012), and claustrophobia (El-Kordi et al., 2013). However, the mechanism of action of M6a remains unknown. Many studies have attempted to determine the importance of M6a structure. The anti-M6a monoclonal antibody M6a-mAb, which recognizes specifically the major external loop of M6a (EC2), reduces migration of cerebellum neurons (Lindner et al., 1983). Lagenaur et al. (1992) reported that this antibody inhibits neurite extension in mouse cerebellum neurons. Accordingly, we found that the structure of EC2 is critical to induce filopodium outgrowth and synaptogenesis (Fuchsova et al., 2009) and that mutations in M6a intracellular sites, which are motifs for protein kinase C (PKC) and casein kinase-2 phosphorylation, decrease the motility of filopodia induced by M6a (Brocco et al., 2010). The zebrafish M6a ortholog, which lacks a phosphorylation site for PKC, fails to induce neurite extension in PC12 cells (Huang et al., 2011). Also, M6a depends on membrane lipid microdomain association and requires the

activity of Src kinases and mitogen-activated protein kinases (MAPK) for its filopodium induction (Scorticati et al., 2011).

Our hypothesis is that M6a receives external stimuli through its EC2 that might produce homo- or heteroassociation of M6a in specific membrane microdomains and that this association facilitates its stabilization, induces autophosphorylation in specific C-terminal residues facing the cytoplasm, and propagates the signal cascade leading to neuronal plasticity.

Huttlin et al. (2010) performed a phosphorylation study and found that the intracytoplasmic tyrosine 251 (Y251) of M6a is phosphorylated in the mouse brain. Phosphorylation of intracytoplasmic Tyr residues is specifically recognized by proteins that possess Src homology domains (SH2; Lim et al., 2009), such as the Src kinases. Phosphatidylinositide 3-kinases (PI3K), in addition to the Src kinases, are involved in cell growth, proliferation, and differentiation (Cantley, 2002; Kalia et al., 2004). Src is known to be able to activate PI3K in different models (Bromberg et al., 2008; Cao et al., 2009). Many PI3K functions are related to its ability to activate protein kinase B (AKT) through the SH2 domain. Moreover, downstream activation of AKT involves Ras and MAPK activation, thus promoting neuronal plasticity (Kalia et al., 2004; Duric et al., 2010).
Here we analyzed whether the phosphorylation state of M6a at the Y251 residue contributes to neurite/filopodia induction and whether Src/PI3K/AKT/Ras pathways are involved. By using phosphorylated and non phosphorylatable mutants of M6a at Y251, we determined that phosphorylation at Y251 is required for neurite extension but not for filopodia formation. Furthermore, phospho-M6a at Y251 involves Src/PI3K/ AKT activation and rescues external inhibition by anti mAb treatment.
The schematic representation of tetraspan neuronal glycoprotein M6a and the possible signaling pathways it's shown below. A: Based on the predicted computational model, M6a contains four transmembrane domains, a small extracellular loop (EC1), a short intracellular loop, a large extracellular loop (EC2), and the N- and C-terminal cytoplasmic tails. B: Partial amino acid sequence of the C-terminus of M6a and its Y251 mutants Y251A, a nonphosphorylatable form, and Y251D, a constitutively phosphorylated form.



