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Resource

Dissecting the effects of mtDNA variations on complex traits using mouse conplastic strains

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Previous reports have demonstrated that the mtDNA of mouse common inbred strains (CIS) originated from a single female ancestor and that mtDNA mutations occurred during CIS establishment. This situation provides a unique opportunity to investigate the impact of individual mtDNA variations on complex traits in mammals. In this study, we compiled the complete mtDNA sequences of 52 mouse CIS. Phylogenetic analysis demonstrated that 50 of the 52 CIS descended from a single female *Mus musculus domesticus* mouse, and mtDNA mutations have accumulated in 26 of the CIS. We then generated conplastic strains on the C57BL/6J background for 12 mtDNA variants with one to three functional mtDNA mutations. We also generated conplastic strains for mtDNA variants of the four *M. musculus* subspecies, each of which contains hundreds of mtDNA variations. In total, a panel of conplastic strains was generated for 16 mtDNA variants. Phenotypic analysis of the conplastic strains demonstrated that mtDNA variations affect susceptibility to experimental autoimmune encephalomyelitis and anxiety-related behavior, which confirms that mtDNA variations affect complex traits. Thus, we have developed a unique genetic resource that will facilitate exploration of the biochemical and physiological roles of mitochondria in complex traits.

[Supplemental material is available online at www.genome.org.]

The mammalian mitochondrial genome (mtDNA) is a closed, circular, double-stranded DNA with genes encoding mitochondrial oxidative phosphorylation (OXPHOS) enzyme complexes that are essential for ATP production (Anderson et al. 1981). In humans, mtDNA variations can be rare pathogenic mutations or deletions that cause maternally inherited mtDNA disorders or common mtDNA variants that lead to functional changes and thus predispose individuals for polygenic diseases (Wallace et al. 1988; Taylor and Turnbull 2005). However, unique genetic characteristics of mammalian mtDNA, for example, the lack of recombination and the transmission as one haplotype, hamper efforts to identify precisely the variations responsible for traits and effects and to define the biochemical and physiological consequences of individual variations. The same characteristics complicate the genetic manipulation of mtDNA. Although several transmitochondrial mice have been produced, a mouse with a single mtDNA mutation has not yet been generated (Pinkert and Trounce 2002).

An unexpected observation provided a potential solution to the limitations of mtDNA. In 1982, Ferris et al. (1982) demonstrated that most mouse common inbred strains (CIS) had the same mtDNA RFLP pattern, which suggested that mouse CIS were descended from a single female. This conclusion was confirmed by sequencing the mtDNA of CIS (Johnson et al. 2001; Bayona-Bafaluy et al. 2003; Mathews et al. 2005; Goios et al. 2007), which also demonstrated that several mutations had accumulated during the establishment of the strains, with a higher ratio of non-synonymous/synonymous mutations than in wild mice (Goios et al. 2007). Those mutations are of potential functional importance, as has been demonstrated in recent studies (Johnson et al. 2001; Mathews et al. 2005; Moreno-Loshuertos et al. 2006). These findings suggest that the systematic production of conplastic strains (mitochondrial substitution strains) with CIS mtDNA carrying one or more functional mutations would constitute a genetic resource to investigate the roles of individual mtDNA variations in complex traits.

In the present study, we report the generation of a unique public resource of 16 conplastic strains that carry potential functional mtDNA mutations on a C57BL/6J genetic background. In humans, mtDNA variations are associated with many neurological diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis (MS), and bipolar disorder (Shoffner et al. 1993; Kalman and Alder 1998; Kato et al. 2001; van der Walt et al. 2003). Recently, we reported that the mtDNA allele nt13708A increases susceptibility to MS (Yu et al. 2008). To test the usefulness of this genetic resource, we investigated the conplastic strains with regard to nervous system phenotypes, in particular experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, and anxiety-like behavior as an aspect of bipolar disorder. We show that mtDNA variations affect both phenotypes, which suggests that conplastic strains are a useful genetic resource for investigating the role of mtDNA in complex traits.

