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Early onset of aging-like changes is restricted to cognitive abilities and skin structure in Cnr1$^{-/-}$ mice

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Abstract

Genetic deletion of the cannabinoid 1 (CB1) receptor leads to an early onset of learning and memory impairment. In the present study we asked whether the lack of CB1 receptors accelerates aging in general or is selective for cognitive functions. We therefore compared the onset and dynamics of age-dependent changes in social memory, locomotor activity, hearing ability, and in the histopathology of peripheral organs between wild-type and Cnr1 knockout (Cnr1$^{-/-}$) mice. We observed deficits in social memory already in 3-month-old Cnr1$^{-/-}$ mice. In contrast, wild-type animals showed such deficits at the age of 6 months. Sensory and motor functions were similar between the genotypes. Thus, hearing loss for higher frequencies and the development of hypomotility showed a similar age-dependent course. In the periphery we detected an early onset of aging-like histological changes in the skin, but not in other organs. We conclude that the lack of CB1 receptor does not induce accelerated aging in general, but induces changes in cognitive function and in skin structure that resemble those associated with aging.

Keywords: Cannabinoid 1 receptor; Skin aging; Testis; Hearing loss; Locomotor activity; Knockout mouse model; Learning and memory

1. Introduction

Aging is associated with a general decline in fitness, as evident from a reduction in sensory, motor, and cognitive abilities, as well as characteristic histological changes. Healthy aged individuals exhibit deficits in cognitive functions, like attention (Muir et al., 1999; Verhaeghen and Cerella, 2002), memory (Jennings and Jacoby, 1997; Muir et al., 1999), and perception (Baltes and Lindenberger, 1997; Ison et al., 2007). The onset and progression of such age-related changes shows a high individual variability in humans (Grady and Craik, 2000) and in animals (Dean et al., 1981; Gallagher and Nicolle, 1993). Thus, many senior citizens still enjoy their cognitive abilities, whereas others may show signs of cognitive impairments already early in their life. The biological basis of this variation is not fully understood, but differences in the antioxidative capacity (Skaper et al., 1999), stress hormone levels (Magri et al., 2006), and glutamate neurotoxicity (Beal, 1992) are considered to be significant contributing factors. The activity of the endocannabinoid system might influence age-related physiological changes, because endocannabinoids have a strong impact on all these factors. The activity of the endocannabinoid system influences hormonal stress reactivity (Steiner and Wotjak, 2008), regulates the activity of glutamatergic neurons (Hampson and Grimaldi, 2001), and en-

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docannabinoids protect neurons from oxidative damage (Grundy et al., 2001).

Endocannabinoids are retrograde transmitters synthesized on demand from their lipid precursors. The most abundant and widely studied endocannabinoids are 2-arachidonoyl-glycerol (2-AG) and anandamide. 2-AG is a full agonist of the cannabinoid 1 (CB1) receptor, while anandamide is a partial agonist of this receptor (Reggio, 2006). To date, 2 cannabinoid receptors have been cloned and characterized. The cannabinoid 2 (CB2) receptor is more abundant in the periphery, whereas its expression in the central nervous system is very low and mostly restricted to glial cells. On the other hand, the CB1 receptor is widely present in the brain and probably represents one of the most abundant G protein coupled receptor in the mammalian central nervous system. CB1 receptors are also found in peripheral organs, with the highest density in the skin and in the testis. In skin it is mainly expressed by keratinocytes (Maccarrone et al., 2003), but is also present in neuronal fibers and immune cells (Onaivi et al., 2006). In testis, CB1 receptors are localized in spermatids, spermatozoa, and also in Leydig cells, suggesting an important role of the receptor in spermatogenesis (Cacciola et al., 2008).

Endogenous and exogenous cannabinoids are known to affect learning and memory processes. Acute exposure to cannabinoid receptor agonists detrimentally affects learning and memory functions in humans (Lichtman et al., 2002) and in animals (Lichtman et al., 1995; Nava et al., 2001). Accordingly, the acute pharmacological blockade of the CB1 receptor has a beneficial effect on the memory (Terranova et al., 1996; Wolff and Leander, 2003). Behavioral analyses of mice with a deletion of the CB1 receptor gene Cnr1 (henceforth referred to as $Cnr1^{-/-}$ mice) support the pharmacological effects on memory function. Collectively, previous studies showed that learning and memory functions are altered in $Cnr1^{-/-}$ mice, as indicated by an enhanced memory retention in the object-recognition task (Maccarrone et al., 2002; Reibaud et al., 1999), a deficit in reversal learning in the water-maze test (Varvel and Lichtman, 2002), and a delayed extinction learning in a fear-conditioning paradigm (Marsicano et al., 2002; Martin et al., 2002). However, these experiments were mostly performed with young mice.

The endocannabinoid system is subjected to age-related changes. Old rats for example showed lower CB1 receptor densities and messenger RNA (mRNA) expression levels in various brain areas compared with young rats, which were most prominent in the basal ganglia and the cerebellum (Berrendero et al., 1998; Romero et al., 1998). Otherwise, the brainstem of aged rats showed a substantial increase in CB1 mRNA levels. Comparison of 2 age groups of mice (26–48-week-old vs. 6–10-week-old) did not reveal any differences in CB1 receptor levels, but showed a reduced receptor coupling in the limbic forebrain of older animals (Wang et al., 2003). Also a small but significant region-specific reduction in endocannabinoid levels has been described in aged animals (Maccarrone et al., 2002; Wang et al., 2003). The functional consequences of these age-related changes remain to be clarified, but it has been suggested that they contribute to behavioral changes observed in aged animals, such as the age-dependent decline in food intake. Thus, the fact that anorexia occurs more frequently in old age (Morley, 2001) could be a result of an age-dependent change in the endocannabinoid system (Wang et al., 2003).

