Expression Pattern and Functional Analysis of Mouse Stam2 in the Olfactory System

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ABSTRACT

Gene trap mutant mice Stamgt1Gaj were investigated in order to elucidate in vivo function of Stam2 (signal transducing adaptor molecule 2) gene, which was in vitro implicated in sorting cargo marked by monoubiquitination toward degradation in the lysosomes. The expression analysis showed high Stam2 expression in the brain including the regions related to olfaction, and in the olfactory epithelium, but not in the respiratory part of nasal mucosa. To test mouse olfaction, ability to find chocolate hidden under the sawdust in the cage was examined. When food was given ad libitum before trials, mutants needed more time and failed more frequently to find the chocolate. In contrast, when the mice were fasted overnight before trial, there were no differences between mutants and wild type mice. No changes in morphology of olfactory mucosa were observed. The obtained results showed the existence of phenotype differences between mutants and wild type mice. However, different results of two approaches aimed to test olfaction, with and without food deprivation, currently do not enable to assign the particular function of Stam2 to olfaction. This emphasizes how slight modification of experimental setup in behavioural testing can cause important differences on the obtained results.

Key words: Stam2, olfactory test, olfactory mucosa, X-gal staining, mouse

Introduction

Two main directions of transport within the cell are secretory and endocytic pathways. The endocytic pathway starts by endocytosis, an intake of material into the cells, and it is continued via early and late endosomes (frequently referred as multivesicular bodies) toward lysosomes. Along this pathway STAM2 (signal transducing adaptor molecule 2) is implicated to have a role in selecting cargo marked by monoubiquitination toward degradation in the lysosomes. STAM2 is a 67-kDa protein with an NH2-terminal VHS (VPS27/HRS/STAM) domain, a UIM (ubiquitin interacting motif), an SH3 (Src homology 3) domain, a coiled-coil motif and an ITAM (immunoreceptor tyrosine-based activation motif) domain. It was together with STAM1 identified as associated with HRS (hepatocyte growth factor-regulated tyrosine kinase substrate)1. HRS and STAMs (via the tandemly located VHS and UIM domains) bind ubiquitin and ubiquitinated cargo and in this way sort the ubiquitinated cargo proteins for trafficking to the lysosome5.

One way to elucidate Stam2 function in vivo is to analyse the corresponding mouse mutants. Stam2gt1Gaj mutant was generated by gene trap method. In this method lacZ gene was inserted in frame of the investigated gene, which enables the visualisation of Stam2 expression pattern3. Moreover the gene trap vector insertion was expected to be mutagenic and the production of normal mRNA of the affected gene impaired. The importance of vesicular transport and intercellular signalling in the neurons, and the preliminary studies of the expression pattern indicated the possible role of Stam2 in the brain. In case of Stam1, its loss of function causes the loss of CA3 hippocampal neurons4. The role of Stam2 remained elusive as the complete loss of function seemed to result with no phenotype changes5. In order to investigate the consequences of Stam2 gene trap mutation and to get insight in Stam2 in vivo function the phenotype of homozygous carriers of gene trap mutation was analysed. Our hypothesis was that the mutation would affect...
the olfactory system, a dynamic area of the brain, which includes numerous receptors and generation/integration of new neurons.

The olfactory mucosa, consisting of the olfactory neuroepithelium and the underlying lamina propria, lines the posterodorsal nasal cavity in terrestrial mammals. The epithelium is composed of a limited number of cell types, among which are olfactory sensor neurons that are bipolar in shape and extend 12 or more modified cilia splaying out over the surface of the epithelium. Their thin unmyelinated axons join the fascicles of the olfactory nerve running to the main olfactory bulb. The axons of bulbar output neurons project in the olfactory tract to several higher-order brain structures, among them the olfactory nucleus projects to the medial dorsal nucleus of the thalamus, which in turn projects to the orbitofrontal cortex, the region of cortex involved in the conscious perception of smell.

Stam2 was highly expressed along the olfactory pathway and homozygous mice performed olfactory test worse than the wild type controls. However, the modification of olfactory test with overnight food deprivation changed the outcome and the difference in olfaction could not be observed any more, which made the possible role of Stam2 in olfaction questionable.

