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OXIDATIVE STRESS MARKERS IN THE
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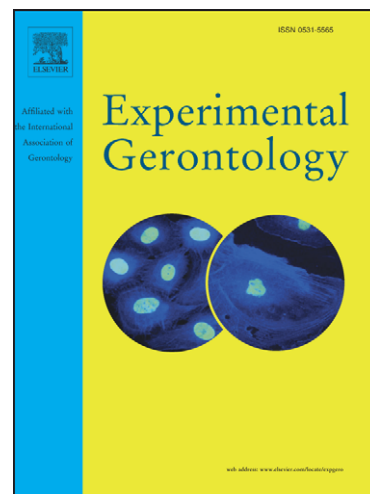
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DELETION OF THE LIFE SPAN DETERMINANT P66^{SHC} IMPROVES PERFORMANCE IN A SPATIAL MEMORY TASK, DECREASES LEVELS OF OXIDATIVE STRESS MARKERS IN THE HIPPOCAMPUS AND INCREASES LEVELS OF THE NEUROTROPHIN BDNF IN ADULT MICE

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Running title

Reduced oxidative stress and behavior

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Abstract

Deletion of the p66^{Shc} gene in mice results in reduced levels of oxidative stress and longer lifespan. Reactive oxygen species (ROS) can lead to tissue damage, particularly in the brain. In this study we extended previous findings on the behavioral phenotype of the p66^{Shc-/-} mice. Cognitive performance of adult and old p66^{Shc-/-} and p66^{Shc+/+} mice was tested in a Morris water maze (MWM) task while general reactivity and pain sensitivity were assayed at adulthood respectively in an open field and by means of a tail flick test. Levels of Brain-derived neurotrophic factor (BDNF), a neurotrophin involved in several aspects of synaptic plasticity, emotionality and pain sensitivity, were assessed in selected brain areas. P66^{Shc-/-} adult subjects, compared to WT, overall showed a better performance in the MWM, lower emotionality and a higher pain threshold, in addition to increased basal levels of BDNF in the hippocampus, as well as decreased levels of oxidative stress markers in the same brain area. Although all aged subjects failed to learn the cognitive task, aged p66^{Shc-/-} subjects **were** characterized by a better physical performance. These results suggest an interaction between the p66^{Shc} gene and specific signaling pathways involved in behavioral adaptation to stress and aging.

Keywords: p66^{Shc}; Oxidative stress; Mice; Morris water maze; Open field; Anxiety; Pain sensitivity; BDNF; Aging; Neurodegeneration

1. Introduction

Deletion of the p66^{Shc} gene in the 129Sv/Ev mouse strain (p66^{Shc-/-}) results in increased resistance to oxidative stress and in an extension of lifespan of about 30% (Migliaccio et al., 1999). P66^{Shc} knock out mice show no obvious phenotypical or histopathological abnormality and are characterized by a decreased incidence of aging-associated pathologies such as cardiovascular diseases and cancer (Francia et al., 2004; Migliaccio et al., 1999). P66^{Shc} is encoded by the ShcA gene together with the two cytoplasmic adaptor proteins p52^{Shc}/p46^{Shc} and has a specific role in the intracellular pathway(s) that regulates reactive oxygen species (ROS) metabolism and apoptosis (Giorgio et al., 2005; Migliaccio et al., 1999; Trinei et al., 2002). In the adult mouse brain p52^{Shc} and p46^{Shc} are present only in proliferative regions, while p66^{Shc} is expressed, though at very low levels, also in total brain lysates (Cattaneo and Pelicci, 1998). The role of p66^{Shc} in differentiated tissues still remains to be elucidated.

ROS are generated as a by-product of normal metabolism (Chavko et al., 2003). Both pathophysiological conditions and emotional stress may increase their generation, leading to a condition of oxidative stress which represents an important mechanism contributing to aging in a wide range of organisms (Liu and Mori, 1999; Madrigal et al., 2006). The mammalian brain is characterized by poor antioxidant defenses, high metabolic rate, and reduced capacity for cellular regeneration resulting particularly susceptible to oxidative stress insults (Floyd and Hensley, 2002). In addition, ROS and oxidative stress have been found to be involved in the neuropathology of several neurodegenerative disorders associated with cognitive impairment (Finkel and Holbrook, 2000). Intracellular ROS levels are decreased in the fibroblast of p66^{Shc-/-} mice as revealed by the reduced oxidation of ROS-sensitive probes and by the reduced accumulation of endogenous markers of oxidative stress (Francia et al., 2004; Napoli et al., 2003; Trinei et al., 2002). In this scenario, we hypothesized that p66^{Shc-/-} mice might be protected from the negative effects of free radicals since birth and characterized by a more efficient homeostatic control and by better abilities to cope with changes in the internal milieu. This could result in improved brain function and more adaptive behavioral and cognitive performances, especially under stressful situations (Coyle and Puttfarcken, 1993; Siesjo et al., 1989). The Morris water maze (MWM) is a hippocampal-dependent spatial memory task (Morris, 1984). Cognitive performance in this test can be affected by emotional reactivity and by Brain-derived neurotrophic factor (BDNF), a neurotrophin involved in neuronal survival and differentiation as well as in specific aspects of behavioral plasticity, emotionality and pain sensitivity (Cirulli et al., 2004; Thoenen, 1995). In this study, 4- and 24-months-old p66^{Shc-/-} mice were tested in a MWM. To characterize the neurobiological variables involved in spatial memory performance, experimental subjects were assayed for basal levels of BDNF in selected brain areas, in addition to levels of isoprostanes 15-F_{2t}-IsoP to monitor the extent of oxidative stress in the same regions.

