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Research report

The combined impact of IgLON family proteins Lsamp and Neurotrimin on developing neurons and behavioral profiles in mouse

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ABSTRACT

Cell surface neural adhesion proteins are critical components in the complex orchestration of cell proliferation, apoptosis, and neuritogenesis essential for proper brain construction and behavior. We focused on the impact of two plasticity-associated IgLON family neural adhesion molecules, Neurotrimin (Ntm) and Limbic system associated membrane protein (Lsamp), on mouse behavior and its underlying neural development. Phenotyping neurons derived from the hippocampi of *Lsamp*$^{-/-}$, *Ntm*$^{-/-}$ and *Lsamp*$^{-/-}$*Ntm*$^{-/-}$ mice was performed in parallel with behavioral testing.

While the anatomy of mutant brains revealed no gross changes, the *Ntm*$^{-/-}$ hippocampal neurons exhibited premature sprouting of neurites and manifested accelerated neurite elongation and branching. We propose that Ntm exerts an inhibitory impact on neurite outgrowth, whereas Lsamp appears to be an enhancer of the said process as premature neuritogenesis in *Ntm*$^{-/-}$ neurons is apparent only in the presence of Lsamp. We also show interplay between Lsamp and Ntm in regulating tissue homeostasis: the impact of Ntm on cellular proliferation was dependent on Lsamp, and Lsamp appeared to be a positive regulator of apoptosis in the presence of Ntm. Behavioral phenotyping indicated test-specific interactions between Lsamp and Ntm. The phenotypes of single mutant lines, such as reduced swimming speed in Morris water maze and increased activity in the elevated plus maze, were magnified in *Lsamp*$^{-/-}$*Ntm*$^{-/-}$ mice.

Altogether, evidence both from behavioral experiments and cultured hippocampal cells show combined and differential interactions between Ntm and Lsamp in the formation of hippocampal circuits and behavioral profiles. We demonstrate that mutual interactions between IgLON molecules regulate the initiation of neurite sprouting at very early ages, and even cell-autonomously, independent of their regulation of cell-cell adhesion.

1. Introduction

The initiation and maintenance of robust functional neuronal circuits depends on the delicate regulation of cellular proliferation, apoptosis, and the establishment of precise neuronal connections. The outgrowth and elongation of neurites at the appropriate times and in the correct directions provide the basis of functional neural connectivity (da Silva and Dotti, 2002), and thus, cognitive and behavioral functions of the brain. Structural alterations during neuritogenesis are associated with abnormal neural circuit formation in psychiatric disorders such as autism spectrum disorders (Bakos et al., 2015) and schizophrenia (Lang et al., 2014). Disrupted functional brain connectivity, reflecting impairment of the integrity of white matter fiber tracts, has been found both in the patients with schizophrenia or bipolar disorder (Argyelan et al., 2014; Rashid et al., 2014; Li et al., 2017). Cell membrane molecules, such as the IgLON proteins, are critical for the formation of correct interactions between neural cells (Tan et al., 2017). The IgLON superfamily of cell adhesion molecules (CAMs) are the most abundantly expressed GPI-anchored neural cell surface glycoproteins (Salzer et al., 1996) in the neural cell membrane, and these proteins include Lsamp (limbic system associated membrane protein), Ntm (neurotrimin), Opcml (opioid-binding cell adhesion molecule),

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Kilon/Negr1 (neuronal growth regulator 1) and IgLON5 (Vanaveski et al., 2017). In the brain, Lsamp, Negr1 and Opcml are expressed both in neurons and oligodendrocytes except for NTM, which has been found to be enriched specifically in neurons (Sharma et al., 2015). IgLONs facilitate the assembling of the tuned groups of neurons during the formation of neuronal circuits (Kolodkin and Tessier-Lavigne, 2011; Schmidt et al., 2014). In particular, IgLONs have been shown to promote growth cone migration, axon target guidance, synapse formation, and dendritic tree formation, throughout development and adulthood (Miyata et al., 2003; Hashimoto et al., 2009; Yamada et al., 2007; Akeel et al., 2011; Pischedda et al., 2014; Sanz et al., 2017; Singh et al., 2018). Several studies have shown functional alterations of IgLON family members in several malignancies both in the brain and outside central nervous system (Chen et al., 2003; Ntougkos et al., 2005; Minhas et al., 2013; Kim et al., 2014), suggesting further functions in cellular homeostasis.