In a previous study we examined the role of the endocannabinoid system in age-related learning paradigms in mice and provided evidence for an accelerated age-related decline of learning and memory abilities in the absence of CB1 receptors (Bilkei-Gorzo et al., 2005). Although 5–8-week-old $Cnr1^{-/-}$ mice performed better than age-matched $Cnr1^{+/+}$ controls in the rotarod- and partner recognition tests, 4–6-month-old $Cnr1^{-/-}$ animals showed significant learning deficits and performed at levels comparable to those of much older mice. The accelerated decline in learning performance in mature $Cnr1^{-/-}$ animals is probably related to the absence of CB1 receptor-mediated (Veldhuis et al., 2003) neuroprotective effects of endocannabinoids (Panikashvili et al., 2001; Sinor et al., 2000). We found a significant reduction in neuronal densities in the CA1 or CA3 regions, which are particularly sensitive to oxidative stress and neurotoxic effects (Cervos-Navarro and Diemer, 1991; Pulsinelli, 1985). In contrast, the relatively resistant CA2/CA3 region and the dentate gyrus showed no neuronal loss.

In the present study we asked whether the early onset of aging-like changes in $Cnr1^{-/-}$ mice is specific for learning and memory functions or is rather a result of a generally accelerated aging process. To answer this question, we first compared the onset and progression of age-related changes in locomotor activity and hearing ability, which are generally observed in aging mammals (Tou and Wade, 2002; Van Eyken et al., 2007). CB1 receptors are also expressed in peripheral organs and it is thought that the central nervous system regulates the aging process of extraneural tissues (Bishop et al., 2010). These facts led us to question if a lack of CB1 receptors accelerates aging in peripheral organs. Thus, the presence of age-related histological changes like appearance of calcium deposits in soft tissues (Kurosu et al., 2005; Wong et al., 1994), atrophy of seminiferous tubules in the testis (Chigurupati et al., 2008), reduction of the subdermal fat layer in the skin (Coleman et al., 2009; Sommer et al., 2006), and increase in the alveoli diameter in the lung (Janssens et al., 1999) was tested in wild-type and Cnr1 knockout animals. We demonstrate that deletion of CB1 receptors does not accelerate aging in general, but rather specifically affects learning and memory functions in the brain and induces changes in skin structure that resemble those associated with skin aging.

2. Methods

2.1. Animals

We used offspring obtained from a heterozygous *Cnr1*$^{+/-}$ breeding colony on a congenic C57BL/6J background. The test groups consisted of male and female homozygous wild-type and knockout littermates of different ages. Mice received water and food ad libitum. They were housed as single sex littermates in groups of 3–5 and were kept in a reversed light-dark cycle (dark period between 9 AM and 7 PM). Female animals were used in the partner recognition paradigm, while male and female mice were used for assessing the age-dependent changes in locomotor and hearing abilities. Behavioral studies were conducted during the active phase of the animals in a dimly lit, low-noise environment. Animal care and animal experimentation conduction followed the guidelines of the German Animal Protection Law (issued in 1998).

2.2. Partner recognition test

We exclusively tested female mice in the partner recognition test, because our previous studies showed that intermale aggression could be a confounding factor in this paradigm. The trials were conducted in an open-field arena (44 cm × 44 cm) in a dimly lit, sound-isolated environment. Initially, the test animals were habituated to the arena 5 minutes daily for 5 days. The sawdust covering the floor of the arena was saturated with mouse odor, because we did not change it during the experiments. Thereby the mice felt familiar in the test environment and the social behavior was not influenced by the differences in anxiety levels (Haller et al., 2002; Thiemann et al., 2009). The test trials consisted of 2 sessions: First, we put the animals into the familiar arena, where a young female DBA/2J-Penk1$^{-/-}$ mouse was presented. We have previously demonstrated that DBA/2J-Penk1$^{-/-}$ mice have very low social activity (Bilkei-Gorzo et al., 2004) thus rarely initiating social contacts. Therefore, the overall level of social interactions largely depends on the partner test mouse. The activity of the mice was videotaped for 5 minutes and the time spent with investigation of the partner was calculated using "The Observer" software (Noldus, Wageningen, Netherlands) by an investigator unaware of the genotype. In the next session, the test was repeated with the same partner and in the same arena, but with variable time intervals between the sessions. In the first trial, the interval between the sessions lasted 2 hours. This time interval was doubled in the consecutive trials until the animals did not recognize the previously presented partner, i.e., the time spent with social interactions was not significantly lower in the second session (Student paired *t*-test). If mice did not recognize their partner in the first trial, we shortened the time interval to 1 hour in the second trial. Animals were left undisturbed for at least 24 hours between trials.

2.3. Locomotor activity

Male and female mice were placed individually into the center of an open field apparatus (44 × 44 × 30 cm). Their locomotor activity was measured using infrared beams placed 3 cm above the floor level. An automatic monitoring system (Actimot, TSE Systems GmbH, Bad Homburg, Germany) tracked the movements for 20 minutes and analyzed the raw data. Distance traveled (m) was used for statistical evaluation. Mean values and standard errors of mean were calculated for each group. Age-dependent changes in motor activity were evaluated using 3-way analysis of variance (ANOVA; main factors: genotype, age, sex). The onset of age-dependent reductions in locomotion was tested separately in the strains using 1-way ANOVA followed by a Bonferroni *t*-test (Statistica 7, Statistica Inc., Gaithesburg, MD, USA).