Results

Sequence and phylogeny of mtDNA in mouse inbred strains

We sequenced the complete mtDNA of 27 common inbred strains: 129P3/J, BPH/2J, BPL/1J, BPN/3J, BUB/BnJ, C57BL/10J,

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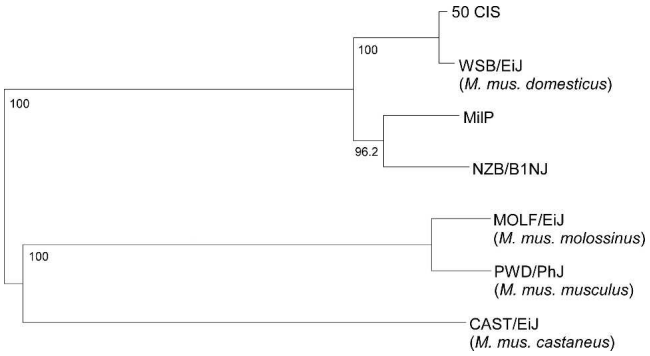


Figure 1. Phylogenetic tree of mtDNA from mouse inbred strains. The tree was generated with ClustalW software on the basis of the mtDNA sequences of 56 mouse inbred strains, including 52 classic inbred strains (CIS) and four wild-derived inbred strains (WIS). For the four WIS, the taxonomic classification is indicated. Numbers correspond to bootstrap values (percentage of 1000 total bootstrap replicates).

CE/J, I/LnJ, LG/J, LP/J, LT/SvEi, MA/MyJ, MRL/MpJ, NOR/LtJ, P/J, PL/J, RF/J, RIIIS/J, SB/LeJ, SEA/GnJ, SJL/J, SM/J, SOD1/EiJ, STR/ort, SWR/J, TALLYHO/JngJ, and YBR/EiJ. Including the previously published sequences of 29 inbred strains (Loveland et al. 1990; Johnson et al. 2001; Bayona-Bafaluy et al. 2003; Mathews et al. 2005; Goios et al. 2007), the complete mtDNA sequences of 56 inbred mouse strains were collected. The strains consisted of 52 CIS and four wild-derived inbred strains (WIS). A phylogenetic tree based on the mtDNA sequences demonstrated that all 52 CIS, with the exception of NZB/B1NJ and MilP, shared a subbranch related to WSB/EiJ, a WIS belonging to the *Mus musculus domesticus* subspecies (Fig. 1). This confirmed the previous conclusion that most CIS were descended from one *M. musculus domesticus* female mouse (Ferris et al. 1982).

To further characterize the 50 CIS with the same maternal origin, we generated a parsimonious tree for all mtDNA variations in these strains. The mtDNA sequences of the 50 mouse strains formed a star-like network, with a main group of 18 strains in the center (Fig. 2). The other 32 strains were one to three variations apart from the central group. Four mtDNA variations—nt9348 G/A, nt9416 C/T, nt13053 T/C, and nt14698 T/C—were carried by at least two strains, which indicates that they occurred in the early stage of development of CIS. Each of the other variations was carried by only one strain, which suggests that most mtDNA variations in CIS were recent mutations. In total, 26 mtDNA sequence variations were identified in the 52 CIS. Four of the variations were located in tRNA- or rRNA-coding regions, and 22 were in protein-coding regions, including 15 nonsynonymous and seven synonymous variations (Table 1).

Generation of mouse conplastic strains

Next, we generated conplastic strains for potentially functional mtDNA mutations by introducing the mtDNA variant from the donor into recipient strains with successive backcrossing for 10 or more generations. The 15 nonsynonymous and four RNA variations were considered to be potentially functional mtDNA mutations. We generated conplastic strains for mtDNA variants from 12 CIS, each of which differs from the reference mtDNA by only one to three polymorphisms. Those 12 mtDNA variants encompass 14 potentially functional mtDNA variations, including 12 nonsynonymous polymorphisms and two polymorphisms in tRNA genes. Owing to the unavailability of mtDNA donor strains, we did not generate conplastic strains for the other five potentially functional mtDNA mutations. We also generated conplastic strains for the mtDNA variants of four *M. musculus* subspecies, each of which carries hundreds of sequence variations compared with the reference sequence. The strain C57BL/6J was used as the recipient strain for all conplastic strains. Altogether, 16 conplastic strains on the C57BL/6J genetic background were generated or are in production (Table 2).

