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Increased neurodegeneration during ageing in mice lacking high-affinity nicotine receptors

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We have examined neuroanatomical, biochemical and endocrine parameters and spatial learning in mice lacking the β2 subunit of the nicotinic acetylcholine receptor (nAChR) during ageing. Aged $\beta2^{-/-}$ mutant mice showed region-specific alterations in cortical regions, including neocortical hypotrophy, loss of hippocampal pyramidal neurons, astro- and microgliosis and elevation of serum corticosterone levels. Whereas adult mutant and control animals performed well in the Morris maze, 22- to 24-month-old $\beta2^{-/-}$ mice were significantly impaired in spatial learning. These data show that β2 subunit-containing nAChRs can contribute to both neuronal survival and maintenance of cognitive performance during ageing. $\beta2^{-/-}$ mice may thus serve as one possible animal model for some of the cognitive deficits and degenerative processes which take place during physiological ageing and in Alzheimer's disease, particularly those associated with dysfunction of the cholinergic system.

Keywords: ageing/gliosis/homologous recombination/neurodegeneration/nicotinic acetylcholine receptor

Introduction

Alterations in cholinergic systems are a common feature of normal ageing in mammals (Bartus *et al*., 1982; Decker, 1987). In addition, marked impairment in cholinergic systems is a highly consistent finding in human dementias (Aubert *et al*., 1992; Kasa *et al*., 1997). Among cholinergic markers, high-affinity binding sites for nicotine decrease by up to 80% in the telencephalic regions of patients suffering from Alzheimer's disease (Nordberg and Winblad, 1986; Whitehouse *et al*., 1988). It has been proposed that the nicotinic deficit in demented patients contributes to their cognitive impairment. This hypothesis is based on evidence that treatment with nicotinic antagonists impairs performance in spatial memory and other cognitive tasks in rodents (McGurk *et al*., 1989; Elrod and Buccafusco, 1991) and that experimentally induced lesions of the cholinergic system result in cognitive alterations which can be rescued by treatment with nicotinic agonists (McGurk *et al*., 1991). In addition, nicotine improves vigilance and rapid information processing in humans (Levin, 1992).

More recently, it has been proposed that nicotinic deficits may contribute to the development of age-related structural alterations of brain tissue. For example, both *in vivo* and in culture, nicotine protects striatal, hippocampal and cortical neurons against the neurotoxicity induced by excitotoxic amino acids (Marin *et al*., 1994; Borlongan *et al*., 1995) as well as the toxicity caused by β-amyloid, the major component of senile plaques (Kihara *et al*., 1998). Whereas no single mechanism for nicotine-mediated neuroprotection has been determined, studies have shown that nicotine increases the levels of neuronal growth factors (Maggio *et al*., 1997; Belluardo *et al*., 1998), decreases the levels of nitric oxide generated in response to neuronal injury (Shimohama *et al*., 1996) and inhibits glutamate-evoked arachidonic acid release from cultured striatal neurons (Marin *et al*., 1997). It has also been reported that smoking is negatively correlated with the incidence of Parkinson's disease and Alzheimer's disease (Jarvik, 1991).

Taken together, these data implicate nicotinic acetylcholine receptors (nAChRs) in protection against both pathological structural changes in the brain and cognitive deficits associated with ageing. However, no direct evidence for the impact of chronic loss of nAChRs on neural tissue structure is available. In this context, transgenic mice lacking nAChRs offer a unique opportunity to study the effects of chronic absence of these receptors on brain structure and behaviour. It is of particular interest to determine what role the various combinations of nAChR subunits play during normal ageing in a model system (Le Novère and Changeux, 1995; Role and Berg, 1996). A line of mice with a deletion of the gene encoding the β2 subunit of the nAChR has been generated by homologous recombination (Picciotto *et al*., 1995). These mice lack high-affinity binding for nicotine (Zoli *et al*., 1998), show alterations in the passive avoidance task (Picciotto *et al*., 1995) and are resistant to the reinforcing effects of nicotine in a self-administration paradigm (Picciotto *et al*., 1998). We have extended these studies to examine neuroanatomical, biochemical, behavioural and endocrine markers of ageing in these animals.

Results

Loss of β2-containing nAChR results in neural tissue atrophy and neuronal loss

Structural markers of brain ageing include neuronal degeneration and loss in some brain regions, including the cortex, substantia nigra and, most consistently, hippocampus.