2.4. Acoustic startle response test

The hearing ability was assessed as the reactivity to startle-eliciting auditory stimuli. We measured the reactivity of wild-type and Cnr1$^{-/-}$ male and female mice from 4 different age groups (2, 3, 4, and 5-month-old). One test session lasted approximately 15 minutes and involved a random presentation of different stimuli after a 5-minute habituation period. Stimuli of 9, 12, 15, 18, and 21 kHz with 100 dB were applied for 40 ms, 3 times each. The intertrial time was set between 20 and 40 seconds. The startle response apparatus (TSE-Systems GmbH, Bad Homburg, Germany) contained a cage of Plexiglas and wire mesh (11 × 5.5 × 6 cm), with metal grid floor (4 mm diameter, 6 mm apart). Each cage was located in a ventilated, sound-attenuated chamber (inner dimensions: 35 × 32 × 35 cm) on a vibration-sensitive platform. Two speakers located on both sides of the cage delivered a continuous background white noise (65 dB) and the startle-eliciting signals. Groups were compared using 3-way ANOVA (main factors: genotype, sex, age) separately for each frequency. The onset of significant reductions in hearing ability was tested separately in the strains using 1-way ANOVA followed by a Bonferroni *t*-test (Statistica 7, Statistica Inc., Gaithesburg, MD, USA).

2.5. Age-dependent histological changes

We deeply anesthetized 2, 5, and 12–14-month-old Cnr1$^{-/-}$ and Cnr1$^{+/+}$ mice (4 males and females in each age group) with isoflurane. After transcardial perfusion with ice-cold phosphate buffered saline followed by Zamboni fixative, we removed the aorta, kidney, stomach, lung (collapsed), and testis for histological examinations. Each organ was postfixed for 4 hours in the fixative, cryoprotected in 10% sucrose overnight and frozen in isopentane chilled with dry ice. Skin probes were embedded in paraffin and sliced into 12-μm sections using a microtome. Other tissue samples were embedded in Tissue-Tek (Sakura Finetek, Zoeter-

woude, Netherlands) and cut into 16-μm sections using a cryostat. The histological structure of testis, skin, and lung was compared between mice of different age and genotype after hematoxylin/eosin (HE) staining. Briefly, the sections were first stained for 10 minutes in 1% hematoxylin solution, rinsed in tap water and subsequently stained with a 0.1% eosin solution for 5 minutes. The presence of calcium deposits in the aorta, kidney, and in the abdominal wall was investigated using *Von Kossa* staining (Troiano et al., 2009). Briefly, sections were incubated in 1% silver nitrate solution for 20 minutes under ultraviolet light. Unreacted silver was removed with 5% sodium thiosulfate, rinsed, and counterstained with nuclear fast red. After washing in water and dehydrating with increasing concentrations of ethanol and xylol, the slides were covered and stored at 4 °C. Quantitative analyses of the sections was done by an experienced researcher blind to the experimental groups. Images of the sections were taken using a standard light microscope (Axioplan 2 imaging, Carl Zeiss GmbH, Jena, Germany) connected to a digital camera (KYF-75U, JVC, Yokohama, Japan) and a PC system with Nis Elements imaging software (NIS Elements 3.0, Nikon, Tokyo, Japan). The surface of 16–20 neighboring tubules and the cell-free central region in the tubules was measured in each section. The cell-free area within the seminiferous tubules was calculated as a percentage of the total area.

In the lung, the volume of alveoli was assessed as mean area of 8–10 neighboring alveoli per section. Width of the subdermal fat layer was calculated as a mean of 5 width values for each section. We determined means and standard error of means for the cell-free area of the seminiferous tubules in testis and lung specimens and the width of subdermal fat by evaluating 5 sections per animal. Statistical analysis of the data was performed using 3-way ANOVA (lung and skin; main factors: genotype, sex, age) or 2-way ANOVA (testis; main factors: genotype, age) followed by a Bonferroni *t*-test (Statistica 7, Statistica Inc., Gaithesburg, MD, USA).

3. Results

3.1. Partner recognition test

We assessed the ability of mice to recognize a previously seen partner as a reduction of social interaction time during a second presentation (Fig. 1). Both genotypes showed an age-dependent decrease in the partner recognition ability. This was significantly different between wild-type and Cnr1$^{-/-}$ mice for each intertrial time (2 hours: $F[8,162] = 10.51$, $p < 0.001$; 4 hours: $F[5,108] = 5.155$, $p < 0.001$; 8 hours: $F[3,72] = 6.233$, $p < 0.001$). Wild-type mice up to 8 months of age recognized the previously presented partner after a 2-hour interval, whereas only Cnr1$^{-/-}$ mice up to 5 months of age recognized the partner animal (Fig. 1A).