Effects of mtDNA variants on EAE

To evaluate the usefulness of this unique genetic resource, we performed some phenotypic analyses on a subset of the available conplastic strains. The C57BL/6J strain is used widely for induction of EAE by immunization with MOG 53-55 peptides. In humans, it has been suggested that mtDNA variations affect susceptibility to MS (Yu et al. 2008). Therefore, we investigated the effect of mtDNA variants on EAE. For the EAE model, we used five conplastic strains: C57BL/6J, C57BL/6J-mt$^{AKR/J}$, C57BL/6J-mt$^{FVB/N}$, C57BL/6J-mt$^{PWD/Ph}$, and C57BL/6J-mt$^{NZB/B1N}$. Male and female mice from each conplastic strain were used equally in the experiments. No difference in the clinical score between males and females was observed. Thus, to perform the analysis, we combined data from both sexes for each conplastic strain. After EAE induction, the disease began on day 3, reached a peak on days 11–17, then started to subside and reached the chronic phase on day 30 after immunization. All five conplastic strains showed a similar course of disease. However, the C57BL/6J-mt$^{NZB/B1N}$ strain was more resistant to EAE than were the other four conplastic strains (Fig. 3A). Further analysis demonstrated that the resistance was due to a lower disease incidence in C57BL/6J-mt$^{NZB/B1N}$ strain (~64%) than in the other four strains (100%) (Fig. 3B). Therefore, we concluded that mtDNA variations affect the susceptibility to EAE.

Effects of mtDNA variants on behavior

The C57BL/6J mtDNA differs from AKR/J mtDNA only in nucleotide 9821 (nt9821; an A-repeat variation), and the FVB/N mtDNA differs from AKR/J mtDNA only in nt7778 (G/T variation), the latter of which lies in the mitochondrial ATPase subunit-8 gene. In humans, mutations in the ATPase subunit-6 and subunit-8 genes were found in patients with a family history of bipolar disorder with manic and depressive episodes, for which maternal transmission was suspected (Kato et al. 2001). Therefore, C57BL/6J, C57BL/6J-mt$^{AKR/J}$, and C57BL/6J-mt$^{FVB/N}$ strains were used to investigate the role of single mtDNA polymorphisms in such complex traits as anxiety-like behavior and related neurophysiological markers. We examined anxiety by testing the mice in the elevated plus maze. Interestingly, C57BL/6J-mt$^{FVB/N}$ mice were significantly more anxious than C57BL/6J-mt$^{AKR/J}$ and C57BL/6J mice (Fig. 4A). They spent less time in the open arms of the maze than C57BL/6J mice and walked shorter distances in open arms than C57BL/6J-mt$^{AKR/J}$ and C57BL/6J mice.

To test whether this effect was due to overall reduced locomotor activity, we counted total arm entries, entries into closed arms, and distances walked in closed arms. C57BL/6J-mt$^{FVB/N}$ showed less total arm entries and entries into closed arms but no difference in distances walked in closed arms (Fig. 4B). The C57BL/6J-mt$^{FVB/N}$ mice did not leave closed arms as often as the

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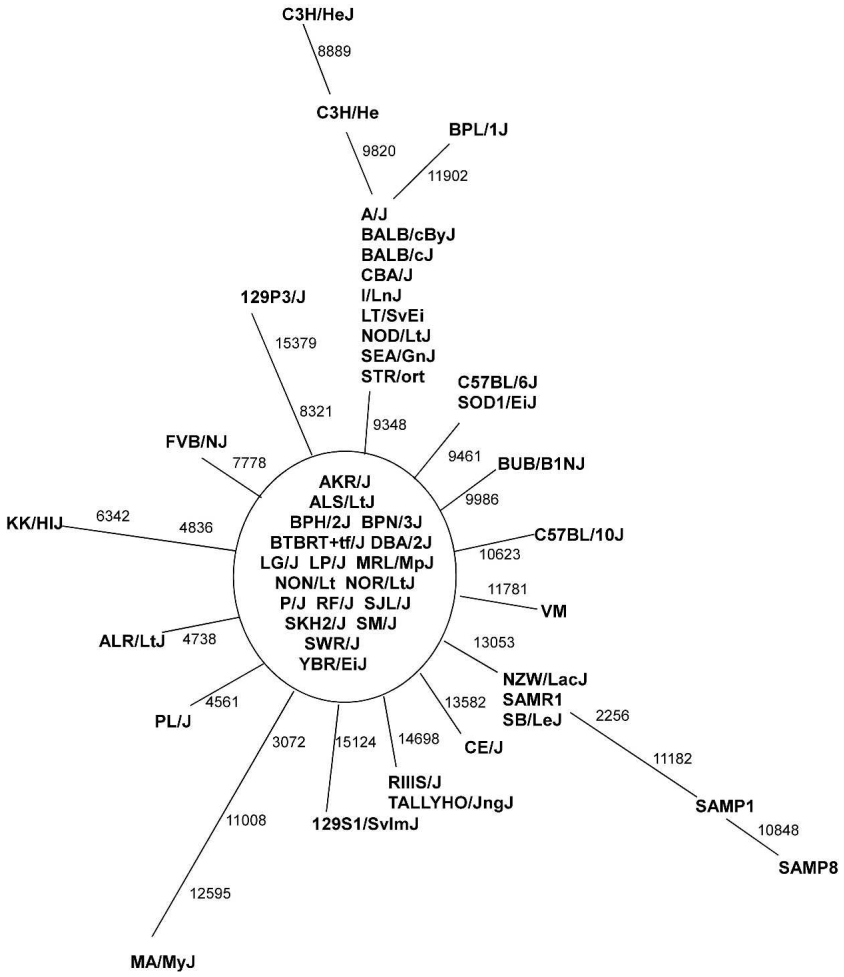


