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Subcellular localization of the carbohydrate Lewis^x adhesion structure in hippocampus cell cultures

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ABSTRACT

The Lewis^x (Le^x) epitope (Gal(β1–4)[Fuc(α1–3)]GlcNAc-R) has been associated with the development of the central nervous system of diverse species including human and rodents. In this work, Le^x has been found in the tetanus neurotoxin insensitive vesicle-associated membrane protein (TI-VAMP) compartment of rat hippocampus neurons in culture, at 7 days in vitro (DIV), when neurite extension is abundant. The TI-VAMP compartment is known to be associated with neurite outgrowth. Le^x was found predominantly in neurites but also in somata and in growth cones. Abundant Le^x-carrier glycoproteins specific to neurons have been identified at this stage of differentiation. At a later stage of differentiation, at 14 DIV, Le^x appeared in extrasynaptic sites of GABAergic neurons, and in synaptic sites of glutamatergic neurons.

1. Introduction

The fucosylated carbohydrate Lewis^x (Le^x) determinant (Gal(β1–4)[Fuc(α1–3)]GlcNAc) is temporally and spatially regulated in the developing central nervous system (CNS) of diverse species, including human, rat, mouse, chicken, and *Xenopus* (Dasgupta et al., 1996; Gotz et al., 1996; Yoshida-Noro et al., 1999), where it is synthesized by fucosyltransferase IX (FUT9) (Brito et al., 2007; Kudo et al., 2007). Complex-type *N*-glycans with terminal Le^x are among the most abundant neutral carbohydrates detected in human brain (Chen et al., 1998). Le^x has been shown to participate in neuronal adhesion and neurite outgrowth (Brito et al., 2007; Gotz et al., 1996; Yoshida-Noro et al., 1999). In the CNS, Le^x expression also identifies stem cells and highly prolific progenitor cells (Capela and Temple, 2006). The knock-out mouse FUT9$^{-/-}$ showed a lack of Le^x in the brain, concomitant with behavioral alterations, but without obvious pathological changes (Kudo et al., 2007). The animal exhibited anxiety-like behavior, possibly due to a decreased number of GABAergic neurons from basolateral amygdala.

Le^x has been detected in mouse cerebral primary cultures, at the surface of cell bodies, processes and growth cones of neurons, as well as in cerebellum sections (Nishihara et al.,

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Abbreviations: ara-c, cytosine arabinoside; CNS, central nervous system; DAPI, 4′,6-diamidino-2-phenylindole; DIV, days in vitro; FUT9, fucosyltransferase IX; LAMP-1, lysosomal associated membrane protein-1; Le^x, Lewis^x; MAPs, anti-microtubule associated proteins; TI-VAMP, tetanus neurotoxin insensitive vesicle-associated membrane protein

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2003). Le^x has been shown to participate in carbohydrate–carbohydrate interaction in homophilic binding (de la Fuente et al., 2005), and also in carbohydrate–protein interactions, as it is a ligand for C-type lectins (Taylor and Drickamer, 2007). In NT2N neuronal cells, Le^x expression was increased during neuronal differentiation in vitro, where it was found at the cell surface, in lysosomes, and for the first time, in the tetanus neurotoxin insensitive vesicle-associated membrane protein (TI-VAMP) compartment (Brito et al., 2007). The TI-VAMP compartment has been proposed to define an exocytic pathway involved in neurite outgrowth (Alberts et al., 2003; Martinez-Arca et al., 2000; Martinez-Arca et al., 2001).

In vitro cell cultures of CNS neurons obtained from embryonic hippocampus have been well characterized (Danglot et al., 2003; Dotti et al., 1988). The maturation of the culture is composed of five stages. At stage 1 (0.25 days in vitro (DIV)), the neurons are round and extend lamellipodia, and at stage 2 (0.5 DIV), the neurons extend neurites. Axon and dendrite differentiation occurs respectively at stage 3 (1.5 DIV) and stage 4 (4 DIV). Maturation of the synapse takes place at stage 5 (>7 DIV).

In the present work, Le^x has been detected subcellularly in neurons from rat hippocampus cultures. It was found in the TI-VAMP compartment at 7 DIV. Later, at 14 DIV, it appeared in extrasynaptic sites of GABAergic neurons, and in synaptic sites of glutamatergic neurons.