The partner recognition ability of older animals became worse after increasing the time between the partner presentations. After a 4-hour interval, only wild-type mice at the age of 6 months or younger recognized the previously seen partner (Fig. 1B). After an 8-hour intertrial time, only 5-month-old mice or younger could still recognize the pre-

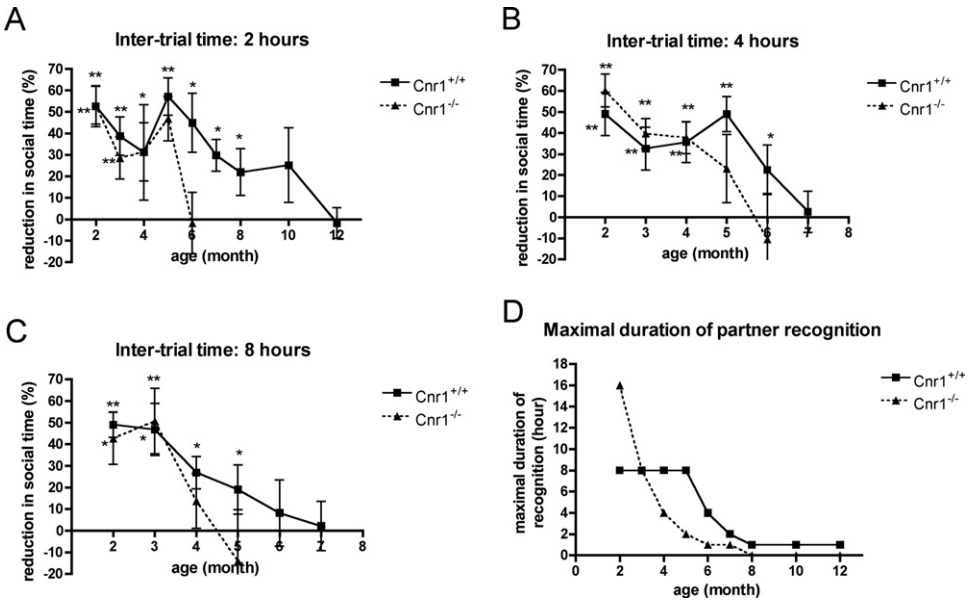

Fig. 1. Partner recognition ability in Cnr1$^{+/+}$ and Cnr1$^{-/-}$ mice. Reduction in time spent with social interactions in 2 consecutive trials is indicated as a function of age. The intertrial time was (A) 2 hours, (B) 4 hours, and (C) 8 hours. The sign of recognition is a significant difference in social time between the 2 presentations. Mean and standard error of the mean (SEM) of the percentage reduction in social time is shown. (D) Duration of partner recognition is reduced in 3-month-old Cnr1$^{-/-}$ animals, whereas only 6-month-old wild-type animals showed first deficits in partner recognition. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; difference in social time between the first and second presentation (Student paired *t*-test).

viously met partner (Fig. 1C). For Cnr1$^{-/-}$ mice, the oldest age group, which recognized the partner animal after a 4-hour interval was 4 months old, whereas for the 8-hour interval it was 3 months old. Thus Cnr1$^{-/-}$ animals showed an early onset of recognition ability loss, independent from the severity of the task.

The maximal duration of the partner recognition ability is depicted in Fig. 1D. Two- to 5- month-old wild-type mice recognized the partner mouse up to 8 hours after the first presentation. With an increasing age (6–12 months), the recognition ability gradually decreased and 8-month-old or older animals recognized the partner maximally 1 hour after the first encounter. Although 2-month-old Cnr1$^{-/-}$ mice exhibited a better social memory than the age-matched wild-type animals, this difference disappeared at the age of 3 months. Thus, Cnr1$^{-/-}$ animals showed a much earlier onset in the reduction of partner recognition ability. At 4 months of age, the partner recognition ability of Cnr1$^{-/-}$ mice was worse than that of wild-type mice.

3.2. Locomotor activity

Analysis of the locomotor activity by 3-way ANOVA revealed a significant main effect for the factor age ($F[6,163] = 4.939$; $p < 0.001$), but not for genotype ($F[1,163] = 1.690$; $p > 0.05$) or sex ($F[1,163] = 0.602$; $p > 0.05$). This age-dependent change in locomotor activity was significantly different between the strains (age × genotype: $F[6,163] = 2.457$; $p < 0.05$; Fig. 2), but was not influenced by the sex of the animals (age × sex: $F[6,163] = 1.476$; $p > 0.05$). The lack of interaction between the main factors (age × genotype × sex: $F[6,163] = 1.809$; $p > 0.05$) suggests that the sex did not influence the genotype effect on the age-dependent change in motor activity. A significant genotype effect was only seen in 3-month-old animals. Later on, the locomotor activity decreased similarly in Cnr1$^{+/+}$ and Cnr1$^{-/-}$ animals.

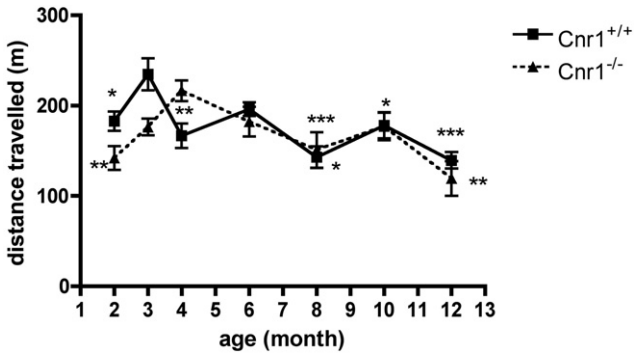

Fig. 2. Age-dependent changes in locomotor activity were measured as distance traveled in the open field. Although strains reached their maximum locomotor activity at different ages, the age-dependent decline in activity was similar between the genotypes. Mean values are shown, error bars represent standard error of the mean (SEM). * $p < 0.05$: ** $p < 0.01$; *** $p < 0.001$; significantly reduced activity compared with the age group with the highest locomotor activity (3-month-old in Cnr1$^{+/+}$ and 4-month-old in Cnr1$^{-/-}$ mice) according to 1-way analysis of variance (ANOVA) followed by Bonferroni *t*-test. + $p < 0.05$; difference between wild-type and null-mutant animals according to 2-way ANOVA followed by Bonferroni *t*-test.