Neuronal hypotrophy and loss is moderate during physiological ageing of both humans and rodents (Coleman and Flood, 1987), but is more clear-cut in cases of pathological ageing such as that seen in patients with Alzheimer's disease. In humans, a prominent marker of normal and, more specifically, pathological ageing is the accumulation of senile plaques formed by an amyloid core encircled by astrogliosis and microgliosis (Selkoe, 1994). In rodents, there is no evidence of amyloid deposition during ageing, but gliosis has been proposed as a rodent homologue of senile plaque formation (McMillian *et al.*, 1994). We therefore used anatomical staining methods to assess neuronal loss and neuronal tissue hypotrophy, as well as gliosis in $\beta2^{-/-}$ and their control siblings during ageing.

Atrophy was assessed in several di- and telencephalic regions. A modest (~10%) but highly significant ($p < 0.01$, Mann–Whitney *U*-test) decrease in cortical area/section (Figure 1A–C) was observed in several neocortical areas (including somatosensory and motor cortex at both anterior and posterior levels) of 22- to 24-month-old $\beta2^{-/-}$ mice as compared with their control siblings, whereas the area of the thalamus and of the basal ganglia did not change (Figure 1C). Reduced cortical area could be characterized as a widespread decrease in cortical thickness. Reduction in thickness of a similar extent was present in both external (II–III) and internal (V–VI) cortical layers of frontoparietal somatosensory and motor areas (not shown). A slight but significant (~5%, $p < 0.05$, non-parametric test for multiple comparisons) cortical atrophy was present in 22- to 24-month-old mice with respect to 4- to 6-month-old control mice (Figure 1D). Whereas no change in neocortical area was detected in $\beta2^{-/-}$ young adult (4- to 6-month-old) mice, a significant decrease in neocortical area was already observed in 10- to 12-month-old mutant mice (~5%, $p < 0.01$, non-parametric test for multiple comparisons, Figure 1D) when compared with age-matched control siblings.

The neuronal area and cell number in the various hippocampal subfields was also determined using Nissl and Feulgen staining, respectively (Figure 2A and B). A significant decrease (20 and 15% in CA1 and CA3, respectively, $p < 0.01$, non-parametric test for multiple comparisons) in the number of Feulgen positive neuronal nuclei was detected in the pyramidal layer of the CA1 and CA3 fields of 22- to 24-month-old as compared with 4- to 6-month-old control mice (Figure 2C). Neither staining method revealed a difference in neuronal cell number between 4- to 6-month-old $\beta2^{-/-}$ and control mice in either the CA1 or CA3 subfields of the hippocampus. In contrast, a moderate but highly significant decrease in the number of neuronal nuclei per field (as visualized by Feulgen staining, ~15%, $p < 0.01$, non-parametric test for multiple comparisons, Figure 2A–D) as well as thickness of the pyramidal layer (as visualized by Nissl staining, ~7%, $p < 0.01$, Mann–Whitney *U*-test, not shown) was selectively observed in the CA3 field of the hippocampus in 22- to 24-month-old $\beta2^{-/-}$ mice as compared with age-matched control siblings. A significant decrease in the number of Feulgen positive neuronal nuclei in CA3 was already seen in 10- to 12-month-old $\beta2^{-/-}$ mice (Figure 2C).

Aged C57Bl/6, but not DBA/2, mice have been shown to develop periodic acid Schiff (PAS)-positive granules