2. Results and discussion

2.1. *Le^x expression during the differentiation of rat hippocampal cultures*

In order to characterize Le^x expression during the development of rat hippocampal neurons at the cellular level, we monitored Le^x by immunofluorescence microscopy in rat hippocampal cultures. In the first hours after plating, Le^x was detected at the surface of many adherent cells (Le^x+), some of which were also labeled with anti-microtubule associated proteins (MAP+), whereas others were not (MAP–) (Fig. 1A). As differentiation proceeded from 18 h to 3 DIV, the Le^x staining became restricted to MAP– cells (Fig. 1B). However, at 7 DIV, the culture contained a sub-population of MAP+Le^x+ cells with a high degree of neuritic arborisation, and another sub-population of MAP+Le^x– cells (Fig. 1C). Both sub-populations probably consisted of neurons since they were MAP+. At this time of culture, 22±3% of total cells was MAP+ of which 46±1% expressed the Le^x determinant. Cell quantification was performed as described in "Experimental procedures". At 14 DIV, 34±3% of the cells were MAP+, and a higher proportion expressed the Le^x determinant (72±14%) (Figs. 1D, E). At 7 and 14 DIV Le^x was also detected in MAP– cells, possibly glial cells visualized in Figs. 1D, F. Since maturation of synapses occurs after 7 DIV, this increase in Le^x is probably associated with synapse formation.

2.2. *The Le^x determinant is localized in the TI-VAMP compartment of rat hippocampus neurons*

To investigate if Le^x could be detected in the TI-VAMP compartment of rat primary cultures of hippocampus neurons, colocalization analysis with TI-VAMP or lysosomal associated membrane protein-1 (LAMP-1) was performed in 7 DIV cultures. At this developmental stage, there is high neurite extension in the hippocampus neurons, which implicates the TI-VAMP compartment in bringing new components to the tip of the neurites to promote their outgrowth, including L1 cell adhesion molecule (Alberts et al., 2003) and synaptotagmin VII (Arantes and Andrews, 2006). In MAP stained neurons, Le^x colocalized with TI-VAMP in the soma and most extensively in puncta along the neurites (Fig. 2A, shown with arrowheads in magnified inset). The detection of Le^x in locations that did not overlap with TI-VAMP was possibly due to carriers present in different vesicles along the neurites. Le^x and TI-VAMP positive puncta were also detected in growth cones, but with a lower degree of colocalization (Fig. 2B, shown with arrowheads in magnified inset). In MAP-negative cells labeled with Le^x there was also TI-VAMP staining, although no colocalization was observed (results not shown). These results suggest that Le^x-carriers are cargo of the TI-VAMP compartment in neurons.

Le^x was also detected in LAMP-1-positive puncta, mainly in the cell body where LAMP-1 labeling was more intense and colocalization was almost total (Fig. 2C). There was also some degree of colocalization in the neuritic processes (Fig. 2C, shown with arrowheads in magnified inset). The possibility that LAMP-1, a lysosomal and highly glycosylated protein (Carlsson et al., 1988), could be a carrier of the Le^x epitope in rat hippocampal primary cultures, was ruled out by Western blot analysis (see below, Fig. 3). LAMP-1 has an apparent molecular mass of approximately 120 kDa (Carlsson et al., 1988) and no Le^x-reactive bands were detected in homogenates of hippocampus primary cultures in that molecular mass range (Fig. 3A).

The detection of Le^x in LAMP-1 positive structures might be partially due to the observation that the TI-VAMP compartment is partially LAMP-1 positive and shares some properties with lysosomes (Proux-Gillardeaux et al., 2005).