3.3. Acoustic startle response test

The hearing ability of the mice was significantly reduced with aging over a broad frequency range. The genotype had no influence on the startle reactivity at any of the frequencies tested and there was neither an interaction between genotype and age, nor between genotype, age, and sex. These data suggest that a lack of CB1 receptors does not influence the age-related loss of hearing ability (Fig. 3). Detailed results from the statistical analysis are summarized in Supplementary Table 1.

3.4. Age-dependent histological changes

The age-dependent histological changes in the testis are depicted in Fig. 4. The size of cell-free areas within the seminiferous tubules was significantly larger in Cnr1$^{-/-}$ male mice in each age group (genotype effect: $F[1,114] = 62.14$; $p < 0.001$; Fig. 4A). In addition, an age-dependent increase of the cell-free area was detected (age effect: $F[2,114] = 5.337$; $p < 0.01$), which was similar between the strains (age × genotype interaction: $F[2,114] = 2.359$; $p > 0.05$).

The age-dependent histological changes in the skin are depicted in Fig. 5. The width of the subdermal fat layer was significantly smaller in Cnr1$^{-/-}$ mice (genotype effect: $F[1,468] = 29.80$; $p < 0.001$), independent of age or sex (genotype × sex interaction, $F[2,468] = 3.154$; $p > 0.05$). While the subdermal fat layer increased in older wild-type animals, we observed an atrophy of the subdermal fat layer in 12-month-old Cnr1$^{-/-}$ mice (genotype × age interaction $F[2,468] = 8.240$; $p < 0.001$; Fig. 5). This phenotype was observed in both sexes (genotype × age × sex interaction $F[2,468] = 0.032$; $p > 0.05$).

In the lung, the size of the alveoli did not differ between the strains ($F[1,228] = 0.134$; $p > 0.05$) or sexes ($F[1,228] = 3.157$; $p > 0.05$) and also the age-dependent increase in alveolar size ($F[1,228] = 66.77$; $p < 0.001$) was similar between the groups (age × genotype $F[2,228] = 2.584$; $p > 0.05$; age × sex $F[2,228] = 0.074$; $p > 0.05$ and age × sex × genotype $F[2,228] = 2.093$; $p > 0.05$, data not shown). Also, we did not find calcium deposits in the aorta, kidney, or stomach wall in mice at an age of 2, 5, and 12 months (data not shown).

4. Discussion

Genes that are known to influence aging are often involved in the maintenance of homeostasis including in antioxidative defense (Hekimi and Guarente, 2003; Liu et al., 2003), DNA repair and maintenance (Hasty et al., 2003; Tomas-Loba et al., 2008), insulin-like growth factor signaling (Cohen and Dillin, 2008; Kurosu et al., 2005), and