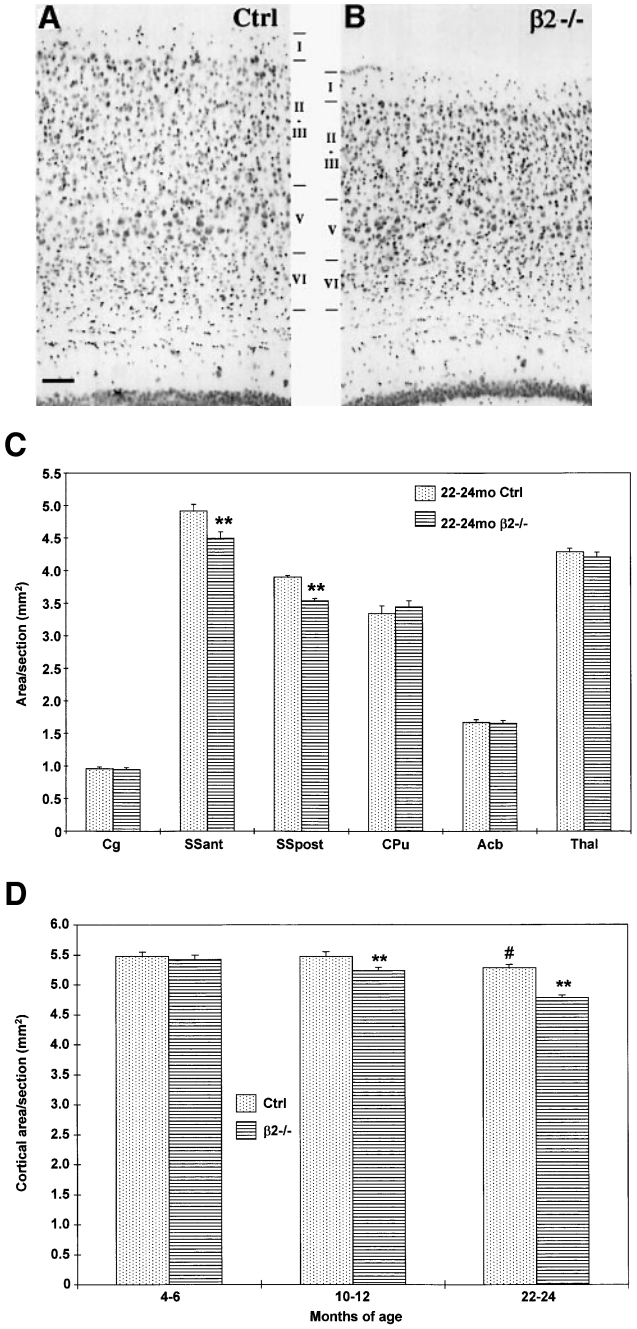

Fig. 1. Regional atrophy in aged $\beta2^{-/-}$ mice. (**A** and **B**) Nissl staining of the somatosensory cortex in aged $\beta2^{-/-}$ mice and their control (Ctrl) siblings. Bregma level = –1.7 mm according to Franklin and Paxinos (1997). Calibration bar, 100 μm. (**C**) Quantitation of the surface/section of different brain regions of aged $\beta2^{-/-}$ mice and Ctrl siblings. Cingulate cortex (Cg), caudate-putamen (CPu), nucleus accumbens (Acb) and anterior somatosensory cortex (SSant) were measured at bregma level + 1 mm, whereas posterior somatosensory cortex (SSpost) and thalamus (Thal) were measured at bregma level –1.7 mm. Mean ± SEM values are shown ($n = 14$ for Ctrl and $n = 11$ for $\beta2^{-/-}$). Statistical analysis according to Mann–Whitney *U*-test, $^{**}p < 0.01$. (**D**) Neocortical area at different ages in $\beta2^{-/-}$ mice and Ctrl siblings. The entire area of neocortex/section was measured in coronal sections at bregma level –1.7 mm. Mean ± SEM values are shown (4 to 6 months: $n = 14$ for Ctrl and $n = 5$ for $\beta2^{-/-}$; 10 to 12 months: $n = 16$ for Ctrl and $n = 22$ for $\beta2^{-/-}$; 22 to 24 months: $n = 14$ for Ctrl and $n = 11$ for $\beta2^{-/-}$). Statistical analysis according to non parametric test for multiple comparisons, $^{**}p < 0.01$ $\beta2^{-/-}$ versus age-matched Ctrl, # $p < 0.05$ versus 4- to 6-month-old Ctrl.

