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Enhanced fear expression in Spir-1 actin organizer mutant mice

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ABSTRACT

Spir proteins nucleate actin filaments at vesicle membranes and facilitate intracellular transport processes. The mammalian genome encodes two Spir proteins, namely Spir-1 and Spir-2. While the mouse *spir-2* gene has a rather broad expression pattern, high levels of *spir-1* expression are restricted to the nervous system, oocytes, and testis. *Spir-1* mutant mice generated by a gene trap method have been employed to address Spir-1 function during mouse development and in adult mouse tissues, with a specific emphasis on viability, reproduction, and the nervous system. The gene trap cassette disrupts Spir-1 expression between the N-terminal KIND domain and the WH2 domain cluster. *Spir-1* mutant mice are viable and were born in a Mendelian ratio. In accordance with the redundant function of Spir-1 and Spir-2 in oocyte maturation, *spir-1* mutant mice are fertile. The overall brain anatomy of *spir-1* mutant mice is not altered and visual and motor functions of the mice remain normal. Microscopic analysis shows a slight reduction in the number of dendritic spines on cortical neurons. Detailed behavioral studies of the *spir-1* mutant mice, however, unveiled a very specific and highly significant phenotype in terms of fear learning in male mice. In contextual and cued fear conditioning experiments the male *spir-1* mutant mice display increased fear memory when compared to their control littermates. Our data point toward a particular function of the vesicle associated Spir-1 actin organizer in neuronal circuits determining fear behavior.

Introduction

In multicellular organisms cell diversity, spatial organization, and communication are strongly dependent on the dynamic actin cytoskeleton. Actin filaments assemble from monomeric G actin proteins into a double stranded helical filament. The dynamic assembly and disassembly of actin filaments combined with motor proteins of the myosin family are driving forces of morphological cell dynamics and cell motility throughout the animal and plant kingdoms of metazoans (Pollard and Cooper, 2009). Actin related functions also regulate intracellular vesicle transport processes and therefore orchestrate the composition of receptors, ion channels, and adhesion molecules in the cellular membrane, allowing proper functionality and communication of cellular networks. The spatial and temporal initiation of actin polymerization is an essential regulatory element for actin to fulfill its different cellular functions (Pollard and Cooper, 2009). Due to the relative instability of the actin dimer, nucleation factors are needed; they overcome the kinetic barrier to form a filament from free actin monomers, a process called nucleation. Three major classes of actin nucleation factors have been identified to date, namely the Arp2/3 complex and its nucleation promoting factors, the FH2 domain containing nucleators of the formin superfamily, and nucleation factors containing one or multiple WH2 actin binding domains (Pollard and Cooper, 2009). The Spir proteins are the founding members of WH2-containing actin nucleators (Quinlan et al., 2005).

Spir actin nucleation activity occurs in a cluster of four consecutive actin-binding WH2 domains in the central region of the

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protein (Quinlan et al., 2005). Although Spir proteins alone are capable of nucleating actin polymerization *in vitro*, they were also found to act in cooperation with the distinct actin nucleators of the formin subgroup (FMN) of formins encompassing *Drosophila melanogaster* Cappuccino protein and its two vertebrate homologs formin-1 (Fmn-1) and formin-2 (Fmn-2) (Pechlivanis et al., 2009; Quinlan et al., 2007). The *Drosophila spire* and *cappuccino* mutants were identified in the same genetic screen and have an identical phenotype (Manseau and Schupbach, 1989; Wellington et al., 1999). Later it was shown that both proteins regulate a common actin meshwork in the *Drosophila* oocyte, which suppresses premature cytoplasmic streaming (Dahlgaard et al., 2007).

The mammalian genome encodes for both *spir-1* and *spir-2* (Kerkhoff, 2006). Recently mouse Spir-1 and Spir-2 were described to cooperate with the mouse Cappuccino-homolog formin-2 in regulating a similar actin meshwork in mouse oocytes, which is required for asymmetric spindle positioning during meiosis (Pfender et al., 2011). The knock down of both mouse Spir proteins is necessary to generate an actin meshwork and spindle migration phenotype, indicating a redundant function of Spir-1 and Spir-2 in oocyte maturation. In addition, the Spir and formin-2 proteins co-localize at the cleavage furrow during polar body extrusion, a process found to be dependent on Spir function (Pfender et al., 2011). The Spir/formin cooperation is therefore considered a major regulatory function in mammalian oocyte maturation and is thought to be an essential mechanism in human reproduction. Hypofertility was consistently identified as a major phenotype of Fmn2$^{-/-}$ mice (Leader et al., 2002). Additionally, it has been revealed that the Spir/formin regulated ooplasmic actin meshwork is assembled on Rab11-containing vesicles (Schuh, 2011). Moreover, Spir and Rab11 highly colocalize when transiently expressed in somatic cells (Kerkhoff et al., 2001). Both proteins function in exocytic vesicle transport processes. Rab11 is localized at the trans-Golgi network, post-Golgi vesicles, and the recycling endosome and operates in exocytic and recycling processes of cell membrane proteins (Sonnichsen et al., 2000).