Materials and Methods

Animals

Mouse line Stam2(+/+) was obtained by gene trap method and kept on C57BL/6NCrl genetic background. Homozygous animals of Stam2(+/+) line were obtained by intercrosses of heterozygotes. Genotyping of transgenic animals was performed by PCR on tail DNA. In behavioural experiments homozygous and wild type mice from the same litter were used. All animals were group-housed in single-sex cages, maintained in 12:12 light:dark light cycle, with food and water available ad libitum. The subjects were 7 weeks old at the beginning of the test, and the testing was done during a 4 week period. A total of 19 wild type and 16 homozygous mice were subjected to olfactory test with food given ad libitum. A subgroup of 9 wild type and 5 homozygous animals was subjected to overnight fasting before testing.

Expression studies X-gal staining

Mice were anesthetized with Avertin (0.5 g/kg), perfused with fixative containing 2% formaldehyde and 0.2% glutaraldehyde in PBS. Brain and nasal mucosa were isolated and fixation was continued by immersion. X-gal (C14H15BrClNO6, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) staining was done as a whole mount for nasal mucosa. The brain was sectioned by vibratome (200 µm section thickness). Specimens were washed in PBS, and stained overnight in the dark at 37 °C in the mixture of 1 mg/mL X-gal (Merck), 2 mM MgCl2, 10 mM K2Fe(CN)6, 10 mM K3Fe(CN)6, 0.01% Igepal and 0.01% Na-deoxycholate in PBS. The stained specimens were cleared in 70% glycerol and photographed under the stereomicroscope (Olympus AX70).

Olfactory test

Olfactory testing was performed in a standard cage filled with sawdust. The cage was kept at the same place in the room during every trial. Eight grams of chocolate was grinned, placed on a small plate and buried in the sawdust always at the same location in the cage. Mice were placed in the opposite corner to the chocolate, and the latency to locate the hidden chocolate was recorded. Cut off time was eight minutes.

Two different approaches were applied. In the first approach, food was given ad libitum until the beginning of the test; in another, before every trial mice were food deprived overnight before testing, testing was repeated 2 times with each mouse, one trial every three days. Tests without fasting were repeated at least 4 times with each mouse, one trial every three days, and in the test with fasting tests were repeated twice with each mouse. Test scores have been attributed to each trial (finding chocolate in less than 1 minute – score 1; between 1 and 5 minutes – score 2; more than 5 minutes or chocolate not found – score 3). Score attributed to every mouse was calculated as an average score of the repeated trials. The scores were graded and compared using the Wilcoxon-Mann-Whitney two sample rank sum test.

Histology and electron microscopy

Homozygous and wild type mice were anesthetized with Avertin (0.5 g/kg), perfused first with PBS and subsequently with 4% paraformaldehyde in PBS for histology, or with 6% glutaraldehyde in PB for electron microscopy. For histology, mouse heads were decalcinated with EDTA, embedded in paraffin, and sectioned with microtome. The sections were stained with haematoxylin and eosin. For electron microscopy, the olfactory mucosa was dissected, and the specimens were postfixed in OsO4, dehydrated in ascending concentration of ethanol, and embedded in Durcupan (Fluka). The ultrathin sections stained with uranyl acetate and lead citrate were observed under transmission electron microscope (Zeiss 902A).

Results

Stam2 expression pattern

To determine the expression pattern of Stam2 along the olfactory pathway β-galactosidase presence, which mirrors the expression pattern of Stam2 was determined. Stam2 was expressed along the olfactory pathway in mouse, from the olfactory mucosa to the brain cortex. Stam2 was expressed in the olfactory region, but not in the respiratory region of septal nasal mucosa (Figure 1a and b). In the main olfactory bulb Stam2 was expressed in the glomerular, mitral cell, and granule cell layers. Expression of Stam2 was present in the anterior olfactory nucleus, in the tenia tecta, and in the piriform cortex (Figure 1c and d).
Olfactory tests

Two different approaches were applied in olfactory testing. In the first, food was given to mice ad libitum before the test; in the second, the mice were fasted overnight before being tested. The mutant and the wild type mice general health, including weight, senses of vision, hearing and touch, appearance of the coat, skin, whiskers, nails, and pads, was checked before the olfactory test.

In case of trials where food was given ad libitum before olfactory testing, the wild type mice found the chocolate more frequently than Stam2\(^{gt1Gaj/gt1Gaj}\) mutants. The proportion of successful chocolate finding for Stam2\(^{gt1Gaj/gt1Gaj}\) was 0.53 whereas the proportion of successful chocolate finding trial for Stam2\(^{+/+}\) was 0.88. The difference in frequency of successful finding between homozygous and wild type mice was significant (\(\chi^2=30.94, p<2.7\times10^{-8}\)).