The number of potential interactions between IgLONs is extremely high due to their ability to form homophilic and heterophilic inter-family associations both in *cis* and *trans* orientations on the cell surface. In the current study we investigated the single and combined effects of two IgLONs, Lsamp and Ntm, on hippocampal development and mouse behaviors. On the plane of the neuronal membrane, heterophilic *cis*-interaction between Lsamp and Ntm has been shown to be one of the most likely IgLON combinations. Likewise the affinity between Lsamp and Ntm has been shown to be the highest when pairs of IgLONs were compared for their potential to form *trans*-interactions between neurons (Reed et al., 2004). Behavioral studies in *Lsamp*$^{-/-}$ mice indicate the significance of Lsamp in the regulation of complex emotional and social behavior (Innos et al., 2011, 2012, 2013a, 2013b; Philips et al., 2015) whereas Ntm$^{-/-}$ mice exhibit deficit in emotional learning (Mazitov et al., 2017). Lsamp has been shown to be implicated in hippocampal plasticity and adaptation in changing environments (Qiu et al., 2010; Heinla et al., 2015). Polymorphisms and expressional alterations in the Lsamp gene in humans have been linked to a spectrum of neuropsychiatric disorders (Must et al., 2008; Behan et al., 2009; Koido et al., 2012, 2014) whereas polymorphisms in the Ntm gene have been found to be associated with cognitive functions (Liu et al., 2007) and intelligence (Pan et al., 2010) and expressional alterations in the dorsolateral prefrontal cortex have been found in schizophrenic brains (Karis et al., 2018).

On the cellular level, IgLON cell adhesion molecules have been shown to modify specific neuronal projections that are relevant in modulating behavioral reactions. Ntm has been shown to participate in directing the thalamocortical and pontocerebellar projections (Struyk et al., 1995; Chen et al., 2001). Lsamp is involved in the establishment of thalamic, septo- and intrahippocampal circuits (Keller et al., 1989; Pimenta et al., 1995; Mann et al., 1998); and it is critical in the fasciculation of dopaminergic afferents from the midbrain to lateral habenula (Schmidt et al., 2014).

The neuroanatomical distribution of two IgLON members, Lsamp and Ntm, is highly heterogeneous throughout the brain, and it has been proposed that they are expressed by distinct complementary subpopulations of neurons with co-expression at a few sites (Philips et al., 2015; Gil et al., 2002). The brain areas expressing both Lsamp and Ntm include sensory and sensory-motor cortex, entorhinal cortex, hippocampus, amygdala, thalamus (ventral posteromedial, lateral geniculate nucleus and lateral dorsal nuclei), pyriform cortex, cerebellum, brain stem nuclei, spinal cord and dorsal root ganglia, (Philips et al., 2015; Struyk et al., 1995; Gil et al., 2002). In cultured hippocampal neurons, the co-expression of Lsamp and Ntm has been shown at the level of single neurons (Gil et al., 2002). Despite overlapping expression distribution and high affinity, Ntm and Lsamp have been shown to have differential effects on behavior and antagonistic functions in several cell culture studies (Hashimoto et al., 2009; Ntougkos et al., 2005; Mazitov et al., 2017). Therefore, the IgLON CAMs Ntm and Lsamp are an attractive couple to study for their distinct and combined abilities to affect neuronal function and the corresponding behavioral correlates.