We have previously shown that p66^{Shc-/-} mice, both at adulthood and during aging, are characterized by reduced emotionality (elevated plus maze test) and increased pain threshold (hot plate test) (Berry et al., 2007). Thus a further aim of this study was to refine this analysis at adulthood evaluating both spontaneous behavior and peripheral pain sensitivity in these mutants, respectively by means of an open field and a tail flick test.

2. Materials and Methods

Animals

Experimental subjects were non-littermate 4- (adult) and 24- (old) months-old p66^{Shc+/+} (WT) and p66^{Shc-/-} (KO) male mice of the 129Sv/Ev strain generated as previously described (Migliaccio et al., 1999), bred in the animal facility of the Section of Behavioral Neuroscience at the Istituto Superiore di Sanità (Rome, Italy). Animals were kept under standard conditions: housed in an air-conditioned room (temperature 21 ± 1 °C, relative humidity $60 \pm 10\%$) with a white-red light cycle (lights on from 08:30 to 20:30). Home cages were Plexiglas boxes (42 x 27 x 14 cm) with sawdust as bedding. Pellet food (Enriched Standard Diet purchased from Mucedola, Settimo Milanese, I-20019, Italy) and tap-water were continuously available. Different batches of animals were used for each behavioral test. For the Morris water maze test (MWM) experimental subjects were 20 adult (10 WT and 10 KO) and 17 old mice (7 WT e 10 KO).

The open field test (with and without an object) was performed on 7 WT and 6 KO adult mice, while the tail flick nociceptive test was performed on 10 subjects for each genotype (adult mice). Behavioral performances were video recorded using a digital video-camera connected to a professional Sony videocassette recorder V0-5800PS (Model TR 7000E, Sony, Tokyo). The behavioral analysis was carried out from the videotapes, using commercial softwares (“Ethovision 1.9” for the MWM and “The Observer 3.0” for the open field tests – Noldus, The Netherlands). All scores were assigned from the same observer blind to the genotypes. At the end of each behavioral session, apparatuses were thoroughly cleaned (cotton pads wetted with a 70% solution of ethanol and water). To evaluate basal levels of BDNF, and isoprostanes (15-F_{2t}-IsoP) cortices and hippocampi of adult (6WT and 6 KO) and old (6WT and 6 KO) subjects, which were not exposed to the behavioral procedures, were dissected and immediately frozen until usage. All behavioral tests were conducted under dim light between 9:30 and 13:30, i.e., during the white-light period.

All experimental procedures were carried out in accordance with the EC guidelines (EC Council Directive 86/609 1987) and with the Italian legislation on animal experimentation (Decreto L.vo 116/92). For pain assessment see (Cirulli et al., 2000b).

Morris Water maze

The apparatus used consisted of a white Plexiglas circular pool 88 cm in diameter and 33 cm in height. The pool, placed in the middle of an experimental room, was filled with water kept at a temperature of 24-26°C. A plastic platform (8 cm in diameter) was placed 0.5 cm below the water surface with its edge at 10 cm from the edge of the pool. Experimental subjects were trained to learn the position of a hidden platform in a 4-days acquisition phase (Balogh et al., 1999). Each day mice underwent 4 consecutive trials during which they were allowed to freely swim for 45 seconds (cut-off time) or until they found and climbed onto the platform. On experimental day 5, all subjects were tested for memory retention in a 45 seconds probe trial during which the platform was removed from the pool and the time spent in the target quadrant of the maze (where the platform was located during the acquisition phase) was scored as a reliable measure of memory retention. On the same day, one hour following the end of the probe trial, mice were tested in a 4-trials reversal task during which the platform was moved to a reversal quadrant diametrically opposed to the acquisition one. During this phase experimental subjects had to learn the novel position of the platform. The latency

to reach the platform was scored both during acquisition and reversal phases. The ability of both adult and old subjects to identify and reach a visible platform was tested in a pilot study (cued version of the task).

Open field

WT and KO subjects were individually placed in a novel environment (cubic open-field box: 44 cm wide, 44 cm deep and 44 cm high, made of grey Plexiglas, ideally divided in 25 squares) for 15 minutes and their spontaneous behavior was scored (latency, frequency and duration of squares crossings with both fore and hind paws, rearing and grooming episodes, and immobility). Furthermore thigmotaxis and risk assessment behavior (stretched-attend posture) were taken into account as indexes of emotionality. For thigmotactic behavior, the arena was ideally divided into a peripheral (external perimeter 44 cm, inner perimeter 26.4 cm) and a central part (26.4 x 26.4 cm) and time spent in each portion was scored. To further investigate behavioral reactivity, following 2 day of habituation to the experimental context (day 4) during which behavioral performance was not recorded, mice were re-introduced in the open field, a novel object (a small Chinese *sake* cup: 4.5 external diameter and 4 cm height) was positioned in the center of the arena and the behavior was videotaped and successively scored as on day 1; exploration of the object (sniffing and physical contacts) was also scored.