2.3. *Le^x-carrier glycoproteins are expressed by neurons in rat hippocampal cultures*

Le^x-carriers have been described as glycoproteins and glycolipids of the developing rat brain (Allendoerfer et al., 1999). In order to identify Le^x-carrier glycoproteins from rat hippocampal neurons, differentiating primary cultures were grown in the presence of the mitosis inhibitor cytosine arabinoside (ara-C), to limit glial proliferation and to obtain a culture enriched in post-mitotic neurons (Fig. 3). Cellular extracts of these cultures were subsequently probed by Western blot with anti-Le^x. Eight hours after plating, a single band that migrated near the 71 kDa marker was detected (Fig. 3A, arrowhead). This band did not correspond to a cleaved form of L1, a cargo molecule of the TI-VAMP compartment (Alberts et al., 2003), as confirmed by re-probing of the membrane with an antibody that recognizes the extracellular domain of L1 (Rathjen and Rutishauser, 1984) (Fig. 3B). At 7 DIV, three Le^x-carriers were detected: one heavier than 460 kDa (Fig. 3A, arrow 1), a second of 460 kDa (Fig. 3A, arrow 2) and an abundant third carrier of apparent molecular mass between 71 and 117 kDa (Fig. 3A, arrow 3). These bands did not consist

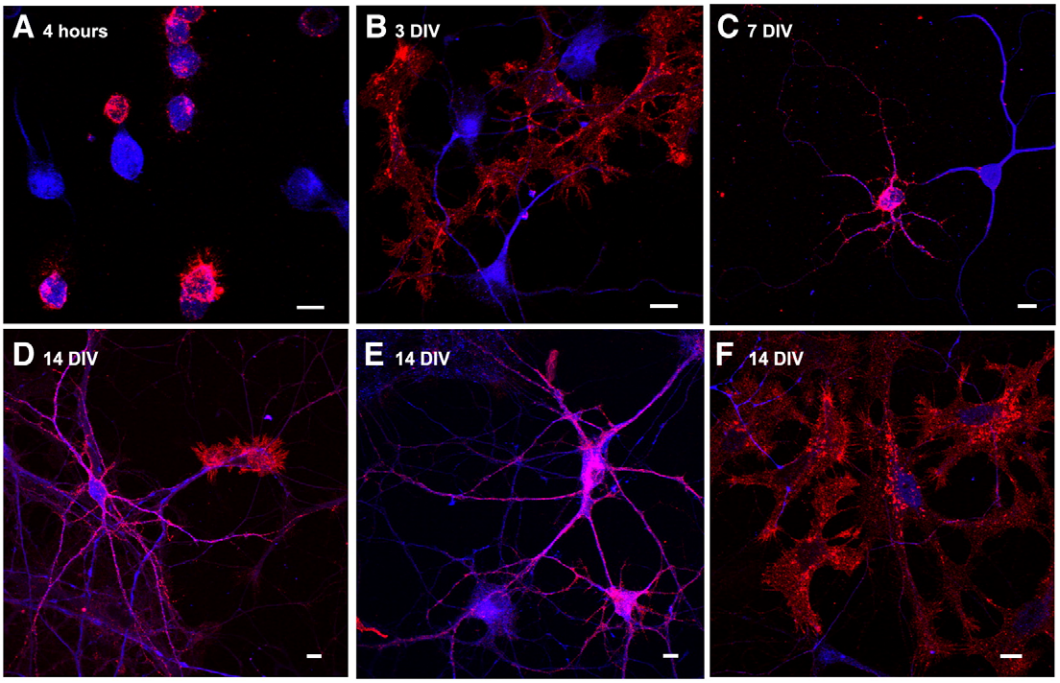

Fig. 1 – Detection of the Le^x determinant along rat hippocampal cultures development. Primary cultures from rat hippocampus were fixed at different times: 4 h (A); 3 DIV (B); 7 DIV (C); 14 DIV (D–F). Permeabilized cells were probed with anti-Le^x (shown in red) and anti-MAPs (shown in blue). Maximum intensity z-projections of 10 to 15 confocal optical sections of 0.5 μm are shown. Scale bars = 10 μm. The experiment was performed with at least two independent cultures.

of L1 (Fig. 3B). In 7 DIV cultures that were not incubated with ara-C, allowing glia overgrowth, the three carriers were not detected (Fig. 3A). Thus, the glycoproteins detected with anti-Le^x in rat hippocampal primary cultures were likely expressed by neurons, since they were only detected in the culture enriched in post-mitotic neurons. The three bands peaked at 7 DIV after which the detection levels of the carriers decreased markedly (Fig. 3A). The variations observed during the in vitro culture were not due to differences in total protein content, as shown by the detection of unvarying levels of β-tubulin (Fig. 3C, arrowhead).