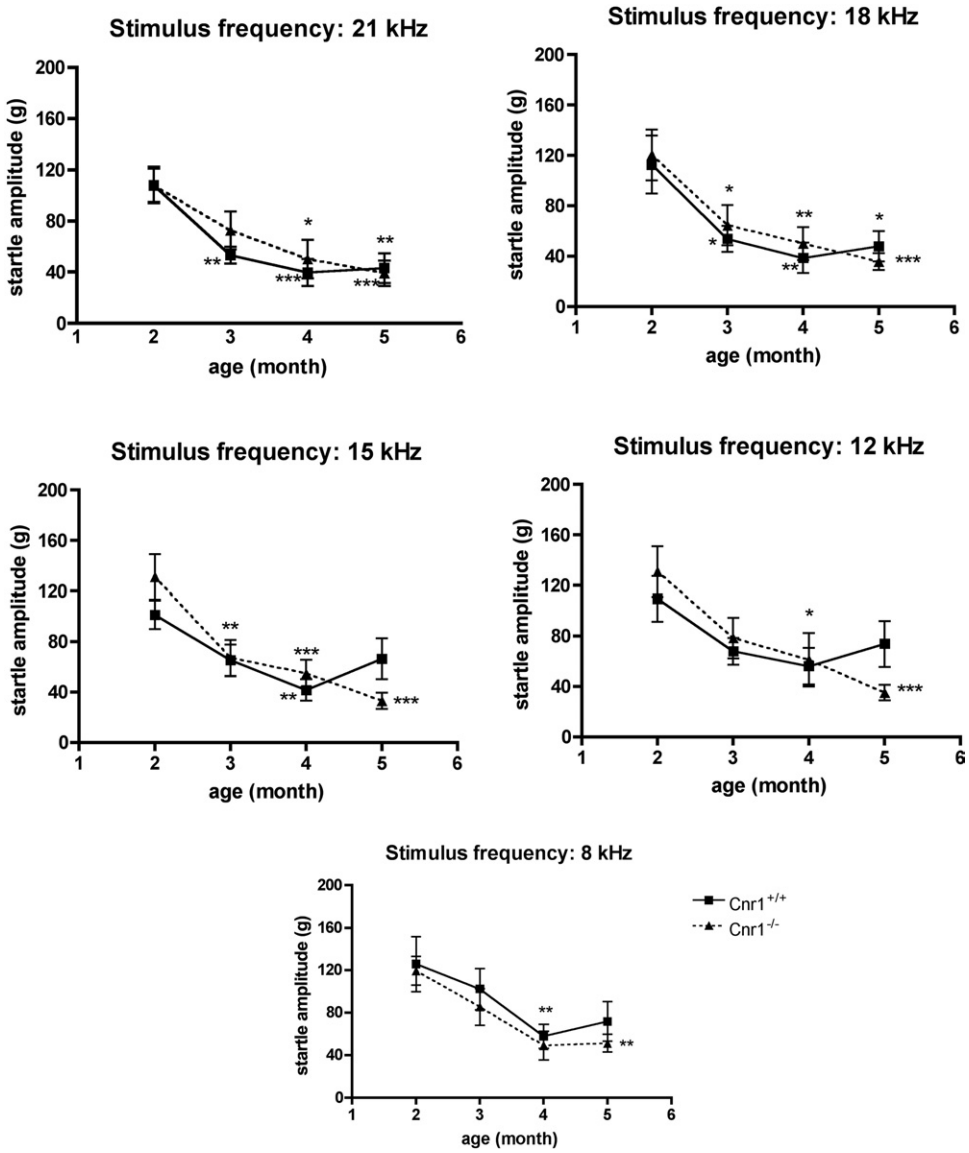

Fig. 3. The age-dependent loss of hearing ability measured as the acoustic startle response intensity that was elicited by a 100 dB sound was similar between the genotypes in a wide frequency range. Mean ± standard error of the mean (SEM) is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ significantly reduced activity than 2-month-old animals from the same genotype according to 1-way analysis of variance (ANOVA) followed by Bonferroni *t*-test.

cellular stress resistance (Longo and Kennedy, 2006; Niedernhofer et al., 2006). It was therefore unexpected that animals lacking the endocannabinoid receptor-encoding gene Cnr1 showed an early onset of learning and memory disabilities and neuronal degeneration in the hippocampus (Bilkei-Gorzo et al., 2005). This phenotype may reflect the fact that the endocannabinoid system is involved in the regulation of stress sensitivity (Griebel et al., 2005; Steiner et al., 2008), glutamate release (Hajos et al., 2001; Shen and Thayer, 1998), and that endocannabinoids have antioxidative capacity (Garcia-Arencibia et al., 2007; Underdown et al., 2005). Each of these processes is known to be involved in aging, therefore we hypothesized that deletion of the CB1 receptor may generally accelerate aging of the brain and the body. In the brain, however, the accelerated aging-like changes were restricted to the learning and memory abilities, whereas locomotor activity and hearing was not affected in CB1 null mutants.

The fact that the aging process of cognitive, but not of sensory or motor functions is affected in Cnr1$^{-/-}$ mice possibly reflects differences in the sensitivity of neuronal populations to cellular stress. The possible reason of this region-specific difference in cellular resistance is the variations in intrinsic oxidative damage repair capacity in the brain. Hippocampus, key region of learning and memory functions, is especially sensitive to oxidative stress (Wang et al., 2005), whereas regions involved in central sensory or motor processes are relatively resistant (Sava et al., 2006).