Figure 2. The most parsimonious tree for all mouse mtDNA variations found in 50 common inbred strains. The mtDNA sequence of the AKR/J strain (AB042432) was taken as the reference sequence, with the positions of the mtDNA variations indicated. nt9821, an A-repeat variation, and some other heteroplasmic variations were not included.

other strains, not even via the central platform to the opposite closed arms (data not shown), and thus rarely re-entered closed arms. These differences cannot be explained by reduced mobility of the C57BL/6J-mt$^{FVB/N}$ mice because the distance walked in closed arms was not affected by the mtDNA mutation.

To determine whether behavioral differences were reflected by stress hormones or brain activity, serum corticosterone and brain neurotransmitter levels were analyzed. There were no differences between mouse strains, which indicated that the behavioral differences were based on different pathways (Supplemental Tables 1, 2). Taken together, these results indicate that single mtDNA variations can affect anxiety-related behavior.

Discussion

In this study, we generated a panel of conplastic strains on the C57BL/6J genetic background. Compared with the control strain, the 16 new strains carried either few or numerous mtDNA variations. We showed that the mtDNA variations could affect susceptibility to EAE. This observation is consistent with those in humans, in which mtDNA variations are associated with susceptibility to MS (Yu et al. 2008). Our results provide confirmatory evidence for the role of mitochondria in MS. Furthermore, we provide a mouse model for investigating the mechanism of the mitochondrial contribution to MS.

Evidence exists for the effects of mtDNA variations on central nervous system functions. mtDNA deletion affects the regulation of diurnal rhythms in humans and mice and the estrus cycle in mice. A single study has searched for associations between mtDNA variations and cognitive functions in humans (Skuder et al. 1995). A mouse study associated mtDNA variations with differences in cognitive functions and exploratory behavior but not with anxiety-like behavior (Roubertoux et al. 2003). In the present study, we show that a single mtDNA variation can affect anxiety-like behavior. This discrepancy with the prior study can be explained by the fact that Roubertoux and colleagues used different mouse strains than those employed here. In particular, their mice did not have nonsynonymous variations in the genes mt-A*tp*6 and mt-*tp*8, which are the genes that have been associated with bipolar disorder in humans (Kato et al. 2001). The C57BL/6J-mt$^{FVB/N}$ mice carry a mutation in the *mt-Atp8* gene that could be responsible for the increased anxiety-like behavior observed in these mice. Thus, our mitochondrial substitution strains represent a promising resource to study anxiety as an aspect of bipolar disorder.

In humans, a growing body of evidence suggests that mtDNA polymorphisms are associated with complex diseases or traits such as diabetes, Alzheimer's disease, Parkinson's disease, MS, cancer, aging, and bipolar disorder (De Benedictis et al. 1999; McMahon et al. 2000; Kato et al. 2001; Poulton et al. 2002; van der Walt et al. 2003, 2004; Canter et al. 2005; Mohlke et al. 2005; Kalman 2006; Kazuno et al. 2006). However, dissection of the role of individual mt gene variants has been hampered by the lack of appropriate experimental models. The conplastic strains generated in this study provide at least a partial solution to that difficulty because the C57BL/6J strain is the most widely used mouse strain and has been used as an animal model for many diseases and traits. The conplastic strains also could be used to investigate interactions between mtDNA and nuclear genomes with available transgenes that carry known disease-linked mutations. Mitochondrial–nuclear DNA epistasis is a common phenomenon that profoundly affects complex traits (Johnson et al. 2001; Roubertoux et al. 2003; Del Giudice et al. 2005; Capri et al. 2006; Gusdon et al. 2007).