Until now, only two proteins are known to be associated with the TI-VAMP compartment: L1, a cell-cell adhesion molecule involved in axonal growth, whose surface expression and recycling was shown to be TI-VAMP mediated (Alberts et al., 2003), and synaptotagmin VII (SYT7), a 45.6 kDa Ca^{2+}-sensing SYT (Arantes and Andrews, 2006). The results obtained here suggested that the Le^x-carrier glycoproteins identified at day 7 are new components of the TI-VAMP compartment, which is actively involved in neurite outgrowth at that developmental stage. The high molecular mass carriers (bands 1 and 2) detected might correspond to proteoglycans previously identified as Le^x-carriers in the rat brain. For example, Le^x was detected in mannose-linked *O*-oligosaccharides of the chondroitin sulfate proteoglycan phosphocan expressed in the early postnatal rat brain (Krusius et al., 1987), which is a soluble form of a transmembrane, receptor-type protein-tyrosine phosphatase, RPTPβ, that binds neural cell adhesion molecules (Maurel et al., 1994). The reported molecular mass of phosphacan is 800–1000 kDa determined by gel filtration (Faissner et al., 1994). Mannose-linked *O*-oligosaccharides have also been described in rat brain proteoglycans that carry Le^x and the human natural killer-1, HNK-1, epitope, another common carbohydrate antigen in the CNS (Kogelberg et al., 2001; Yuen et al., 1997). In our previous work, a Le^x and HNK-1-carrier of approximately 460 kDa has also been found in human NT2N neuronal cells (Brito et al., 2007).

The Le^x moiety of the carrier must be localized within the lumen of the vesicles or be extracellular since its biosynthesis is catalyzed by several glycosyltransferases localized along the secretory pathway, for example, FUT9 that has been found in the *trans*-Golgi and *trans*-Golgi network (Brito et al., 2008).

2.4. *Subcellular localization of Le^x in neurons from rat hippocampus*

The observation that only a sub-population of hippocampal neurons expressed the Le^x epitope (Fig. 1) led us to hypothesize that those could correspond to a subtype of hippocampal neurons. The establishment of synapses in these cultures is very progressive and depends on the density of the neurons. The first glutamatergic excitatory synapses are seen as soon as 3 DIV, whereas the GABAergic inhibitory synapses begin to be detected at 10 DIV (Danglot et al., 2003). Therefore, to localize Le^x in inhibitory and excitatory neurons, detection was performed at 14 DIV, when both types of neurons were