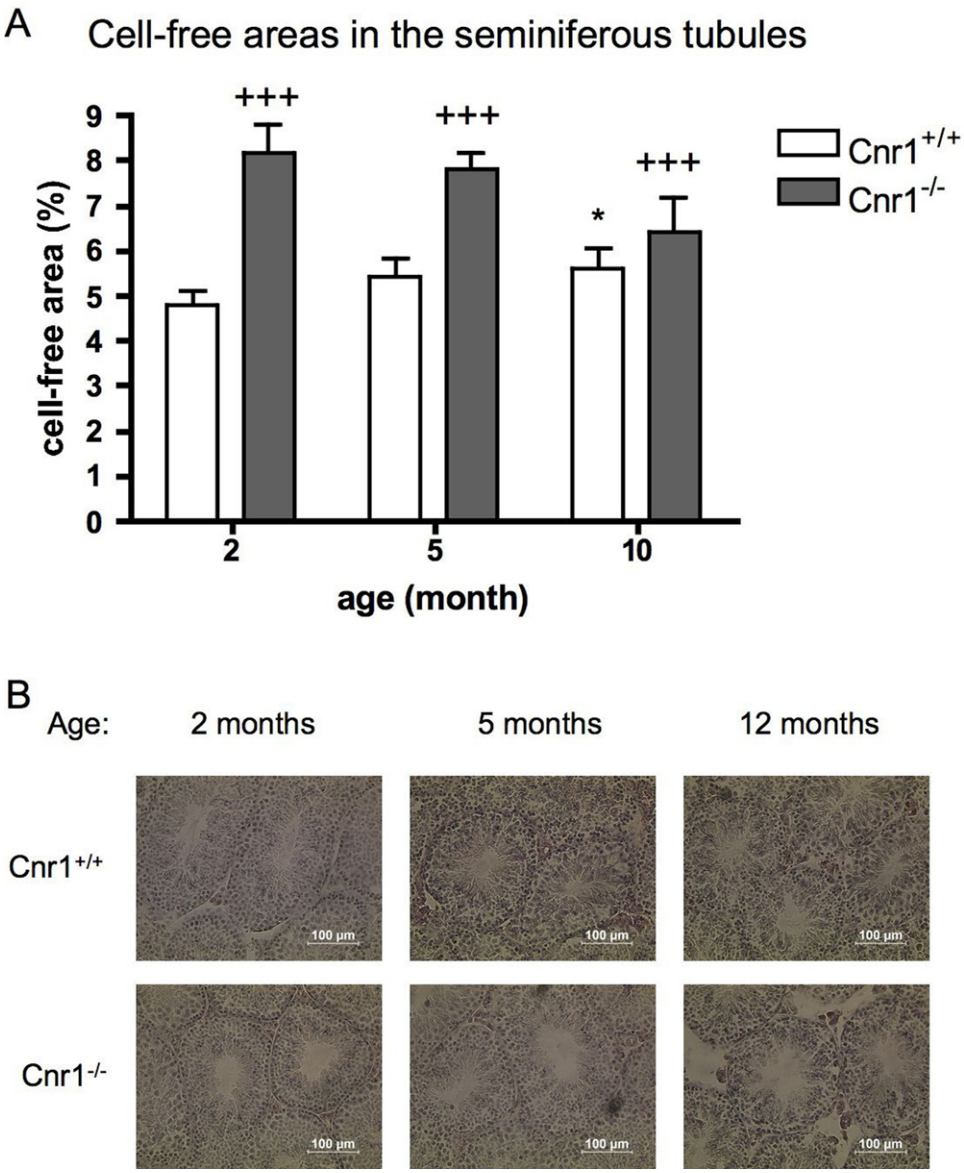

Fig. 4. (A) Atrophy of the testis assessed as percentage of cell-free areas within the seminiferous tubules was present in Cnr1$^{-/-}$ mice in each age group. Significant age-dependent changes were detected only in wild-type mice. Columns represent group means, the error bars represent standard error of the mean (SEM). * $p < 0.05$ significant elevation in cell-free areas compared with 2-month-old animals; $+++$ $p < 0.001$ difference between wild-type and null-mutant animals; both according to 2-way analysis of variance (ANOVA) followed by Bonferroni *t*-test. (B) Representative histological structure of the testis of 2-, 5-, and 12-month old Cnr1$^{+/+}$ and Cnr1$^{-/-}$ animals.

The age-related hearing loss is a result of a progressive degeneration of sensory cells along the basilar membrane (Spongr et al., 1997), and also age-related changes in the stria vascularis could play a significant role (Ohlemiller et al., 2006). The background strain used in our study (C57BL/6J) generally shows an earlier hearing loss than other mouse strains, due to an accelerated loss of hair cells in the basilar membrane (Ding et al., 1999) and neurons in the cochlear nucleus (Willott, 1986). Although a large number of neurons in the cochlear nucleus express CB1 receptors and although it was suggested that they play a significant role in auditory processing (Zheng et al., 2007), our present result suggests that they are not involved in the age-dependent hearing loss.

The development of hypomotility was almost identical in wild-type and knockout animals, although we previously reported an early onset of motor activity changes in Cnr1$^{-/-}$ animals (Bilkei-Gorzo et al., 2005). There is, however, an important difference between the present and our past study. In the present experiments we used animals from a heterozygous breeding colony, whereas in the previous work the wild-type and knockout animals were derived from separate breeding colonies. It is known that intrauterine development and maternal care have a significant impact

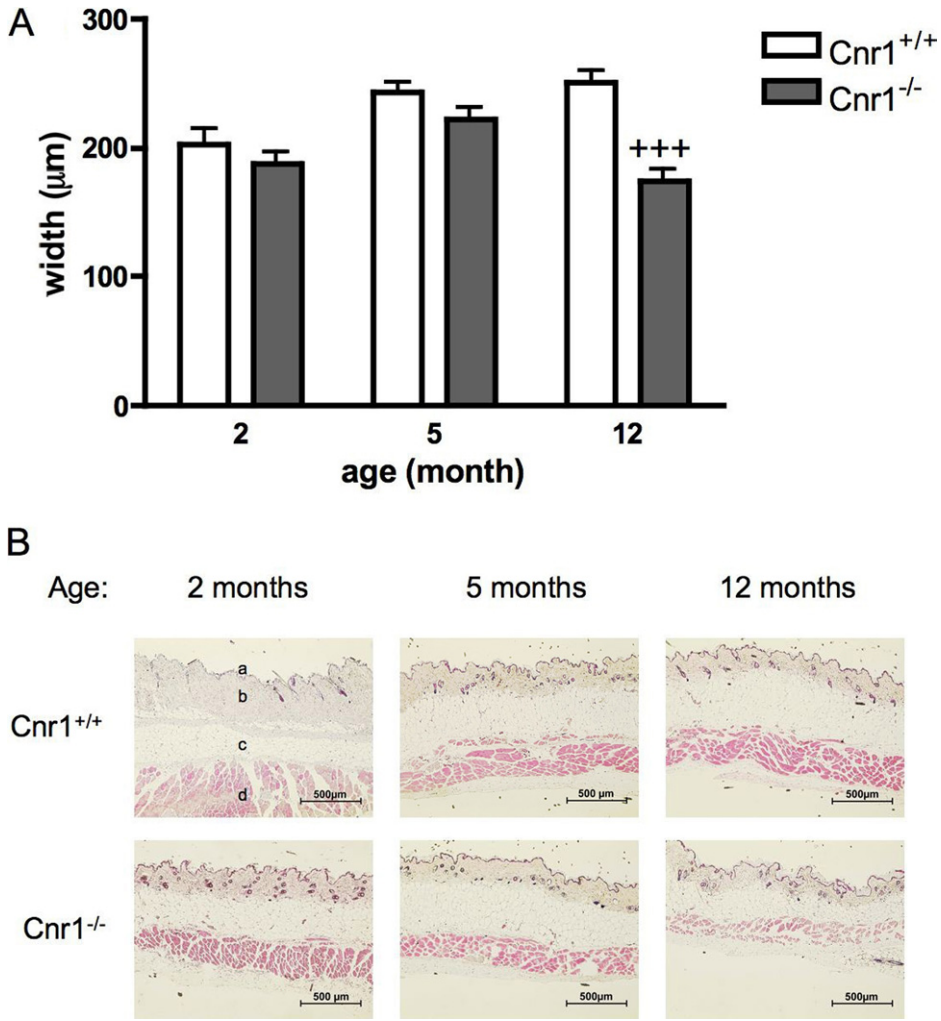

Fig. 5. (A) The width of the subdermal fat layer was significantly reduced in 12-month-old Cnr1$^{-/-}$ mice. Mean ± standard error of the mean (SEM) is shown. *** $p < 0.001$ difference between wild-type and null-mutant animals; according to 2-way analysis of variance (ANOVA) followed by Bonferroni *t*-test. (B) Representative histological structure of the skin of 2-, 5-, and 12-month old Cnr1$^{+/+}$ and Cnr1$^{-/-}$ animals. a, epidermis; b, dermis; c, subdermal fat layer; d, muscle.