In conclusion, we present a novel public genetic resource for investigating mtDNA variations. Using this resource, we demonstrated that mtDNA variations can affect both susceptibility to EAE and behavior. Therefore, these conplastic strains will be useful for investigating the role of mtDNA variations in mammals

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Table 1. List of mtDNA mutations in CIS

Position[a]	Allele[a]	Mutant strains	Gene	Amino acid
nt2256[b]	T/C	SAMP1, SAMP8	*mt-Rnr2*	
nt3072[c]	A/G	MA/MyJ	*mt-Nd1*	Thr-Ala
nt4561[c]	C/T	PL/J	*mt-Nd2*	Phe-Phe
nt4738[b]	C/A	ALR/LtJ	*mt-Nd2*	Leu-Met
nt4836[b]	A/G	KK	*mt-Nd2*	Asn-Ser
nt6342[b]	C/T	KK	*mt-Co1*	Leu-Leu
nt7778[b]	G/T	FVB/NJ	*mt-Atp8*	Asp-Tyr
nt8321[b]	G/A	129P3/J	*mt-Atp6*	Gly-Glu
nt8889[b]	G/A	C3H/HeJ	*mt-Co3*	Ala-Thr
nt9348[b,c]	G/A	A/J, BALB/cJ, BALB/cByJ, BPL/1J, CBA/J, C3H/HeJ, C3H/He, I/LnJ, LT/SvEi, NOD/LtJ, SEA/GnJ, SEA/GnJ, STR/ort	*mt-Co3*	Val-Ile
nt9461[b,c]	T/C	C57BL/6J, SOD1	*mt-Nd3*	Met-Met
nt9820[b]	–/T	C3H/HeJ	*mt-Tr*	
nt9821[b,c]	8A	C57BL/6J, etc.	*mt-Tr*	
	9A	AKR/J, etc.	*mt-Tr*	
	10A	A/J, etc.	*mt-Tr*	
	11A	MRL/MpJ, etc.	*mt-Tr*	
nt9986[c]	G/A	BUB/BnJ	*mt-Nd4l*	Val-Met
nt10623[c]	T/C	C57BL/10J	*mt-Nd4*	Tyr-Tyr
nt10848[b]	G/A	SAMP8	*mt-Nd4*	Gly-Gly
nt11008[c]	G/A	MA/MyJ	*mt-Nd4*	Asp-Asn
nt11182[b]	A/G	SAMP1, SAMP8	*mt-Nd4*	Ser-Gly
nt11781[b]	T/A	VM	*mt-Nd5*	Ile-Met
nt11902[c]	T/C	BPL/1J	*mt-Nd5*	Phe-Leu
nt12595[c]	A/G	MA/MyJ	*mt-Nd5*	Thr-Ala
nt13053[b,c]	T/C	NZW/LacJ, SAMR1, SAMP1, SAMP8, SB/LeJ	*mt-Nd5*	Phe-Phe
nt13582[c]	A/T	CE/J	*mt-Nd6*	Phe-Ile
nt14698[c]	T/C	RIII, TALLYHO/Jng	*mt-Cytb*	Leu-Leu
nt15124[b]	A/G	129S1/SvImJ	*mt-Cytb*	Ile-Val
nt15379[c]	C/–	129P3/J	*mt-Tp*	

[a]mtDNA sequence of AKR/J strain (AB042432) was taken as the reference sequence.
[b]Mutations identified in previous studies.
[c]Mutations identified in this study.

and will facilitate exploration of the biochemical and physiological roles of mitochondria in complex traits.

Methods

Mice

All inbred mouse strains used in this study were obtained from the Jackson Laboratory. To generate the conplastic strains, we crossed mtDNA donor strain females with male C57BL/6J and then backcrossed the female offspring to the male C57BL/6J. In each generation, four female mice were selected randomly and backcrossed to two C57BL/6J males (one male with two females). Such backcrosses were performed for 10 subsequent generations, and the offspring after the tenth generation were regarded as conplastic strains that carried the nuclear genome from the recipient strain and the mitochondrial genome from the donor

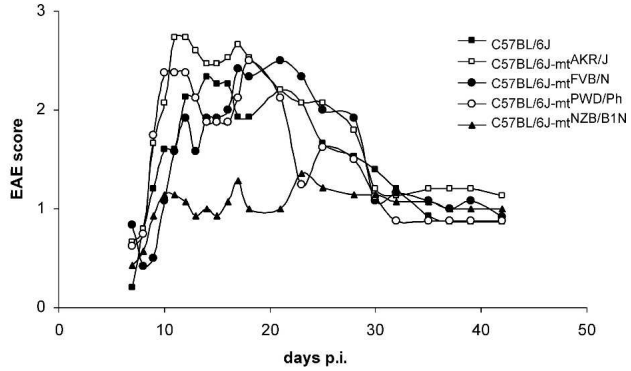
Table 2. List of conplastic strains generated in this study