To decipher the interaction between Lsamp and Ntm at different functional levels of the central nervous system, we investigated the neuronal development, gross anatomy of the brain and behavioral profile in *Lsamp*-deficient, *Ntm*-deficient and *Lsamp/Ntm* double-deficient mice. As the only clearly overlapping change in phenotype for both *Lsamp*$^{-/-}$ and *Ntm*$^{-/-}$ animals has been found to be decreased sensitivity to amphetamine (Innos et al., 2013a, 2013b; Mazitov et al., 2017), the sensitivity to amphetamine in *Lsamp*$^{-/-}$*Ntm*$^{-/-}$ mice was also tested in the current study. The process of neuritogenesis has been well-characterised in cultured hippocampal neurons (Craig and Banker, 1994), which were used in the current study to uncover the IgLON-associated morphological changes during development. The current study is the first to describe the early neuritogenesis and cellular homeostasis of *Lsamp*$^{-/-}$, *Ntm*$^{-/-}$ and *Lsamp*$^{-/-}$*Ntm*$^{-/-}$ hippocampal neurons. Behavioral phenotyping of mice with the same set of genotypes enabled us to gain insight concerning how the alterations in neuronal morphology become manifest at the behavioral level.

2. Materials and methods

2.1. Experimental animals

The generation of *Lsamp*-deficient (*Lsamp*$^{-/-}$) mice with a *LacZ* transgene has been described by Innos et al. (2011). Briefly, exon 1b of the murine *Lsamp* gene was replaced by an in-frame *NLS-LacZ-NEO* cassette resulting in the disruption of all functional *Lsamp* transcripts. *Ntm* gene heterozygous mutant strain (032496-UCD B6;129S5-*Ntm*^{tm1Lex}/Mmucd) was obtained from the Mutant Mouse Regional Resource Centre at UC Davis (https://www.mmrrc.org/catalog/sds.php?mmrrc_id=32496) as described in Mazitov et al., 2017. Briefly, the strategy for the creation of *Ntm*-deficient (*Ntm*$^{-/-}$) mice was analogous to that of *Lsamp*-deficient mice, as exon 1b was deleted, leading to the disruption of all functional *Ntm* transcripts.

We generated double heterozygous mice for *Lsamp* and *Ntm* by crossing *Lsamp*$^{-/-}$ and *Ntm*$^{-/-}$ mice in F2 strain background [(129S5/SvEvBrd × C57BL/6) × (129S5/SvEvBrd × C57BL/6)]. Further crossing of the obtained double heterozygous mice (*Lsamp*$^{+/-}$*Ntm*$^{+/-}$) gave us the entire spectrum of genotypes (including *Lsamp*$^{+/+}$*Ntm*$^{+/+}$, *Lsamp*$^{+/+}$*Ntm*$^{-/-}$, *Lsamp*$^{-/-}$*Ntm*$^{+/+}$, *Lsamp*$^{-/-}$*Ntm*$^{-/-}$) that were used in the behavioral studies. For primary culture, breeding was done separately for all of the genotypes obtained from the above crossings in order to get a sufficient number of new-born pups with particular genotypes and comparable background. The behavioral experiments were performed with male mice 2–4 months of age. Mice were group-housed in standard laboratory cages measuring 42.5 (L) × 26.6 (W) × 15.5 (H) cm, 6–8 animals per cage in the animal colony at 22 ± 1 °C, under a 12:12 h light/dark cycle (lights off at 19:00 h). A 2 cm layer of aspen bedding (Tapvei, Estonia) and 0.5 l of aspen nesting material (Tapvei, Estonia) was used in each cage and changed every week. No other enrichment was used besides nesting material. Tap water and food pellets (R70, Lactamin AB, Sweden) were available *ad libitum*. Breeding and housing of the mice was conducted at the animal facility of the Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia. All animal procedures in this study were performed in accordance with the European Communities Directive (2010/63/EU) and permit (No. 29, April 28, 2014) from the Estonian National Board of Animal Experiments.

2.2. Primary hippocampal neuronal culture

2.2.1. Hippocampal neuronal culture stages followed in the current study

Mouse hippocampal culture was classified into six different stages of development (Baj et al., 2014). At the molecular level, neuritogenesis involves the growth and orientation of cytoskeletal microtubules along actin filament (F-actin) bundles to initiate the growing neurites (Flynn, 2013). The stage 1 was defined as neurite initiation stage, when