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Short communication

Unexpected rescue of alpha-synuclein and multimerin1 deletion in C57BL/6JOlaHsd mice by beta-adducin knockout

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Abstract

Uniform genetic background of inbred mouse strains is essential in experiments with genetically modified mice. In order to assess *Add2* (beta-adducin) function, its null mutation was produced in embryonic stem cells derived from 129Sv mouse and the subsequently obtained mouse mutants were backcrossed for 6 generations with C57BL/6JOlaHsd strain. Comparison of brain proteins between mutated and control animals by two-dimensional gels linked to mass spectroscopy analysis showed expression of *Snca* (alpha-synuclein) in the mutated animals, but unexpectedly not in the control C57BL/6JOlaHsd mice. Comparison between C57BL/6JOlaHsd and C57BL/6NCrl mice confirmed the presence of a deletion encompassing *Snca* and in addition *Mmrn1* (multimerin1) loci in C57BL/6JOlaHsd strain. The segregation of mutated *Add2* together with an adjacent part of the chromosome 6 derived from 129Sv mice, rescued the loss of these two genes in knockout mice on C57BL/6JOlaHsd background. The fact that *Add2* knockout was compared with the C57BL/6JOlaHsd mouse strain, which is actually a double knockout of *Snca* and *Mmrn1* emphasizes a need for information provided by commercial suppliers and of exact denominations of substrains used in research.

Introduction

The widespread presence of inbred strains in rodents allows experimenting with genetically identical animals, not only in the same, but also in different and remote laboratories. Therefore the use of inbred strains has become standard in a wide range of experimental approaches. Their use is essential in gene targeting experiments, where the consequences of the introduced genetic change are best characterized on the uniform genetic background of the inbred strain.

This work deals with gene targeting of beta-adducin (*Add2*), which was performed on MPI-II ES cells derived from 129Sv inbred mice (Voss et al., 1997). The procedure resulted in the loss of function (i.e. knockout) of *Add2* (Muro et al., 2000). To characterize the phenotypic consequences of the introduced mutation and to gain insight into the function of *Add2*, the mice carrying the introduced mutation were bred with C57BL/6 inbred strain for 6 generations to obtain mice that substantially differ from the original C57BL/6

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strain in only one (i.e. *Add2*) locus. Therefore the observed phenotypic changes, including spherocytic elliptocytosis and hypertension, were assigned to loss of *Add2* function (Marro et al., 2000; Muro et al., 2000).

Here we report that *Add2* knockout mice differ substantially from C57BL/6 strain not only in *Add2* locus, but also in alpha-synuclein (*Snca*) and multimerin1 (*Mmrn1*) loci. This was the result of the use of C57BL/6JOlaHsd inbred mouse strain obtained from a commercial supplier (Harlan, Italy), which had a spontaneously arisen deletion of *Snca* gene (Specht & Schoepfer, 2001). As *Snca* locus is located only 6 centiMorgans away from *Add2* locus, segregation of normal *Snca* together with modified *Add2* resulted in the rescue of this deletion. As a consequence, the original C57Bl/6JOlaHsd inbred strain contained a deletion of *Snca*, while *Add2* knockout mice did not. Moreover, together with *Snca*, the deletion includes an additional neighboring gene, *Mmrn1*.

Methods, results and discussion

To analyze the consequences of the null mutation of *Add2* in the brain we searched for differentially expressed proteins between *Add2* knockout mice and the wild type control, C57BL/6JOlaHsd mice derived from Harlan, San Pietro al Natisone, Italy. The comparison of the two dimensional protein gels identified a spot, which was missing in brain extracts from control C57BL/6JOlaHsd mice (Figure 1a). The selected spot on the gel was extracted and analyzed by electrospray ionization mass spectrometry (Finnigan LCQ DECA, Thermo-Finningan Corp) (Porro et al., 2004). Mass spectrometry indicated that it corresponded to SNCA (alpha-synuclein, 56% protein coverage, data not shown). Western blot analysis with anti-SNCA antibody showed a signal only in the extracts prepared from the brains of *Add2* deficient mice, confirming the mass spectrometry results (Figure 1b). As *Snca* was known to be expressed in the brain (Abeliovich et al., 2000), the results could not be explained as a consequence of *Add2* mutation. Bioinformatic examination of the chromosome location of *Snca* gene indicated that it is on the same chromosome 6, only 6 centiMorgans away from *Add2* locus (Figure 2a), with an intergenic region between both genes of about 25 Mbp. Literature search showed that C57BL/6JOlaHsd