Tail flick

The tail flick test is aimed to assess in rodents peripheral pain nociception via a spinal reflex (King et al., 1997). Aged p66^{Shc^{-/-}} mice assayed in the hot plate test show a higher (central) pain threshold compared to WT while a tendency is evident in adult subjects (Berry et al., 2007). To further investigate pain threshold at adulthood, a tail flick test was run in adult subjects in order to characterize whether the peripheral component of pain sensitivity is affected by the genetic manipulation in these subjects. The apparatus was a tail flick unit (Socrel Mod DS-20, Ugo Basile, Italy) with a infrared radiant light source (100 W bulb, 15 V) focused onto a photocell utilizing an aluminum parabolic mirror. Animals underwent the test twice on the same day between 12.00 and 17.00. The latency (s) to move the tail out of the path of a strong beam of focused light was scored as the mean latency recorded on trial 1 and trial 2 if they did not differ significantly from each other. The experimental subjects were gently hand-restrained making sure that they were completely still before focusing the beam. Radiant heat was focused 1–2 cm from the tip of the tail; measurement was terminated if the latency exceeded the cut-off time (15 s at 15 V).

Brain dissection and BDNF and 15-F_{2t}-IsoP extraction and measurement

After decapitation, cortices and hippocampi were immediately dissected out, placed in plastic tubes, weighted, frozen on dry ice and stored at –80 C until metabolite extraction. A detailed procedure for F_{2t}-isoprostane (15-F_{2t}-IsoP) extraction has been described elsewhere (Minghetti et al., 2000). In brief, 10 M of the radical scavenger butylated hydroxy-toluene (stock solution 100x in ethanol) to avoid auto-oxidation was added to each frozen sample, which was quickly thawed, homogenized with a Teflon pestle (Sigma) - 20 cycles in an ice bath – vigorously vortexed and centrifuged at 14000 rpm for 45 min at +4 C. Supernatants were collected and stored at –80 C until analysis. For BDNF extraction, tissue samples were rapidly thawed in ice-cold PBS containing a protease inhibitor cocktail (1:10 of a stock solution in H₂O, prepared following the manufacturer's instructions; Sigma-Aldrich, Italy), and processed as before.

Isoprostanes (15-F_{2t}-IsoP) were measured in tissue extracts by high sensitivity colorimetric enzyme immunoassays (EIA kits, detection limit for 15-F_{2t}-IsoP: 2 pg/mL; Cayman Chemical, Ann Arbor, MI). According to the manufacturers, the cross-reactivity of anti-15-F_{2t}-IsoP for other prostaglandins was less than 1%. All measurements were run at least in duplicate for each sample. Results were expressed as pg/mg of wet tissue. The amounts of free mature BDNF in tissue extracts were measured (duplicate or triplicate) by a high sensitivity colorimetric enzyme immunoassay (EIA kit, detection limit: 7.8 pg/mL, Promega Corporation, Madison, WI), following the manufacturer's instructions. According to the manufacturer's instructions, the cross-reactivity of the anti-BDNF antibody with other related neurotrophic factors (NGF, NT-3, and NT-4) was less than 3%. Results were expressed as pg/mg of wet tissue.

Statistical analysis

Data were analyzed using parametric analysis of variance (ANOVA) with genotype as between-subjects factor (MWM, open field with an object, tail flick test and analysis of BDNF and 15-F_{2t}-IsoP levels) and time blocks as within-subject repeated measures, when appropriate (MWM). Post hoc comparisons were performed using the Tukey's HSD test. Mann and Whitney U test was applied on data on the physical performance of old subjects in the MWM and open field test due to non normality of variables distribution. The Fisher exact probability test was used to analyze the physical performance of old subjects in the MWM and backward walking and rearing behavior in the open field test (two-by-two contingency table).

3. Results

Morris water maze

Since old subjects (24-months-old) failed to reach a learning criterion, data were analyzed separately for the two groups of age.

Both WT and KO adult mice learned to find the platform more efficiently over the subsequent acquisition days (main effect of repeated measures $F(3,54) = 7.363$; $p = 0.0003$, see Fig. 1a). However, during the probe trial, while KO mice spent more than 25% of the allotted time (chance level) searching the platform in the target quadrant, the WT counterpart did not reach the chance level, overall indicating that these subjects did not learn the task properly (interaction between genotype and zone: $F(3,54) = 3.471$; $p = 0.0222$; Fig. 1b, left panel). **Mean velocity over the four acquisition days was 18cm/s for both WT and KO. These data are in line with previous observations collected in our lab on the CD-1 mouse strain and indicate that, in this test, mice used an active searching strategy (data not shown).** No difference was found between the two genotypes during the reversal phase (latency to reach the platform located in the new position: $F(3,54) = 0.059$; $p = 0.9812$).