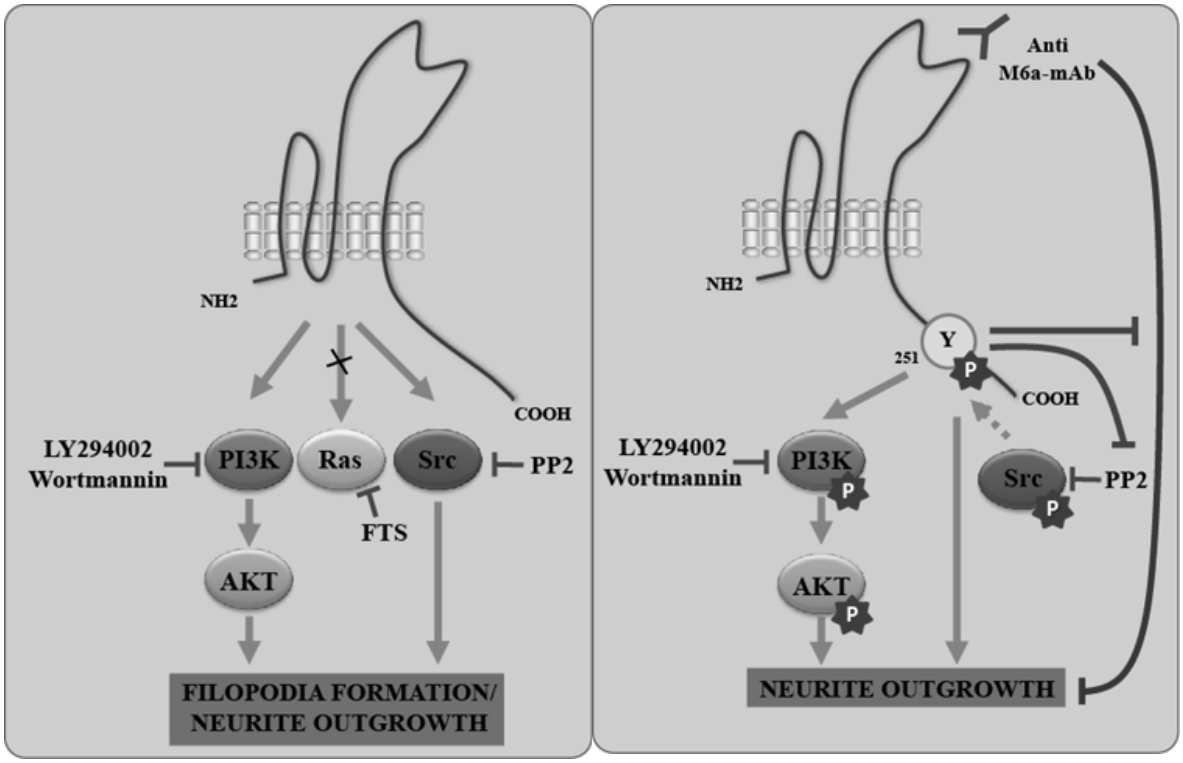
Results

In this work, we provide evidence that the phosphorylation state of Y251 at the C- terminus of M6a is a key regulator of neurite outgrowth in N2a cells and in primary cultures of hippocampal neurons. Like M6a, the constitutively phosphorylated form of M6a (Y251D) induced neuritogenesis and neurite extension. In contrast, abolishing M6a-tyrosine phosphorylation (Y251A) dramatically decreased neurite outgrowth in both hippocampal neurons and N2a cells. We previously reported that the expression of M6a in primary hippocampal neurons induces neuritogenesis (Alfonso et al., 2005a). Here we also demonstrated that M6a induces neurite extension in hippocampal neurons and N2a cells. By using M6a-mAb treatment, we demonstrated a significant decrease in M6a wt-induced neurite elongation in both hippocampal neurons and N2a cells. It is notable that this inhibition was totally rescued when neurite outgrowth was induced by M6a phosphorylated mutant Y251D. The phosphorylation state of M6a at Y251 seems not to be involved in the formation of filopodia-like processes in hippocampal neurons. Moreover, Src and PI3K inhibitors disrupted neurite extension and filopodium formation promoted by M6a. In contrast, M6a-expressing cells exposed to the Ras inhibitor at doses that do not inhibit other enzymes showed no effects. In our hands, the inhibitors did not modify control levels, suggesting that they impaired only the M6a-mediated pathway. We found that M6a and its Y251 mutants colocalized with endogenous Src along the plasma membrane of the cell body and its protrusions in serum-starved N2a cells expressing M6a. Similar results were obtained with hippocampal neurons at 2 DIV and 5 DIV, supporting the idea that this characteristic might be independent of the neuronal development stage and cell type. Both

Y251 mutants, but not wt M6a, colocalized and induced phosphorylation of Src in N2a cells. Conversely, we found that Y251D-expressing cells promoted Src phosphorylation, with the ratio of pSrc/Src significantly higher than those in control cells.

Given that PI3K inhibitors impaired M6a neurite elongation and filopodium induction, the next step was to study whether M6a and its Y251 mutants could modify AKT recruitment and activation. The overexpression of M6a had no effect on membrane recruitment and activation of AKT in any of the cell types studied. However, the Y251 mutants showed strong colocalization with endogenous AKT in the plasma membrane of N2a cells. By using both immunofluorescence and Western blotting experiments, we found that the mutants promoted AKT phosphorylation at Ser473, whereas the ratio of pAKT/AKT in cells transfected with the Y251D mutant was significantly higher. These data suggest that Y251 of M6a recruits and activates not only the Src pathway but also the PI3K/AKT-mediated pathway, leading to neurite extension in N2a cells.

In summary, the present work has analyzed a possible signaling pathway triggered by M6a that leads to neurite and filopodia formation in hippocampal neurons and N2a cells. We found that the phosphorylation state of Y251 in the C-terminus of M6a regulates M6ainduced neurite outgrowth but not filopodia formation. Phosphorylation of M6a at Y251 positively contributes to neurite elongation and requires the PI3K/AKT-mediated signaling independently of external EC2-inhibition stimulus.



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The results were partially published in:

-Tyrosine 251 at the C-terminus of neuronal glycoprotein M6a is critical for neurite outgrowth. Formoso, K., Billi, S.C., Frasch, A.C., **Scorticati, C**., 2015.J. Neurosci. Res. doi:10.1002/jnr.23482.

- Filopodia Formation driven by Membrane Glycoprotein M6a depends on the Interaction of its Transmembrane Domains. Formoso, K., García, M.D., Frasch, A.C., **Scorticati, C**. 2015. J. Neurochem. doi:10.1111/jnc.13153.