Table 1

<table>
<thead>
<tr>
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<th>Total number of trials</th>
<th>Number of successful findings</th>
<th>Percentage of successful findings</th>
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<tr>
<td>Mice fed ad libitum*</td>
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<tr>
<td>wt</td>
<td>108</td>
<td>95</td>
<td>88%</td>
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<tr>
<td>hm</td>
<td>105</td>
<td>56</td>
<td>53%</td>
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<tr>
<td>Mice fasted overnight</td>
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</tr>
<tr>
<td>wt</td>
<td>18</td>
<td>17</td>
<td>94%</td>
</tr>
<tr>
<td>hm</td>
<td>10</td>
<td>10</td>
<td>100%</td>
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*wt – wild type mice, hm – homozygous mice, \(\chi^2=30.94, p<2.7\times10^{-8}\)

Although the observed differences were significant, still the high variability of the results of each animal, and the presence of the wild type mice which did not find the chocolate, suggested that the experimental paradigm could be modified and improved. This was attempted by overnight fasting of the mice before trials, which was expected to increase the mouse motivation to find the chocolate. In those trials where mice were fasted both groups of mice improved their efficiencies in finding chocolate (23/28 trials had score 1; i.e. less than 1 minute to find the chocolate). Moreover, the homozygous animals had the comparable efficiency as the wild type mice (Table 1).

To verify if the differences in olfactory test could be related to some defect present in the mutant mice the morphology of olfactory mucosa was compared in wild
type and mutant mice by light and electron microscopy, what did not reveal any differences (Figure 3).

![Image]

Discussion

The knockout experiments of Stam1 and Stam2 clearly showed the importance of Stam for the mammalian organism. Double knockout resulted with embryo lethality by E11.5, with a defect in ventral folding morphogenesis. Single knockouts had substantially milder consequences, in case of Stam1 there was a loss of CA3 pyramidal neurons, and in case of Stam2 no phenotype changes were detected. This showed that these genes can in most cases compensate the loss of each other. Nevertheless, in the view of importance for the human medicine, where simultaneous mutations of similar genes represent extremely rare events, identifying the consequences of single loss of function with subtle phenotypes resembling human diseases is very valuable. Our findings showed that the phenotype differences between wild type and Stam2 mutant do exist, which implicates a different function of Stam2, which cannot be compensated with Stam1.

The search for phenotype changes and the corresponding Stam2 function was based on its expression pattern. High expression in developing and adult brain, and in particular along the olfactory pathway indicated that Stam2 would have an important brain function. This idea was additionally supported with the brain phenotype of Stam1. The expression pattern analysis showing Stam2 expression not only in the brain regions related to olfaction, but as well in the olfactory region of the nasal mucosa substantiated the need to test olfaction in the mutants. Indeed the test showed the differences between mutants and wild type mice, but the differences were dependent on the test design.

There is an important constant attempt of scientific community to standardize the behavioural tests on rodents. This is of particular significance in the field of functional genetics, as it enables to compare the performance of different, but related mutants. One of the surprising results of our experiments is that the same test paradigm gave substantially different results when a single parameter was changed, i.e. feeding of animals before the test. One could argue that the test should be always performed with overnight fasting of the animals, which gives results with less variability. However, if we would screen the behaviour of our mutants in this way, we would completely miss the fact that the behavioural differences exist. This exemplifies the advantages and disadvantages of the predesigned screens, and could contribute to the idea that every transgenic mouse mutant would show the phenotype if one looks carefully enough.

The fact that the differences between wild type mice and mutants in the olfactory test disappeared when the mice were fasted overnight indicated that currently we cannot clarify if the differences are related to olfaction. The sense of smell could be only slightly affected, which when mice are hungry and highly motivated leaves enough abilities for the mutants to detect the presence and find the hidden chocolate. On the other hand the observed differences could be completely unrelated to olfaction, and a result of changes in motivation. Moreover variety of reasons could distract the attention of mice to look for chocolate some of them even unrelated to brain functioning. Different behavioural tests and further studies of mutant mice are necessary to clarify this fact.

In conclusion, our findings confirmed presence of phenotype changes in Stam2 mutants, and implicated the particular role of Stam2 in mice. Nevertheless, it remained unclear if Stam2 function could be related to olfaction or the mice exhibit another unrelated problem, which was reflected in the olfactory test.

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IZRAŽAJ I ANALIZA ULOGE MIŠJEG GENA STAM2 U NJUŠNOM SUSTAVU

SAŽETAK