As for the old subjects, since they overall failed to learn the task, performance in the acquisition phase was taken into account as a reliable index of physical health. Although the number of KO subjects able to climb onto the platform tended to be higher (two tailed Fisher's exact probability test: $p = 0.1007$; Fig. 1b right panel), more importantly, they were more successful to climb onto the platform when considering the number of times each subject reached the platform with its four paws (over the 16 acquisition trials) ($U(7,10) = 8,000$; $p = 0.0084$; mean values \pm S.E.M. respectively for WT and KO mice: 0.43 ± 0.2 and 2.1 ± 0.38).

Fig. 1 about here

Open field

On experimental day 1 a significant difference was found between WT and KO in the latency to the first sniffing episode ($U(7,6) = 32.0$; $p = 0.0247$) while analysis of the time spent in the central part of the arena just missed statistical significance ($U(7,6) = 6.5$; $p = 0.0600$). In particular $p66^{\text{Shc}^{-/-}}$ mice showed a lower latency to the first sniffing episode and, although they did not differ from WTs in the latency to visit the center of the arena ($U(7,6) = 18.5$; $p = 0.9349$), overall they tended to spend a longer amount of time in it than WTs. Furthermore, during the test, only KO subjects exhibited an uncommon behavior named “backward walking” that involves stepping backwards rather than foreword (see Fig. 2) (two tailed Fisher’s exact probability test $p = 0.0152$). No difference was found in the general locomotor activity (crossing frequency: $U(7,6) = 25.5$; $p = 0.2265$).

Results for day 4 are shown in Fig. 3: $p66^{\text{Shc}^{-/-}}$ mice were overall much more prone to explore both the environment, as shown by the higher number of subjects displaying rearing behavior (two tailed Fisher’s exact probability test $p = 0.0291$), and the object present in the arena (mean frequency of sniffing and contacts with the object: $F(1,11) = 5.924$; $p = 0.0332$). No difference between genotypes was found in locomotor activity (crossings frequency: $F(1,11) = 0.1050$; $p = 0.7519$). As far as emotional reactivity, KO subjects appeared less emotional, performing a lower number of stretched-attend postures (SAP) (main effect of genotype on SAP frequency: $F(1,11) = 4.591$; $p = 0.0554$) and spending a higher amount of time in the center of the arena (interaction between genotype and zone: $F(1,11) = 5.425$; $p = 0.0399$) compared to the WT counterpart.

Fig. 2 and Fig. 3 about here

Tail flick

Results from the tail flick test (Fig. 4) confirmed the tendency to higher pain threshold previously observed with the hot plate test. In fact, KO subjects showed a higher latency to flick their tail out from the light beam compared to the WT counterpart (genotype main effect: $F(1,18) = 6.63$; $p = 0.0188$) being therefore less sensitive (higher pain threshold) to a peripheral thermal noxious stimulus.

Fig. 4 about here

BDNF and 15-F2t-IsoP measurement

In some cases, following the extraction procedure, the amount of tissue available was not sufficient to assay both BDNF and 15-F_{2t}-IsoP, thus the final number of the samples available for each genotype and for each area was between 4 and 6. Since the profiles of the neurobiological variables taken into account were different at the two different ages we decided to analyze separately adult and old subjects. To take into account the increase in type 1 error probability due to separate testing we considered significant only those effects where the significant level was $p < 0.025$ (Bonferroni correction).

Analysis of BDNF levels in adult subjects showed that the hippocampus was characterized by higher levels of BDNF than the frontal cortex (main effect of brain area $F(1,15) = 100.168$; $p < 0.0001$), as previously reported (Yan et al., 1997), and revealed higher levels of this neurotrophin in the KO mice (main effect of genotype $F(1,15) = 9.414$; $p = 0.0078$) especially in the hippocampus (interaction between genotype and brain area $F(1,15) = 7.552$; $p = 0.0149$; post hoc comparisons: $p < 0.01$) (Fig. 5). No main effect of genotype was found for levels of 15-F_{2t}-IsoP in adult subjects ($F(1,16) = 2.687$; $p = 0.1207$) while higher levels of isoprostanes were found in the hippocampus (main effect of brain area $F(1,16) = 9.018$; $p = 0.0084$) compared to the cortex. Higher hippocampal levels of 15-F_{2t}-IsoP were found in WT subjects compared to the KO group (interaction between genotype and area $F(1,16) = 5.865$; $p = 0.0200$; post hoc comparisons: $p < 0.05$) (Fig. 5).

Fig. 5 about here

When cortices and hippocampi of 24-months-old mice were analyzed, no difference was found as a function of genotype for levels of both BDNF ($F(1,18) = 0.041$; $p = 0.8427$) and 15-F_{2t}-IsoP ($F(1,20) = 0.406$; $p = 0.5312$). A main effect of area was found for BDNF ($F(1,18) = 16.337$; $p = 0.0008$) thus, compared to the cortex, also during aging, the hippocampus is characterized by higher levels of BDNF. Levels of 15-F_{2t}-IsoP did not differ between hippocampus and cortex ($F(1,20) = 0.737$; $p < 0.4007$).