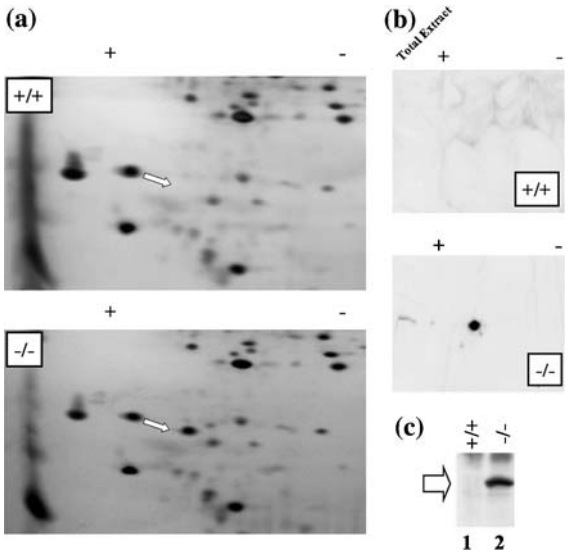

Figure 1. SNCA is absent in C57BL/6JOlaHsd mice. **Panel a.** Two-dimensional gel (2D) analysis (silver stained) of equal amounts of brain protein extracts from the wild type and mutant mice showing a close-up of the region where a spot in the control brain extracts is missing (indicated by the arrow). Mass spectrometry analysis of the spot present in the KO extract identified the spot as SNCA. **Panels b and c.** Western blot analysis of 2D and 1D gels with anti-SNCA antibody (Becton Dickinson), run with the same extracts used in Panel a. 300 μg of total brain extract (control and KO) were loaded in the first dimension (pH range 3–10 non-linear) of the western blot shown in Panel b, while 80 μg of proteins were loaded in Panel c. Each 2D gel shown in Panel b contains a lane of the same extract separated by two-dimensional analysis that was run only in the SDS-PAGE dimension (on the left, indicated as ''Total extract'').

mice originating from Harlan, Bicester, UK had a deletion of *Snca* locus (Specht & Schoepfer, 2001). Therefore, the observed difference in *Snca* expression was not caused by the loss of *Add2* function, but by the absence of *Snca* in the control strain. The normal *Snca* locus present in *Add2* knockout mice originated from 129Sv derived MPI-II ES cells. The segregation of mutated *Add2* together with the adjacent part of the chromosome 6 derived from 129Sv mice unexpectedly rescued *Snca* deletion in knockout mice on C57BL/6JOlaHsd background.

The absence of *Snca* gene in the wild type strain raised the important issue of selection of appropriate controls. To characterize the deletion of *Snca* two subpopulations of C57BL/6 mice, C57BL/6JOlaHsd (derived from Harlan, San Pietro al Natisone, Italy) and C57BL/6NCrl (derived from Charles River, Les Oncins, France),