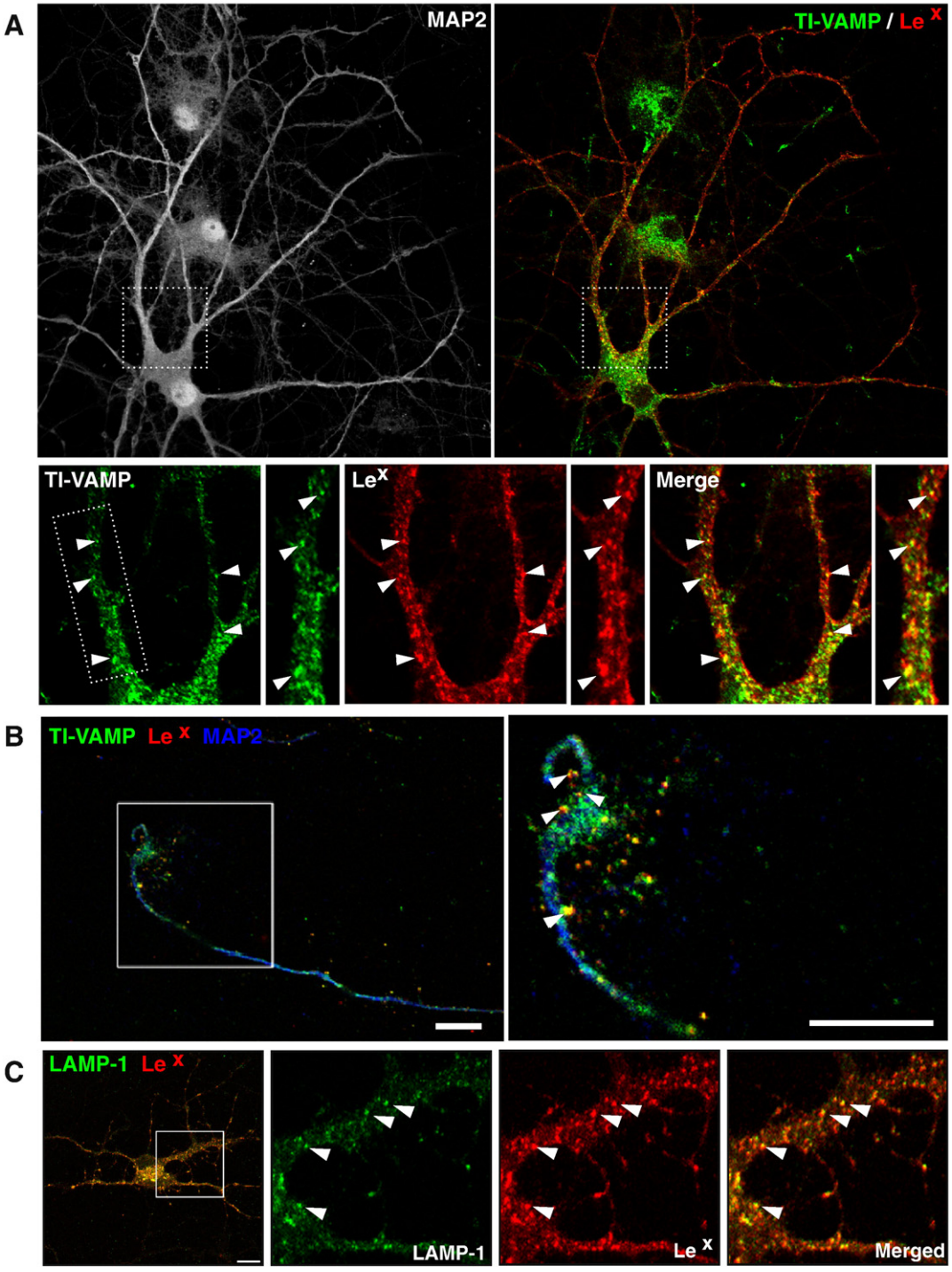

Fig. 2 – Colocalization analysis of Le^x in 7 DIV hippocampus neurons. Primary cultures from rat hippocampus were fixed and permeabilized at 7 DIV and probed with anti-Le^x (shown in red), anti-TI-VAMP (green) and anti-MAPs (white or blue) (A, B) or anti-Le^x (red) and anti-LAMP-1 (green) (C). Single optical confocal sections of 0.5 μm are shown. Scale bars = 10 μm. The insets indicated are presented at higher magnification. The experiments were carried out with at least two independent cultures and for each condition at least five neurons per marker were analyzed by confocal microscopy.

present and synapses were clearly visible. The culture is composed of approximately 10% inhibitory GABAergic interneurons, which was comparable to that previously reported (Benson et al., 1994), whereas the remaining are pyramidal glutamatergic neurons. Thus, aiming to identify the population of neurons that expressed the Le^x determinant, we probed 14 DIV hippocampal cultures with anti-Le^x and anti-GAD, the enzyme that catalyzes the conversion of L-glutamate to GABA