Table 1 about here

4. Discussion

Our data show that deletion of the lifespan determinant p66^{Shc} in adult mice results in better performance in the MWM and increased levels of BDNF in the hippocampus, in addition to reduced emotionality, pain sensitivity and lower levels of oxidative products. Furthermore the lack of this gene results in improved physical performance at old age.

During the probe trial of the MWM p66^{Shc-/-} adult mice spent more time in the target zone compared to WT subjects, a result suggesting that they remembered the platform location, while the WTs did not reach the criterion for memory retention. The genetic background of the mutant mice used for this study is the 129Sv/Ev, one of the most popular strain for knock out mice, although it has been described as being overall characterized by mediocre behavioral performance and poor swimming navigation learning (Wolfer et al., 1998). Since the MWM does not involve the use of food deprivation or foot shock to motivate the animal to solve the task (Morris, 1984), it was chosen as appropriate to assess cognitive abilities in this specific animal model which is characterized by changes in pain threshold and could be more sensitive to food deprivation as a result of the mutation. Nonetheless, the poor performance of the WT allowed us to assess the superiority of the KOs in this easy test where ceiling effects often prevent the assessment of subtle differences in behavioral performance.

As for the mechanism, a growing body of evidence (both in vivo and in vitro) support the concept that ROS may be involved in memory impairment (Abidin et al., 2004; Pellmar et al., 1991; Silva et al., 2004; Williams and Bliss, 1989). In this scenario our data enlarge such a body of evidence showing that a genetic manipulation affecting redox balance in favor of an antioxidant milieu may improve cognitive processes in adult mice.

Both WT and KO subjects, at the old age, failed to learn and remember the platform location in the MWM task. However, KO old mice showed indeed a better physical performance being able to reach the platform and to climb onto it. It is intriguing to speculate that this result may be related to the decreased incidence of aging-associated diseases, such as cardiovascular disease and cancer, that **characterizes** the p66^{Shc^{-/-}} phenotype (Francia et al., 2004; Migliaccio et al., 1999).

When tested in the open field, KO mice exhibited a reduced emotional profile in comparison to their WT counterparts. During experimental day 1, although both WT and KO subjects perceived the novel environment as unfamiliar (no difference in the latency to the first visit to the center), p66^{Shc^{-/-}} subjects were more explorative than the WTs exhibiting a lower latency to the first sniffing episode and tending to spend, on average, a longer amount of time in the center of the open field. This latter result strongly suggests a lower emotional profile of the p66^{Shc^{-/-}} mice. On day 4, following three days of habituation (day 1 plus two further days) to the experimental context, p66^{Shc^{-/-}} mice were more prone to explore both the environment (higher score of the vertical activity) and the novel object (mean number of sniffing plus contacts with the object episodes) compared to WT subjects. In addition, KO subjects spent significantly more time in the center of the arena thus confirming and strengthening the tendency showed during day 1. A further result in favor of a reduced emotional profile of the mutants is provided by the lower frequency of stretched-attend posture (a risk assessment behavior) showed by the p66^{Shc^{-/-}} mice when compared to WTs. We have previously shown that KO mice are characterized by lower levels of emotionality when tested in the elevated plus maze (Berry et al., 2007). With the present study we confirm and strengthen our previous findings in addition to show that this characteristic is already evident at adulthood.

Pain sensitivity has an important adaptive value and responses to emotionally/arousing stimuli are often accompanied by changes in pain threshold. In a previous study we tested pain nociception in p66^{Shc^{-/-}} mice from adulthood to senescence (4-, 11- and 24-months-old mice) and found that pain sensitivity was significantly reduced in old subjects (Berry et al., 2007). In the present study, peripheral nociception was assessed by means of a tail flick test KO subjects showing lower peripheral pain sensitivity already at adulthood. This result suggests that lack of the p66^{Shc^{-/-}} gene might have affected the ontogeny of sensory neurons or neuronal pathways involved in pain sensitivity. Alternatively a reduced exposure to ROS throughout development and adulthood might account for these findings since it has been suggested that ROS contribute to the symptomatology of neuropathic pain disorders (Crisp et al., 2006).

Data on nociception in KO mice are also interesting in that they suggest a link between neuronal pathways underlying pain sensitivity and emotionality. This link has been recently strengthened by means of a functional genomic approach aimed at identifying candidate genes for mood disorders (Ogden et al., 2004). Data from these studies have revealed the existence of a quite intriguing genetic and neurobiological overlap between mood, pain and pleasure pathways. Results obtained in the present study suggest that genes involved in oxidative stress pathways might functionally overlap with these systems.