Strain	mtDNA variants[a]	Gene and amino acid change[b]	Current backcross generation
C57BL/6J-mt$^{AKR/J}$			18
C57BL/6J	nt9821 8A	*mt-Tr*	
C57BL/6J-mt$^{MA/MyJ}$	nt3072G, nt11008A, nt12595G	*mt-Nd1* Thr-Ala, *mt-Nd4l* Asp-Asn, *mt-Nd5* Thr-Ala	6
C57BL/6J-mt$^{ALR/LtJ}$	nt4738A	*mt-Nd2* Leu-Met	10
C57BL/6J-mt^{KK}	nt4836G	*mt-Nd2* Asn-Ser	3
C57BL/6J-mt$^{FVB/NJ}$	nt7778T	*mt-Atp8* Asp-Tyr	19
C57BL/6J-mt$^{129P3/J}$	nt8321A, nt15379 del	*mt-Atp6* Gly-Glu, *mt-Tp*	5
C57BL/6J-mt$^{C3H/HeJ}$	nt8889A,nt9348A, nt9820 ins	*mt-Co3* Ala-Thr, *mt-Co3* Val-Ile, *mt-Tr*	5
C57BL/6J-mt$^{NOD/LtJ}$	nt9348A, nt9821 10A	*mt-Co3* Val-Ile, *mt-Tr*	10
C57BL/6J-mt$^{MRL/MpJ}$	nt9821 11A	*mt-Tr*	3
C57BL/6J-mt$^{BUB/BnJ}$	nt9986A	*mt-Nd4l* Val-Met	6
C57BL/6J-mt$^{BPL/1J}$	nt11902C	*mt-Nd5* Phe-Leu	5
C57BL/6J-mt$^{129S1/SvImJ}$	nt15124G	*mt-Cytb* Ile-Val	5
C57BL/6J-mt$^{NZB/B1nJ}$	*M. mus. domesticus*	85 variations	11
C57BL/6J-mt$^{PWD/PhJ}$	*M. mus. musculus*	390 variations	11
C57BL/6J-mt$^{MOLF/EiJ}$	*M. mus. molossinus*	390 variations	7
C57BL/6J-mt$^{CAST/EiJ}$	*M. mus. castaneus*	379 variations	4

[a]Synonymous variations are not included.
[b]C57BL/6J-mt$^{AKR/J}$ strain was taken as the reference strain.

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A



B

	Incidence	Onset day	Maximal score	AUC
C57BL/6J-mt$^{AKR/J}$	15/15	8.06 ± 1.33	3.6 ± 1.36	41.1 ± 23.4
C57BL/6J	15/15	8.60± 1.29	3.0 ± 1.36	34.0 ± 13.3
C57BL/6J-mt$^{FVB/N}$	12/12	8.08 ± 2.15	2.9 ± 0.99	33.6 ± 12.5
C57BL/6J-mt$^{PWD/Ph}$	8/8	7.75 ± 0.88	3.1 ± 1.29	34.6 ± 11.5
C57BL/6J-mt$^{NZB/B1N}$	9/14***	8.22 ± 1.64	1.9 ± 1.94**	22.5 ± 23.8*

Figure 3. mtDNA variations affect susceptibility to EAE. (*A*) Development of EAE in five conplastic strains. The *x*-axis presents the days post-immunization, and the *y*-axis indicates the EAE score. (*B*) Comparison of EAE clinical phenotypes, including disease incidence, onset day (only the disease mice were included), maximal disease score, and area under the curve (AUC). The C57BL/6J-mt$^{AKR/J}$ strain was taken as the reference strain, and the statistical significance was calculated for each strain compared with the C57BL/6J-mt$^{AKR/J}$ strain. Asterisks indicate significant differences: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

strain. To check the purity of the nuclear genome of conplastic strains, we performed SNP analysis with Illumina Mouse SNP medium-density panels, which contain 1449 SNPs that cover the entire mouse genome. Animals were housed at the animal facility at the University of Rostock, and all procedures were pre-approved by the local Animal Care Committee.