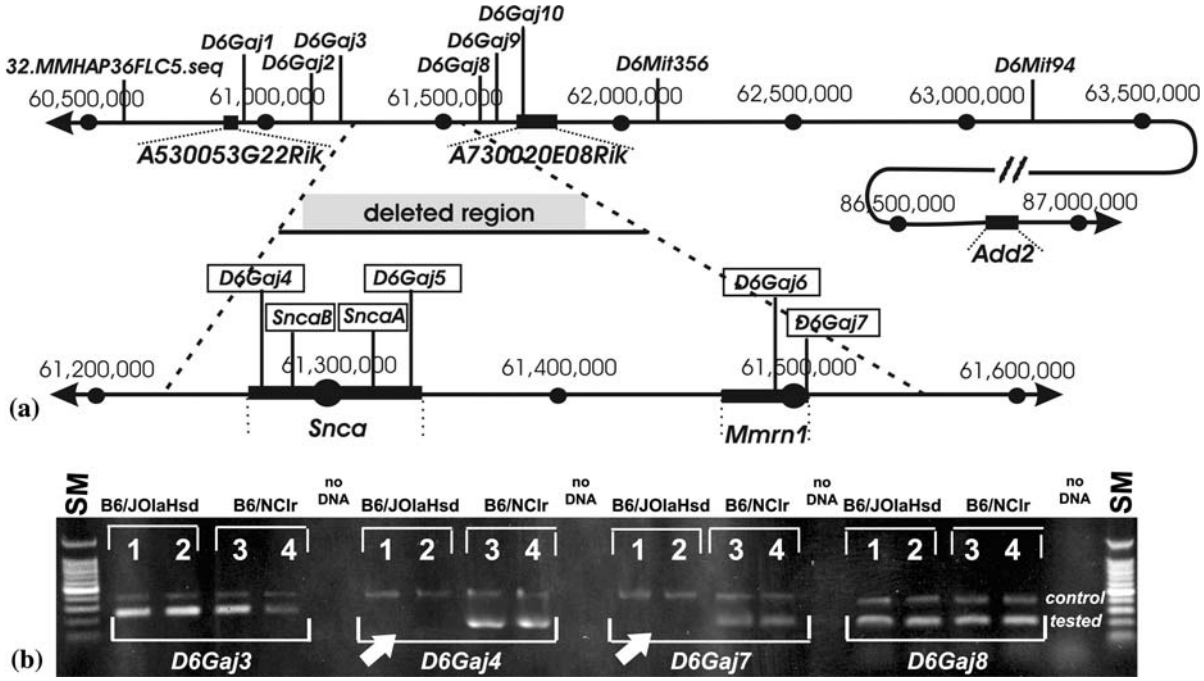

Figure 2. C57Bl/6JOlaHsd mice lack genes *Snca* and *Mmrn1*. **Panel a.** Part of mouse chromosome 6 showing the chromosomal localization of *Snca*, *Mmrn1*, *Add2*, *A530053G22Rik*, and *A730020E08Rik* genes. DNA segments amplified by PCR (*SncaA*, and *SncaB* (Specht & Schoepfer, 2001), *32.MMHAP36FLC5.seq* (UniSTS:123983), *D6Mit356* (UniSTS:130907), *D6Mit94* (UniSTS:130997), *D6Gaj1* (gtacccaatgcacatcatgc, tctagccttcaacaagcctca, 150 bp), *D6Gaj2* (aacaccagagcccaaacaag, aatggcctaggcacacattc, 200 bp), *D6Gaj3* (gccctttcttgcatgatgt, cactcaaaggcagaaccaca, 245 bp), *D6Gaj4* (cagcacacaagaccctgcta, gtcattgcacccaatctcct, 130 bp), *D6Gaj5* (gagctccagcgaacagactc, tggcactcaaatccactctg, 170 bp), *D6Gaj6* (ccatccatctttccgtgagt, agggcggcttgagttttaat, 211 bp), *D6Gaj7* (ctggggatgccttatttgaa, aaaaggagagcatgctggag, 176 bp), *D6Gaj8* (tgtcctctgaaatgcacagc, tgatgcaagaaatgctggac, 185 bp), *D6Gaj9* (catgacacaagggagtgtgg, gtggttgcccctgacttcta, 192 bp) and *D6Gaj10* (accgagggatttgtgaatg, cttccagttccaaggaggag, 150 bp) are marked accordingly, and those, which revealed the presence of a deletion, are boxed. **Panel b.** Gel electrophoresis of PCR amplified DNA segments *D6Gaj3*, *D6Gaj4*, *D6Gaj7* and *D6Gaj8* (lower bands) and the positive control (upper bands, *D6Gaj11*, tctgcctgccttgttttctt, ggacagcagcctccttaga, 401 bp, located in *Nol1* gene on the same chromosome, 30 cM from the investigated region). In comparison to C57Bl/6NCrl mice (B6/NCrl, lanes 3 and 4) it is visible that C57BL/6JOlaHsd mice (B6/JOlaHsd, lanes 1 and 2) lack amplified products *D6Gaj4* and *D6Gaj7* (thick arrows) specific for *Snca* and *Mmrn1*, respectively. no DNA – negative control without DNA, SM – 100 bp ladder as a size marker.

were compared. Polymerase chain reactions (PCR) of genomic DNA with primer pairs specific for *Snca* genomic sequence (*SncaA* and *SncaB* (Specht & Schoepfer, 2001)) showed that the deletion was present in C57BL/6JOlaHsd strain, but not in C57BL/6NCrl animals. PCRs with primer pairs specific for the first exon of *Snca* gene and its 5′ untranslated region (*D6Gaj4*), and with primer pairs specific for the last exon of the gene and its 3′ untranslated regions (*D6Gaj5*) confirmed that the complete coding sequence of *Snca* gene was deleted in these mice (Figure 2).