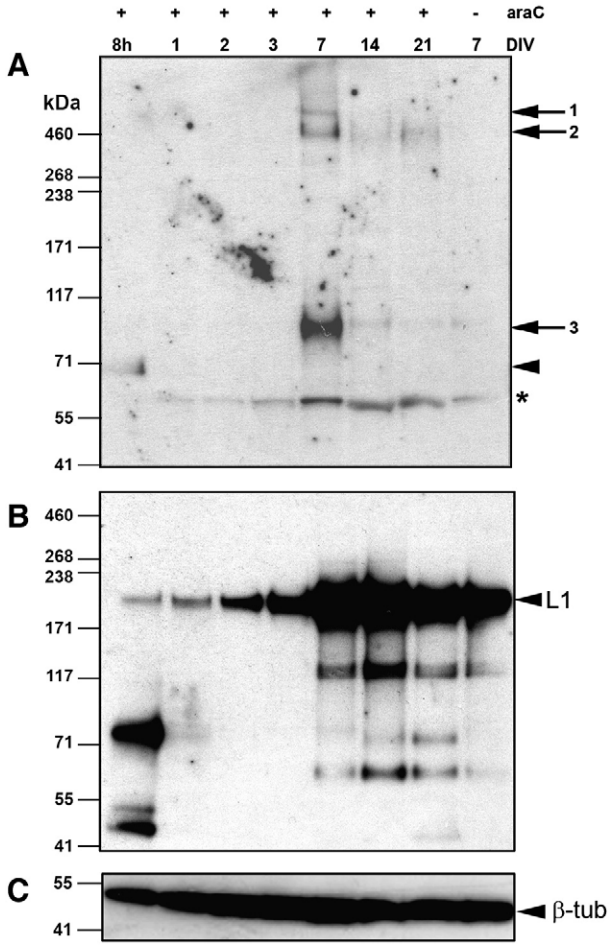

Fig. 3 – Detection of Le^x-carrier proteins from hippocampal cultures. SDS-PAGE and Western blot analysis of cultures with 8 h, 1, 2, 3, 7, 14, and 21 days in vitro (DIV), in the presence of cytosine arabinoside (ara-C), when stated. Equivalent amounts of protein were applied per lane. Primary antibodies were anti-Le^x L5 (A), anti-L1 (B) or anti-β-tubulin (β-tub) (C). The heavy chains of rat IgMs present in the cell extracts were detected by the secondary anti-rat IgM antibody (asterisk).

(reviewed by Varju et al. (2001)). All neurons positive for GAD detected in the cultures also presented Le^x labeling (Fig. 4A), indicating that at 14 DIV hippocampal GABAergic interneurons express Le^x-carriers. Le^x staining at 14 DIV presented a punctate pattern in the cell bodies and in neuritic processes (Figs. 1D and 4A). As shown at higher magnification (Fig. 4A1, A2), the two stainings were interspersed and did not overlap. Thus, Le^x is localized in GABAergic neurons at extrasynaptic sites.

Additionally, Le^x was also detected in MAP-positive cells which remained unlabeled by GAD (Fig. 4B, see arrowhead), which consisted of non-GABAergic neurons, and, therefore, were glutamatergic neurons. Furthermore, a significant degree of colocalization between Le^x and synaptophysin was found (Fig. 4C), indicating that Le^x localized at synaptic sites in excitatory neurons. This was confirmed by the fact that Le^x puncta accumulated near dendritic branchlets (arrowheads in Fig. 4D1–D2), which are usually implied in excitatory synapses (Zhang and Benson, 2000). On the other hand, synaptophysin and GAD colocalize at inhibitory terminals but, since in this culture there was no colocalization between GAD and Le^x, it follows that the synapses where synaptophysin colocalized with Le^x must be excitatory ones. Further studies by electron microscopy will elucidate if the Le^x-carriers are post-synaptic in excitatory neurons.

The distinct subcellular localization suggests that Le^x-carriers of excitatory neurons must be distinct from those from inhibitory neurons. Furthermore, since they are not detected by Western blot at 14 DIV (Fig. 3A) it is plausible to assume that they are distinct from the putative Le^x-carrier from the TI-VAMP compartment, which peaks at 7 DIV, and that they may have a different nature, being, for example, glycolipids.

3. Conclusions

In summary, we found that the Le^x carbohydrate adhesion structure was localized in the TI-VAMP compartment of rat hippocampus neurons in culture with a high activity of neurite extension. Furthermore, Le^x was found at extrasynaptic sites in GABAergic neurons and at synaptic sites of excitatory neurons. This work provides the basis for a better understanding of the relevance of Le^x from the TI-VAMP compartment in neurite outgrowth. Further studies will allow the characterization of the Le^x-carrier and the corresponding candidate receptor, possibly of the family of the C-type lectins, in the CNS. In addition, it provides an ideal system to investigate the relevance of Le^x mediated homophilic interaction or Le^x-carrier/receptor recognition on signaling events associated with neurite outgrowth.