DNA and sequencing

mtDNA sequences of 29 inbred mouse strains were available from http://www.ncbi.nlm.nih.gov or from the literature (Loveland et al. 1990; Johnson et al. 2001; Bayona-Bafaluy et al. 2003; Mathews et al. 2005; Goios et al. 2007). We sequenced mtDNA from an additional 27 inbred mouse strains. DNA samples from all strains in this study were obtained from the Jackson Laboratory, with the exception of DNA from the STR/ort strain, which was prepared from liver with the QIAamp DNA Mini Kit (QIAGEN). We amplified 14 overlapping fragments of ~1.3–2.5 kb covering the entire mtDNA molecule (Supplemental Table 3). PCR reactions contained 1.5 mM $MgCl_2$, 0.25 mM dNTP, 50 ng of template DNA, 1 µM each primer, and 1 unit of Gold AmpliTaq DNA polymerase (PE Applied Biosystems) in a final volume of 50 µL. Amplification conditions were as follows: 95°C for 5 min; two cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 1.5 min; 35 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 7 min. PCR was conducted with the GeneAmp PCR System 9700 cycler (Applied Biosystems, Inc.). PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's protocol, and the purified PCR products were directly sequenced at the QIAGEN genome service center. Sequences were compared using C57BL/6J as the reference sequence. We confirmed all mtDNA variations identified in CIS by sequencing a second PCR product.

Phylogenetic analysis

Phylogenetic analyses were performed with ClustalW software. We constructed a phylogenetic tree based on the mtDNA sequences of 58 inbred strains, and the bootstrap values (percentage of 1000 total bootstrap replicates) were calculated. For the WIS, the taxonomic classifications were retrieved from the Jackson Laboratory or literature as follows: *Mus musculus musculus*, PWD/PhJ; *Mus musculus domesticus*, WSB/EiJ; *Mus musculus molossinus*, MOLF/EiJ; and *Mus musculus castaneus*, CAST/EiJ.

Induction and evaluation of EAE

To induce EAE in C57BL/6 mice, 16-wk-old mice were immunized at the base of the tail with 120 µg of rat myelin-oligodendrocyte glycoprotein (MOG) 35-55 peptide dissolved in 50 µL of PBS and mixed with an equal volume (50 µL) of CFA (IFA with 4 mg/mL *Mycobacterium tuberculosis*). Two hundred nanograms of pertussis toxin dissolved in 100 µL of PBS was injected intraperitoneally immediately after immunization and 48 h later. The clinical scoring of EAE commenced at day 7 after immunization, and animals were monitored for signs of EAE every day during the acute phase and three times per week during the chronic phase. The EAE score was determined as follows: 0, no detectable sign of EAE; 1, tail weakness; 2, tail paralysis; 3, pa-

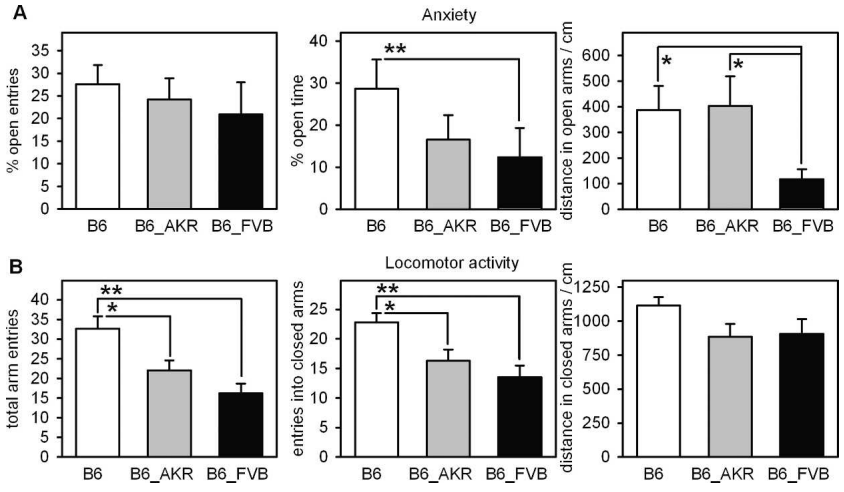


Figure 4. Behavior of C57BL/6J (B6), C57BL/6J-mt$^{AKR/J}$ (B6_AKR), and C57BL/6J-mt$^{FVB/N}$ (B6_FVB) mice on the elevated plus maze. (*A*) Anxiety was measured by the percentage of entries into open arms, the percentage of time spent in open arms, and the distance walked in open arms. (*B*) Locomotor activity was recorded as total entries into open and closed arms, the number of times mice entered closed arms, and the distance walked in closed arms. Data represent means and SEM of nine to 12 mice. Asterisks indicate significant differences between strains; : *$P < 0.05$; **$P < 0.01$; Mann-Whitney test.