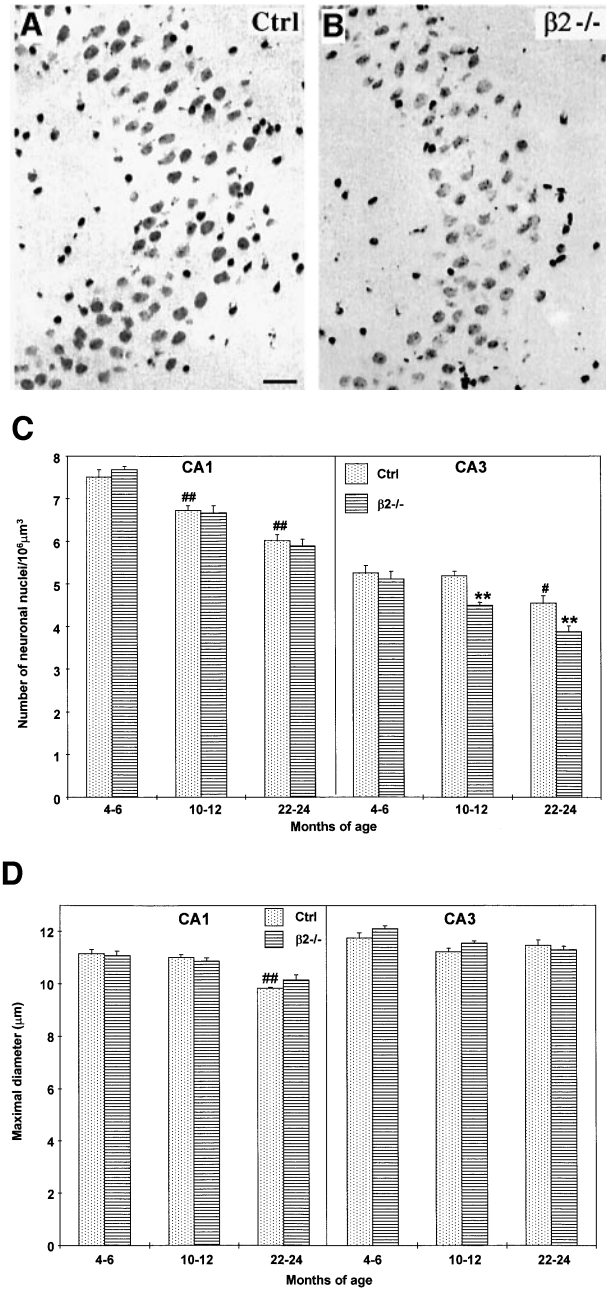

Fig. 2. Loss of pyramidal neurons of the CA3 hippocampal field in $\beta2^{-/-}$ mice. (**A** and **B**) Feulgen-stained nuclei in CA3 hippocampal field of $\beta2^{-/-}$ mice and their control (Ctrl) siblings. Bregma level –1.6 mm. Calibration bar, 25 µm. (**C** and **D**) Number (C) and mean diameter (D) of Feulgen-stained neuronal nuclei of the pyramidal layer of the hippocampal CA1 and CA3 field at different ages in $\beta2^{-/-}$ mice and their control (Ctrl) siblings. Mean ± SEM values are shown (4 to 6 months: $n = 10$ for Ctrl and $n = 5$ for $\beta2^{-/-}$; 10 to 12 months: $n = 16$ for Ctrl and $n = 22$ for $\beta2^{-/-}$; 22 to 24 months: $n = 14$ for Ctrl and $n = 11$ for $\beta2^{-/-}$). Statistical analysis according to non-parametric test for multiple comparisons, $^{**}p < 0.01$, $^{*}p < 0.05$, $\beta2^{-/-}$ versus age-matched Ctrl; ## $p < 0.01$, # $p < 0.05$ versus 4- to 6-month-old Ctrl.

predominantly in the hippocampus and other limbic cortical regions (Jucker *et al*., 1994). No significant difference in the density of PAS-positive granules in hippocampus or piriform cortex was seen between mutant and control animals (not shown). The random distribution of PAS-positive granules in both mutant and control animals implies that we did not select for either a purely DBA/2 or a C57Bl/6 background in either the $\beta2^{-/-}$ population or their control siblings.

Neuronal atrophy in β2$^{-/-}$ mice is accompanied by gliosis

Astro- and microgliosis are common in the aged rodent brain (Perry *et al*., 1993; Kohama *et al*., 1995; Rozovsky *et al*., 1998) and are considered markers of neuropathological changes in the senescent brain (McMillian *et al*., 1994). We therefore looked for changes in astroglial and microglial markers in the brains of $\beta2^{-/-}$ mice as compared with their control siblings, both in regions with signs of increased atrophy or neuronal loss and in regions which are unchanged in comparison with age-matched control mice. Astrocytes and microglia were examined by immunocytochemistry using antisera to glial fibrillary acidic protein (GFAP) (Figure 3A and B) and macrophage surface antigen CD18/CD11b (MAC1) (Figure 4A and B), respectively. In the hippocampal formation a marked increase (~60%, $p < 0.01$, Mann–Whitney *U*-test) in GFAP immunoreactivity (IR) was observed in the stratum oriens of both CA3 and CA1 fields, but not in the polymorph layer of the dentate gyrus, of 22- to 24-month-old $\beta2^{-/-}$ mice with respect to their control siblings (Figure 3C). In the CA1 field the number of GFAP-positive cells also increased significantly (~30%, $p < 0.01$, Mann–Whitney *U*-test, Figure 3D). A marked (~45%, $p < 0.01$, Mann–Whitney *U*-test) and slight (~20%, not significant) increase in MAC1 IR was seen in the CA1 and CA3 field of aged $\beta2^{-/-}$ mice, respectively (Figure 4C).