To determine the range of the deletion, a series of PCR was performed in such a way that the corresponding genomic region was "walked" utilizing several primer pairs (Figure 2). The approximate size of the deleted region was estimated to be between 261,740 and 398,300 bp. Bioinformatic examination suggested that it included another gene, *Mmrn1* (multimerin1), which was situated near *Snca* locus in the direction of the telomere. The deletion of complete *Mmrn1* gene was confirmed with PCR using *D6Gaj7* primers designed within the 5′ untranslated region of *Mmrn1* gene (Figure 2). The flanking genes in both directions, RIKEN cDNA A530053G22 gene (*A530053G22 Rik*) and RIKEN cDNA A730020E08 gene (*A730 020E08Rik*), were preserved and not within the range of the deletion (Figure 2).

A peculiarity of the inbred mouse strains is that due to the sibling breeding, novel mutations will be either lost, or stabilized as homozygous in case they do not produce any obvious phenotype. Nevertheless, considering the exciting advantage

of being able to use experimental animals of constant genetic background around the world, minor attention has been attributed to the possibility that new mutations could appear within an inbred strain and indeed it has been regarded as a minor drawback (Wade et al., 2002). The current work clearly shows that genetic drift occurs not only in an isolated breeding stock in some remote laboratory, but as well in the founding breeders, which via commercial suppliers provided animals for numerous, widely distributed animal facilities (e.g. England, Italy, Croatia).

The described deletion is important, not only because C57BL/6 is commonly used as a background strain for genetically modified mice, but also because of the possibility that C57BL/6JOlaHsd substrain of mice can be used as a substitute for *Snca* knockout in neuroscience research. SNCA is an abundant soluble presynaptic protein in brain neurons, its insoluble fibrillar aggregates are implicated in number of neurodegenerative diseases, referred to as alpha-synucleinopathies, most notably Parkinson's and Alzheimer's diseases (Dev et al., 2003). Indeed, complete *Snca* locus was deleted and the deletion also included apparently unrelated *Mmrn1* gene. *Mmrn1* appears not to be expressed in the brain, but in platelets and is believed to play a role in hemostasis (Hayward, 1997; Leimeister et al., 2002). C57BL/6JOlaHsd strain appeared to be a double knockout for both, *Snca* and *Mmrn1* genes. This finding was recently confirmed on mice kept in England thus validating that the deletion is the same (named *Del(6)Snca1Slab*; Specht & Schoepfer, 2004). No other known or predicted gene is included in the deletion. Although C57BL/6JOlaHsd mice represent a double knockout, they do not show any obvious phenotype changes (Chen et al., 2002; Siegmund et al., 2005). This leads to the remarkable ambivalence of these mice. At the same time they may be used as a wild type control for investigating the consequences of genetic modifications of other genes and conversely as an appropriate model to investigate the function of the two genes affected by the deletion.

The fact that the inbred strains contain an array of recessive homozygous mutations that are largely unknown (e.g. Fenoy et al., 1992; Montagutelli, 2000) could implicate that the awareness of the deletion in C57BL/6JOlaHsd strain does not substantially change its suitability for experimental use as a standard inbred strain and a wild type control. The danger of carrying over flanking genes from 129Sv genome and the possibility of them influencing the phenotype has been long since recognized. Even after 12 generations of backcrossing, the average expected size of the flanking region will be 16 cM or more than 1% of genome (Banbury Conference on Genetic Background in Mice, 1997; Crusio, 2004). Nevertheless, we believe that the presented data seriously question the suitability of the C57BL/6JOlaHsd strain for use as a wild type control in genetic experiments, especially in the field of neuroscience. In the described case due to the rescue of the deletion, the conclusions regarding the Add2 gene function arose from the phenotype comparison of a single knockout strain (*Add2*−/−, *Snca*+/+, *Mmrn*+/+) with that of the unrelated double knockout mice (*Add2*+/+, *Snca*−/−, *Mmrn1*−/−). In the analysis of numerous knockouts done by other researchers, when the targeted loci were not linked to *Snca* and *Mmrn1* deletion, the consequence of the use of C57BL/6JOlaHsd strain was that triple knockouts involving the investigated genes were compared with the double knockout used as the wild type control. Although we have no doubts on the interpretations of the results obtained, it is certainly part of the scrutiny of scientific reasoning to take this fact in the account. As the data on the used substrains of C57BL/6 mice are not frequently present in the publications, it is almost impossible to back trace the extent of this phenomenon.

Keeping all of these arguments in mind, we would like to emphasize that the use of C57BL/6JOlaHsd strain for experimental purposes should be an informed choice. This requires clear denomination of the strain with accompanying information in case of its distribution, and its strict declaration in published works (i.e. C57BL/6JOlaHsd instead of C57BL/6). Moreover the awareness of this deletion in mice provided by commercial suppliers should help to avoid possible mistakes in the interpretation of observed phenotypes of congenic strains on this background.

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