4. Experimental procedures

4.1. *Neuronal culture*

Hippocampal cultures were prepared from 18-day-old, fetal Sprague–Dawley rats, as described previously (Danglot et al., 2003). Neurons were plated in minimum essential medium (MEM) (Gibco) supplemented with 10% horse serum (Sigma), 0.6% glucose (Sigma), 0.2% $NaHCO_3$ (Gibco), 2 mM glutamine (Gibco), and 10 U/ml penicillin-streptomycin (complete MEM). After attachment, neurons were transferred to glia-conditioned media, which was replaced weekly. To obtain glia-conditioned medium glial cells were plated in complete MEM, changed every 3 days until confluence. Conditioned medium was prepared by incubating serum-free neurobasal medium (Gibco) supplemented with B27 (Gibco), 2 mM glutamine (Gibco) and 5 U/ml penicillin–streptomycin (Gibco) with the glial monolayers for 1 week. All cultures were maintained at 37 °C in a 5% CO_2 humidified incubator.

4.2. *Immunofluorescence microscopy*

Cells were processed for immunofluorescence studies as described previously (Danglot et al., 2004). Briefly, cells

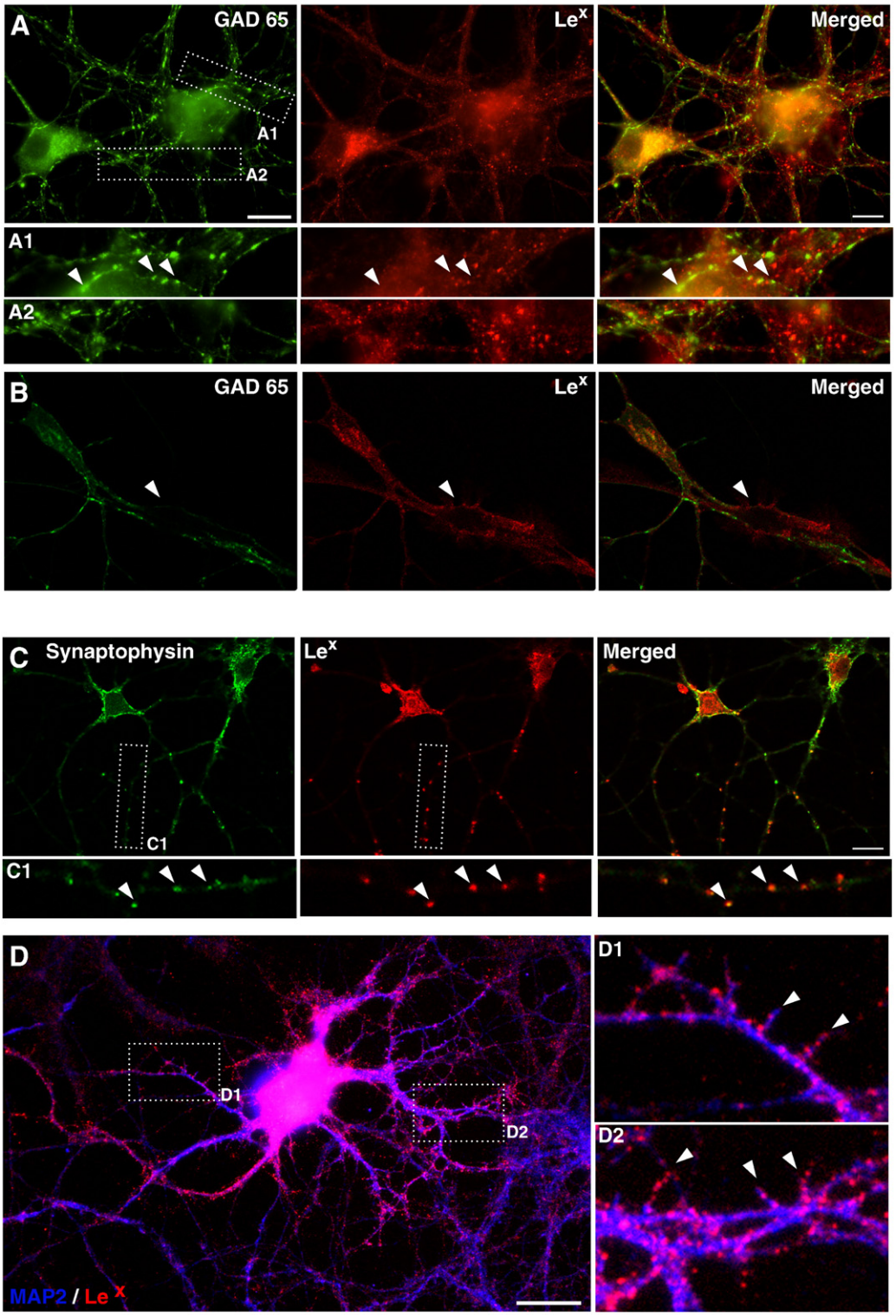

Fig. 4 – Detection of the Le^x determinant in 14 DIV rat hippocampus neurons. Primary cultures from rat hippocampus were fixed and permeabilized at 14 DIV and probed with anti-Le^x (red) and anti-GAD (green, in A and B) or with anti-synaptophysin (green, in C) or anti-MAPs (shown in blue, in D). The insets indicated in (A, C) and (D) are presented at higher magnification in (A1, A2), (C1) and (D1, D2), respectively. Scale bars = 10 μm. The experiment was performed with two independent cultures.

were fixed with 4% paraformaldehyde and 4% sucrose in PBS for 20 min at room temperature, quenched in 50 mM NH_4Cl in PBS for 20 min, permeabilized with 0.1% (w/v) Triton X-100, 0.1% (w/v) fish gelatin in PBS for 4 min, and blocked in 0.25% (w/v) gelatin in PBS for 30 min. They were then incubated with primary antibody in 0.1% (w/v) gelatin