Basal protein levels of BDNF were found to be increased in the hippocampus of KO adult mice. This finding represents a converging point linking together most of the data presented so far. Previous work from this group has shown that a single intrahippocampal administration of BDNF in rats improves behavioral plasticity in the MWM test and that single administrations both in the hippocampus and in the third ventricle are able to decrease pain sensitivity (hot plate test) and emotional reactivity (elevated plus maze) (Cirulli et al., 2000a; Cirulli et al., 2004). Thus it is indeed possible to hypothesize that the higher hippocampal levels of this neurotrophin in the p66^{Shc^{-/-}} mice might underlie their performance in the MWM test and their reduced pain sensitivity and emotionality. The lack of a difference in basal

levels of 15-F_{2t}-IsoP does not exclude that, as a result of an acute stress or insult, deletion of p66^{Shc} might result in reduced production of these markers.

Old subjects did not differ in their basal levels of BDNF and isoprostanes. Changes in BDNF expression appear to occur in Alzheimer's disease patients (Murer et al., 2001) however, evidence to date has been conflicting and BDNF has been reported to be increased (Durany et al., 2000), as well as decreased (Hock et al., 2000) in the hippocampus of human post-mortem Alzheimer's disease brains. Thus, it is possible to hypothesize that the lack of a difference in BDNF levels at this age (24 months) may reflect the progressive lack of homeostatic balance, possibly accompanied by neurodegenerative processes that may occur at senescence. **To further support this hypothesis is the finding that BDNF levels were higher in old subjects, compared to adult mice. An increase in BDNF protein levels in aged rodents has already been reported and has been related to an impaired retrograde transport mechanism from cortex and hippocampus (area of production) to the basal forebrain (area of need) (Bimonte et al., 2003). This unbalance in BDNF levels, which is likely to be independent from changes in oxidative stress, is not accompanied by parallel changes in basal levels of 15-F_{2t}-IsoP. Once again, it cannot be excluded that as a result of an acute stress or insult, old subjects might show a higher increase of this marker of oxidative stress compared to the adults.**

BDNF might not act alone. In this paper a striking result is represented by the specific expression of “backward walking” by p66^{Shc-/-} mice. Such a peculiar behavior has been associated to a hyper-activity of both the serotonergic and dopaminergic systems and to a higher BDNF tone (Bert et al., 2006; Martin-Iverson et al., 1994). BDNF and serotonin (5HT) are both known to regulate synaptic plasticity, neurogenesis and neuronal survival in the adult brain (Mattson et al., 2004). In particular, these two signals co-regulate one another. In addition, impaired 5-HT and BDNF signaling are central to mood disorders since selective serotonin reuptake inhibitors, commonly used to treat anxiety in humans, have been found to increase the expression of BDNF and of its receptor, in the brain (Castren et al., 2007). These data suggest that changes in emotionality and pain sensitivity found in p66^{Shc-/-} mice might be linked to increased serotonergic function, in conjunction with changes in BDNF levels.

In conclusion these data are in line with a previous report showing that deletion of the p66^{Shc} gene, which results in reduced levels of oxidative stress in the brain as well as in peripheral tissues, and increased lifespan, is also able to affect the cognitive/emotional phenotype of the mutants at adulthood and physical performance during aging. Data on central levels of isoprostanes in adult subjects are novel and suggest that the behavioral phenotype shown by the p66^{Shc-/-} mice might be associated to reduced exposure to ROS throughout life. **Further studies are in progress to validate the effects of the p66^{Shc} mutation on the C57/BL6 genetic background.**

These effects, overall, are likely due to complex interactions between the signaling cascades of the p66^{Shc} gene and specific signaling pathways in the brain, including those of BDNF and 5HT, which are highly conserved among organisms, since they play important roles in energy metabolism, adaptation to stress and disease, and in the aging process. **The data presented here point to p66^{Shc} as another target gene to study the basic mechanisms involved in neuronal plasticity and emotional disorders, in addition to successful aging.**

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ACCEPTED MANUSCRIPT

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Figure legends

Fig. 1. Learning and memory performance in the MWM spatial memory test. (A) Mean latency to reach the platform over the 4 acquisition days. (B) Left panel: after removal of the platform (probe trial, day 5) KO adult subjects preferred the target quadrant and spent more time in it than the WT counterpart. Right panel: percent of old subjects able to climb onto the platform at least one time during the acquisition phase. The cut-off time for each trial was 45 seconds. Post hoc comparisons are shown: * $p < 0.05$. Data represent mean values \pm SEM (n = 10 subjects in each group).

Fig. 2. Spontaneous behavior and emotional reactivity in a novel environment (open field arena). The session lasted 15 minutes. Backward walking behavior characterized 90% of the mutants. Post hoc comparisons are shown: * $p < 0.05$. Data represent mean values +SEM (n = 7 WT; 6 KO).

Fig. 3. Open field test with an object. Following 3 days of habituation subjects were re-exposed to the open field arena in the presence of a novel object (day 4). The session lasted 15 minutes. Post hoc comparisons are shown: * $p < 0.05$. Data represent mean values + SEM (n = 7 WT; 6 KO).