on the activity of the animals (Palanza et al., 2001; Weller et al., 2003). The reason of the age-dependent decrease of motor activity is not fully known, but a decline in the number of dopamine receptors in the brain (Inoue et al., 2001), a decrease in leptin (Scarpace et al., 2000), and an increase in body weight (Ahima et al., 1999; Mowrey and Hershberger, 1982) are suggested to play a significant role. Although the activity of the endocannabinoid system modulates each of these 3 factors (Fernandez-Ruiz et al., 2006; Thiebot et al., 2006), we show here that the age-dependent development of hypomotility is normal in animals lacking CB1 receptors up to the age of 12 months. However, we cannot exclude the possibility that loss of CB1 receptors influences the development of sensory or locomotor deficits in a later phase of aging.

We noted that the partner recognition test used in the present study seems to be especially sensitive to age-dependent cognitive decline, because the onset of cognitive deficits is observed earlier than in other paradigms. In wild-type mice, learning and memory deficits are detectable at the age of 20 months in the Morris water maze test (Harburger et al., 2007; Murphy et al., 2006; Zhao et al., 2009) or in the radial arm maze test (Heneka et al., 2006; Marighetto et al., 2008) and of 12 months in the operant or skill-learning test (Bilkei-Gorzo et al., 2005). Here we showed that 3-month-old Cnr$^{-/-}$ and 6-month-old wild-type animals show deficits in the partner recognition test, therefore subsequent studies aiming to elucidate the mechanism of accelerated cognitive aging in Cnr1$^{-/-}$ mice have to be performed with much younger animals than generally used in aging research. These future studies should compare the age-dependent changes in oxidized macromolecules in the brain (Kim et al., 2005), consequences of the elevated steroid levels (Cota et al., 2007) in wild-type and constitutive or glutamatergic neuron specific Cnr1 mutants (Kim et al., 2008).

We also hypothesized that the lack of CB1 receptors may also influence the aging of peripheral organs. Surprisingly, we found differences between the strains in the histological structure of testis and skin, but not in other organs. There was no sign of calcium deposits or early enlargement of lung alveoli in $Cnr^{-/-}$ animals, although these changes are considered as indicators of bodily aging (Kurosu et al., 2005).

An atrophic testis structure, which is also accompanied with aging (Chigurupati et al., 2008), was present in $Cnr1^{-/-}$ animals of each age group. A clear-cut, age-dependent change in the structure of this organ was observed, however, only in the wild-type mice. This result suggests that the gerontomorph histological structure of the testis is probably the result of a developmental deficit and not of accelerated aging. The reduced number of spermatocytes in the $Cnr1^{-/-}$ animals is in line with the proposed role of the endocannabinoid system in the development of adult Leydig cells (Cacciola et al., 2008) and sperm production (Schuel and Burkman, 2006), and is not related to age-dependent changes.

A reduction of the subdermal fat layer, an indicator of skin aging, was present in 12-month-old Cnr1 null mutant animals, but not in wild-type mice. This finding may suggest that the onset of an age-related degeneration of the skin depends on CB1 receptor activity. However, in addition to the reduction of subdermal fat layer, also the quantity and integrity of collagens (Kahan et al., 2009) as well as fibroblast and keratinocyte activity (Knaggs, 2009) are important indicators of skin aging. To decide whether a loss of CB1 receptors leads to skin aging or to histological changes that resembles aging, additional studies are needed. An altered adipocyte metabolism could be responsible for the skin phenotype in $Cnr1^{-/-}$ animals, because the endocannabinoid system has a critical role in the regulation of the energy metabolism (Viveros et al., 2008). However, the atrophy of the subdermal fat layer in old $Cnr1^{-/-}$ animals is clearly not a direct consequence of the leaner body composition of this strain (Ravinet Trillou et al., 2004), because there is no decline, but rather a moderate increase in the body weight in 12-month-old compared with younger knockout mice (data not shown).

Finally we note that we did not find any difference in the course of age-related changes between the sexes. This result was rather unexpected, because the sex is thought to influence lifespan (Tower, 2006) and also skin (Dao and Kazin, 2007) and brain aging (Nithianantharajah and Hannan, 2009). However, the oldest animals we used in this study were 12 months old. Therefore we suggest that gender differences in the progression of brain and body aging do not manifest up to the age of 12 months in mice.

Our results showed that the early onset of cognitive deficits is accompanied by aging-like histological changes in the skin, but not with other signs of early aging in Cnr1 knockouts. We conclude that the lack of CB1 receptor does not induce a generally accelerated aging, but instead induces changes in the skin structure that resembles those associated with aging.

Disclosure statement

All authors declare that they have no actual or potential conflicts of interest.

Animal care and animal experimentation conduction followed the guidelines of the German Animal Protection Law (issued in 1998).

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Appendix. Supplementary data

Supplementary data associated with this article can be found ,in the online version, at doi:10.1016/j.neurobiolaging.2010.07.009.

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