Microgliosis in the neocortex and thalamus was assessed in sections adjacent to those utilized for the assessment of regional atrophy by immunocytochemistry using a MAC1 antibody. A marked (60%) increase in MAC1 IR was seen in neocortical areas but not in the thalamic nuclei (Figure 4C) of $\beta2^{-/-}$ mice as compared with control mice. The increased MAC1 staining was therefore consistent with the hypotrophy present in the neocortex and absent in the thalamus of aged $\beta2^{-/-}$ mice.

Aged β2$^{-/-}$ mice show a spatial learning deficit

Aged rodents undergo a number of behavioural alterations, which include deficits in performance of several cognitive tasks (Gallagher and Nicolle, 1993; Gower and Lamberty, 1993). Among tests for cognitive function, impairment in Morris water maze performance has been repeatedly shown to be associated with ageing in rodents, and is therefore considered to be a standard animal model for age-related cognitive deficits. In addition, treatment with nicotinic agonists can rescue the deficit in Morris maze induced by lesions of the cholinergic telencephalic systems (Decker *et al*., 1994) and improve performance in space learning of normal adult and aged rats (Abdulla *et al*., 1993; Socci *et al*., 1995).

Using the Morris water maze, we measured spatial learning in adult (6–7 and 10 to 12 months of age) and aged (22- to 24 months of age) knockout mice lacking the β2 subunit of the nAChR, as well as their control siblings. Animals were trained to find a hidden platform using three-dimensional cues in the room. An age-related decrease in the performance of control animals was observed in the hidden platform task by 10 to 12 months