Fig. 4. Peripheral pain sensitivity in p66^{Shc^{-/-}} subjects. The latency to flick the tail out of the light beam was calculated as the mean latency recorded over two trials. Cut-off time was 15 seconds. Post hoc comparisons are shown: * $p < 0.05$. Data represent mean values + SEM (n = 10 subjects in each group).

Fig. 5. Hippocampal and frontal cortex levels of BDNF and 15-F_{2t}-IsoP in adult mice. Post hoc comparisons: ** $p < 0.01$ * $p < 0.05$ indicate a significant difference between WT and KO in the hippocampus for BDNF and 15-F_{2t}-IsoP. Data represent mean values + SEM (n = 4 to 6 for WT, and KO).

Table 1. Levels of BDNF and 15-F_{2t}-IsoP in the hippocampus and frontal cortex of old mice. WT and KO subjects did not differ in their levels of BDNF and 15-F_{2t}-IsoP. The Hippocampus of adult subjects was characterized by higher levels of BDNF when compared to cortex (**$p < 0.01$**). Data represent mean values+ SEM (n = 5-6 for WT and KO).

		BDNF (pg/mg)	15-F2t-IsoP (pg/mg)
Old	ctx	WT	36 ± 7
		KO	71 ± 30
	hippo	WT	223 ± 50
		KO	203 ± 49