in PBS for 1 h at room temperature and subsequently incubated with secondary fluorescent antibodies and a 1:500 dilution of DAPI (Invitrogen), for 45 min at room temperature. Cells were washed in PBS and quickly rinsed in distilled water and mounted in Mowiol (Calbiochem). The primary antibodies used were: rabbit IgG anti-L1 cell adhesion molecule ectodomain (Alberts et al., 2003); rat IgM anti-Le^x L5 (Streit et al., 1990); rabbit IgG anti-microtubule-associated proteins (MAPs) 1:200 dilution (Sigma); mouse IgG anti-TI-VAMP clone 158.2 1:200 (Alberts et al., 2003); mouse IgG anti-LAMP-1 1:5 (Ly1C6, Abcam); rabbit IgG anti-synaptophysin MC1 (1:1000) (Chilcote et al., 1995); rabbit IgG anti-glutamate decarboxylase 1:500 (GAD) 65/67 (Chemicon). Secondary antibodies conjugated to Cy5 and AlexaFluor (1:200 dilution from Jackson ImmunoResearch and 1:400 from Molecular Probes, respectively) were used. Fluorescence images were acquired on a Leica DMRD microscope and confocal images on a SP2+AOBS Microscope (Leica).

Quantifications were performed manually using the Cell Counter plug-in of the open source Image J software version 1.41a (http://rsbweb.nih.gov/ij/). Images were taken from random fields, covering all the coverslips. Total cell numbers were determined by counting nuclei, identified by DAPI staining, and the percentage of MAP, GAD and Le^x-positive cells was determined by counting positive cell bodies in each channel. All results given are means ± SEM of experiments from two independent ($n = 2$) hippocampus cultures, with at least 500 cells counted per experiment.

4.3. *SDS-PAGE and Western blot analysis*

When stated, hippocampal neurons were cultured in the presence of 1 μM of ara-C (Sigma) to avoid glial proliferation. Total protein was solubilized and analyzed by Western blot as previously described (Brito et al., 2007). SDS-PAGE was performed in precast NuPAGE Novex Tris-Acetate gels 3–8% (Invitrogen). Anti-Le^x, anti-L1 (a gift of F. Rathjen) and anti-β-tubulin (DSHB) were detected using HRP-labeled secondary antibodies (Jackson ImmunoResearch). Chemiluminescent detection was by the ECL system (GE Healthcare).

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