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ralysis in one hind leg; 4, paralysis in two hind legs but can move; 5, paralysis in two hind legs and cannot move; and 6, moribund.

Behavioral test

The elevated plus maze tests the conflict between the tendency to explore a novel environment and the aversive properties of brightly lit, open, and elevated areas. The elevated plus maze consists of four arms, each 30 cm in length and 5 cm in width. Two opposing arms are enclosed in opaque, 15-cm-high side and end walls. The structure is elevated by 60 cm. Six-week-old male mice of the strains C57BL/6J, C57BL/6J-mt$^{AKR/J}$, or C57BL/6J-mt$^{FVB/N}$ were either received from Harlan-Winkelmann (Borchen, Germany) at 3 wk of age (C57BL/6J) or reared as siblings (C57BL/6J-mt$^{AKR/J}$ or C57BL/6J-mt$^{FVB/N}$) in our facility. We tested anxiety on the basis of the following parameters: (1) percentage of entries into open arms, (2) percentage of time spent in open arms, and (3) distance traveled in open arms. Locomotor activity was determined as (1) total entries into open and closed arms, (2) entries into closed arms, and (3) distance traveled in closed arms. Mice were put into the central position of the maze and left undisturbed for 10 min. Their behavior was recorded by a videocamera and later analyzed by a blinded observer. For all mice, testing was performed between 10 a.m. and 12 p.m. to minimize the influence of the time of day.

Corticosterone and neurotransmitter analyses

Because the elevated plus maze might induce stress in the tested mice, corticosterone and neurotransmitter levels were analyzed in age-matched male mice that were not tested for their behavior. After cervical dislocation, mice were rapidly decapitated, and their trunk blood was collected. Brains were isolated, snap-frozen in liquid nitrogen, and stored at −80°C until neurotransmitter analysis. For all mice, sampling was performed between 9:30 and 9:45 a.m. to minimize the influence of the time of day. Blood was allowed to clot for 2 h and then centrifuged at 2000*g* for 10 min. Sera were stored frozen at −20°C until corticosterone analysis.

Serum corticosterone concentrations were measured in duplicate with a commercially available double antibody rat corticosterone ^{125}I-RIA kit (DRG Diagnostics) according to the manufacturer's instructions. Cross-reactivities of the antibody to potential competing plasma steroids were <0.5%. The test sensitivity was 7.2 ng/mL, and intra- and interassay coefficients of variation (CV) were 6.9% and 8.1%, respectively.

Whole brain concentrations of noradrenalin (NA), its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG), dopamine (DA), its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) were determined in duplicate by high-performance liquid chromatography (HPLC) with electrochemical detection. Brains were weighed and homogenized on ice for 5 min with a hand homogenizer in 4 mL of 0.2 M perchloric acid, followed by centrifugation at 45,000*g* for 10 min at 4°C. After collection of the supernatants, the procedure was repeated. Pooled supernatants of the repeated extractions were again centrifuged at 45,000*g* for 10 min at 4°C. Twenty microliters of each sample was then injected directly in the HPLC system equipped with a 125 × 4-mm reversed-phase column packed with Prontosil C18 AQ (Bischoff Analysentechnik). As the mobile phase, 58 mM sodium hydrogen phosphate buffer containing 1.2 mM octansulfonic acid, 0.3 mM EDTA, 0.2 mM potassium chloride, and 6% methanol (pH 3.8) was used at a flow rate of 1.2 mL/min. Electrochemical detection was achieved by an ISAAC cell with a glassy carbon working electrode set at a potential of 600 mV (Shimadzu). Dihydroxybenzylamine (DHBA) was used as an internal standard for quantification, and tissue contents of the above neurotransmitters and their metabolites were expressed as nanograms per gram of wet tissue weight. As indices of NA, DA, and 5-HT turnover, the MHPG/NA, DOPAC/DA, HVA/DA, and 5-HIAA/5-HT ratios were calculated.

Statistical analysis

Quantitative traits of mouse strains were compared with the nonparametric Mann-Whitney test. Qualitative traits, for example, the incidence of EAE, were compared with the Fisher's exact test. In all statistical tests, $P < 0.05$ was considered to be statistically significant.

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