Table.1

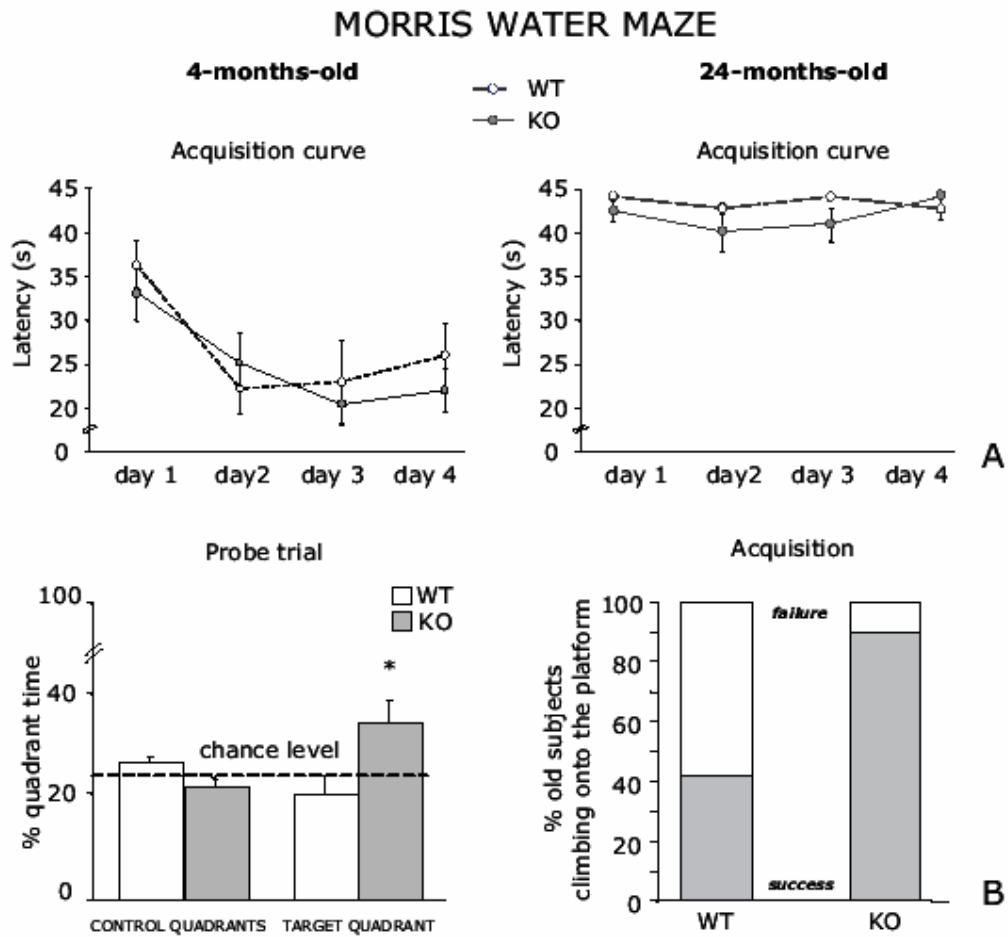


Fig.1

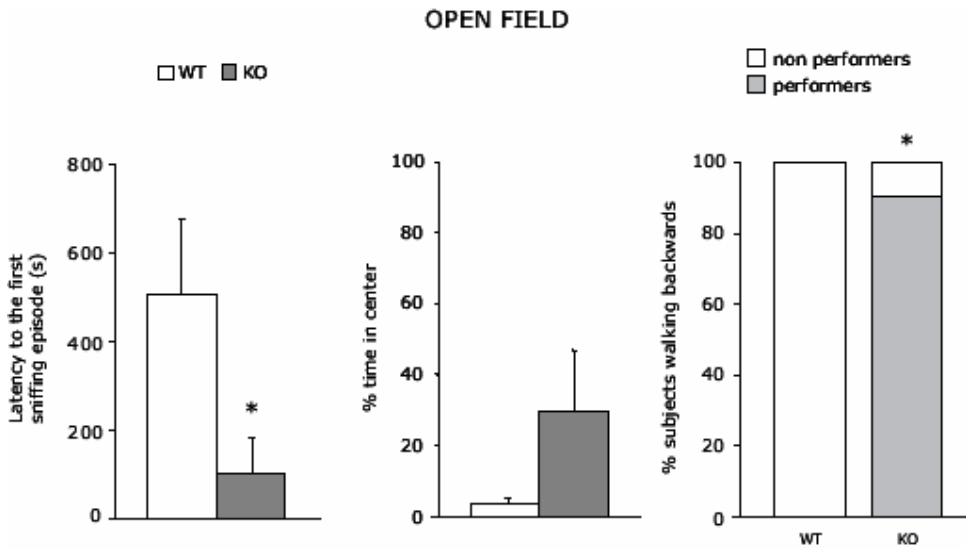


Fig.2

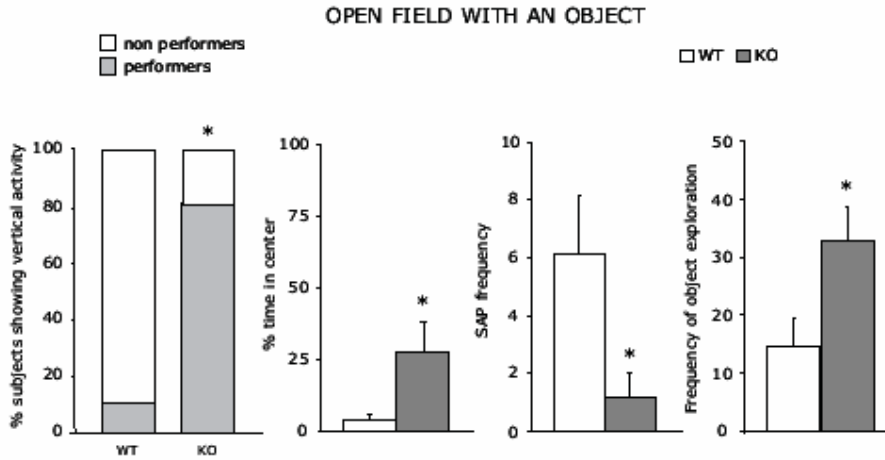


Fig.3

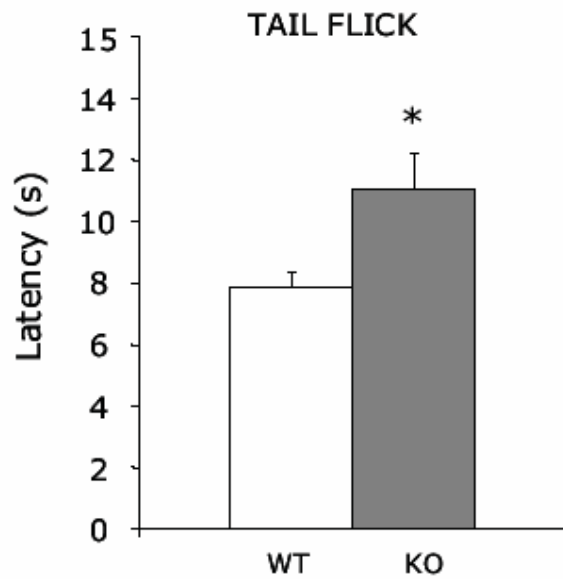


Fig.4

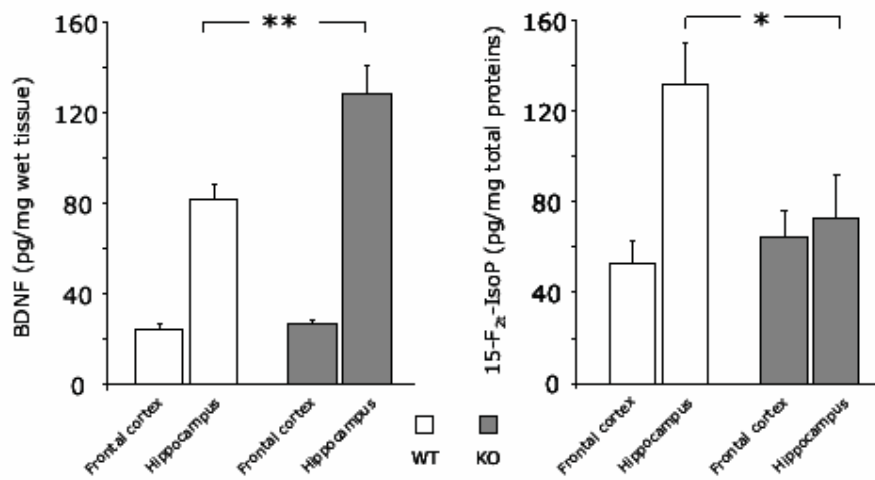


Fig.5