The expression patterns of the mammalian *spir-1* and *spir-2* genes have been studied during mouse embryogenesis and in adult mouse tissues (Pleiser et al., 2010; Schumacher et al., 2004). Highest expression levels of both *spir* genes were detected in mouse oocytes, in which the proteins redundantly function as organizers of ooplasmic actin mesh, and in the assembly of the cleavage furrow during asymmetric meiotic cell divisions (Pfender et al., 2011). During embryogenesis major expression of both *spir* genes was detected in the developing nervous system (Pleiser et al., 2010; Schumacher et al., 2004). In addition, *spir-1* expression was found in the fetal liver, while *spir-2* expression was detected in early stages of intestinal development. In adult tissues the *spir-2* gene shows a rather broad expression pattern, which includes the epithelial cells of the digestive tract, testical spermatocytes, and neuronal cells of the nervous system. In contrast, *spir-1* is mainly expressed in neuronal cells of the nervous system. Minor expression levels were detected in testis and spleen.

Although the nervous system is a major tissue in which *spir-1* is expressed, nothing is known about the neural function of mammalian Spir proteins so far (Pfender et al., 2011; Schumacher et al., 2004). Here we have employed a mouse genetic approach to study the yet unknown function of Spir-1 in the nervous system. Expression of the full-length Spir-1 actin nucleator has been impaired by insertion of a gene-trap cassette into the mouse *spir-1* gene locus on chromosome 18. The overall brain anatomy of *spir-1* mutant mice was not altered, and visual and motor activities of the mice remained normal in both males and females. Behavioral studies, however, revealed a very specific and highly significant phenotype in fear response in the male mutant *spir-1* mice during contextual and cued fear conditioning experiments.

Materials and methods

Mouse strains and genotyping

All mice were kept under standard conventional conditions in the animal facility at the University Hospital of Regensburg, as well as the animal facility of the European Molecular Biology Laboratory at Monterotondo. Food and water were provided *ad libitum*. The *spir-1*$^{gt/+}$ mutant mice were generated by the *Texas A&M Institute for Genomic Medicine* (*TIGM*). Murine embryonic stem (ES) cells from the TIGM 129SvEv gene trap library (clone OST416113), with a gene-trap inserted into the fifth predicted intron of the mouse *spir-1* gene, were employed. The provided mice were intercrossed and pubs were monitored daily until weaning. All Spir-1 mutant mice analyzed were of mixed genetic background (129/SvEvBrd and C57BL/6). For imaging studies of dendritic spines, a GFP-expressing transgenic mouse line (*thy1*-GFP-M), originally devised by Feng et al., 2000, was crossed into the *spir-1*$^{gt/+}$ strain. *thy1*-GFP-M mice were a kind gift from Frank Bradke, DZNE, Bonn, Germany.

Animals were genotyped on tail extracted DNA by standard PCR method using following primer sets:

OST416113-forward (GCATGACTCTTTGGAGAGCATTAGC) and LTR-reverse (ATAAACCCTCTTGCAGTTGCATC) to amplify the mutant allele of *spir-1*$^{gt/gt}$ mice (142 bp fragment, 61 °C annealing) OST416113-forward (GCATGACTCTTTGGAGAGCATTAGC) and OST416113-reverse (AGGCTACAAGAACTGGTCTCCAAGC) to detect the wild type allele (269 bp fragment, 61 °C annealing temperature); *thy1*-GFP-forward (AAGTTCATCTGCACCACCG) and *thy1*-GFP-reverse (TCCTTGAAGAAGATGGTGCG) to amplify the *thy1*-GFP transgene as recommended by the Jackson laboratory (173 bp fragment, 60 °C annealing temperature)

Gene expression analysis by RT-PCR and qPCR

spir-1$^{+/+}$ and *spir-1*$^{gt/gt}$ mice were sacrificed by cervical dislocation and brains were immediately isolated. The telencephalon and the cerebellum were dissected and RNA was extracted using TRIzol reagent (*Invitrogen*). cDNA was generated using the *QuantiTec Reverse Transcription Kit* (*Qiagen*) and used as template in a standard PCR. Following primer sets were used:

KIND-KIND-forward (ATGGCTTGAAGGAAAACGAA) and KIND-KIND-reverse (GACTCAGTTGGGAGGTGAGC) (220 bp fragment, 60 °C annealing temperature); KIND-WH2-forward (CCGGTCCTATCAGGACGTTA) and KIND-WH2-reverse (ATGGGCAGAGGGTTGTACTG) (318 bp fragment, 60 °C annealing temperature).

PCR products were analyzed by agarose gel electrophoreses. DNA was visualized by ethidium bromide staining.

Quantitative analysis of gene expression was performed as described before (Pfender et al., 2011). cDNA generated from brain RNA preparations (5–7 month old mice) were employed to perform real time PCR experiments (qPCR) with the *Roche Light Cycler 480* using SYBR Green. The expression of *β2-microglobulin* was used for normalization. The following primer sets were used: *spire-2*: forward, 5′ aaa tca agc agg agc gga gg 3′, reverse 5′ ggt ggg ggc ttt gag cag ga 3′ (194 bp fragment). *β2-microglobulin*: forward, 5′ atg gga agc cga aca tact g 3′, reverse, 5′ cag tct cag tgg ggg tga at 3′.

Recombinant protein expression

Bacterially expressed recombinant proteins were employed for immunization of rabbits (His6-MBP-Spir-1-KIND) and for GST pulldown experiments (GST-Fmn-2-eFSI). The expression vector encoding a hexa-histidine tagged maltose-binding protein (MBP)/Spir-1-KIND fusion protein (pMalC2-His-hs-Spir-1-KIND,

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