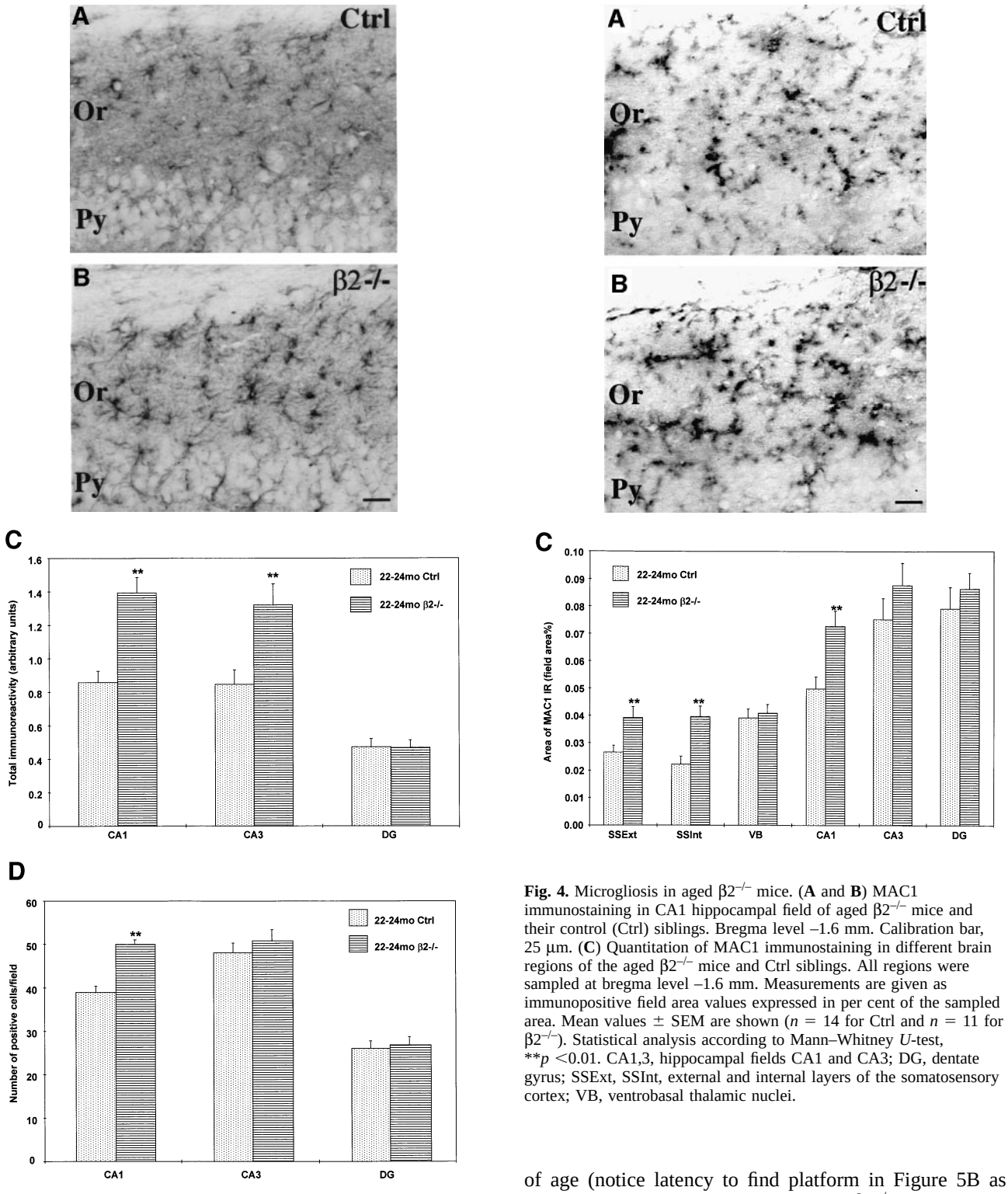

Fig. 3. Astrogliosis in aged $\beta2^{-/-}$ mice. (**A** and **B**) GFAP immunostaining in CA1 hippocampal field of aged $\beta2^{-/-}$ mice and their control (Ctrl) siblings. Bregma level –1.6 mm. Calibration bar, 25 μm. (**C** and **D**) Quantitation of GFAP immunostaining (C) and number of GFAP-positive cells (D) in the hippocampal formation of the aged $\beta2^{-/-}$ mice and Ctrl siblings. In (C), data are expressed as total immunoreactivity values (obtained by multiplying the field area % value by the mean grey tone value of specific profiles). Mean values ± SEM are shown (n = 14 for Ctrl and n = 11 for $\beta2^{-/-}$). Statistical analysis according to Mann–Whitney U-test, $^{**}p < 0.01$. Abbreviations: CA1,3, hippocampal fields CA1 and CA3; DG, dentate gyrus.

Fig. 4. Microgliosis in aged $\beta2^{-/-}$ mice. (**A** and **B**) MAC1 immunostaining in CA1 hippocampal field of aged $\beta2^{-/-}$ mice and their control (Ctrl) siblings. Bregma level –1.6 mm. Calibration bar, 25 μm. (**C**) Quantitation of MAC1 immunostaining in different brain regions of the aged $\beta2^{-/-}$ mice and Ctrl siblings. All regions were sampled at bregma level –1.6 mm. Measurements are given as immunopositive field area values expressed in per cent of the sampled area. Mean values ± SEM are shown (n = 14 for Ctrl and n = 11 for $\beta2^{-/-}$). Statistical analysis according to Mann–Whitney U-test, $^{**}p < 0.01$. CA1,3, hippocampal fields CA1 and CA3; DG, dentate gyrus; SSExt, SSInt, external and internal layers of the somatosensory cortex; VB, ventrobasal thalamic nuclei.

of age (notice latency to find platform in Figure 5B as compared with Figure 5A). Whereas $\beta2^{-/-}$ animals and their control siblings performed identically on this task at both 6–7 and 10- to 12 months of age, 22- to 24-month-old $\beta2^{-/-}$ mice showed a significant impairment in the hidden platform task as compared with their control siblings [$p < 0.02$, two-way analysis of variance (ANOVA) for repeated measures] (Figure 5C). Twenty-four hours after the final training trial, the platform was removed and a transfer test was performed for 60 s. The aged $\beta2^{-/-}$ animals showed a significant impairment in platform crossings ($p < 0.05$, Mann–Whitney U-test) (Figure 5E),

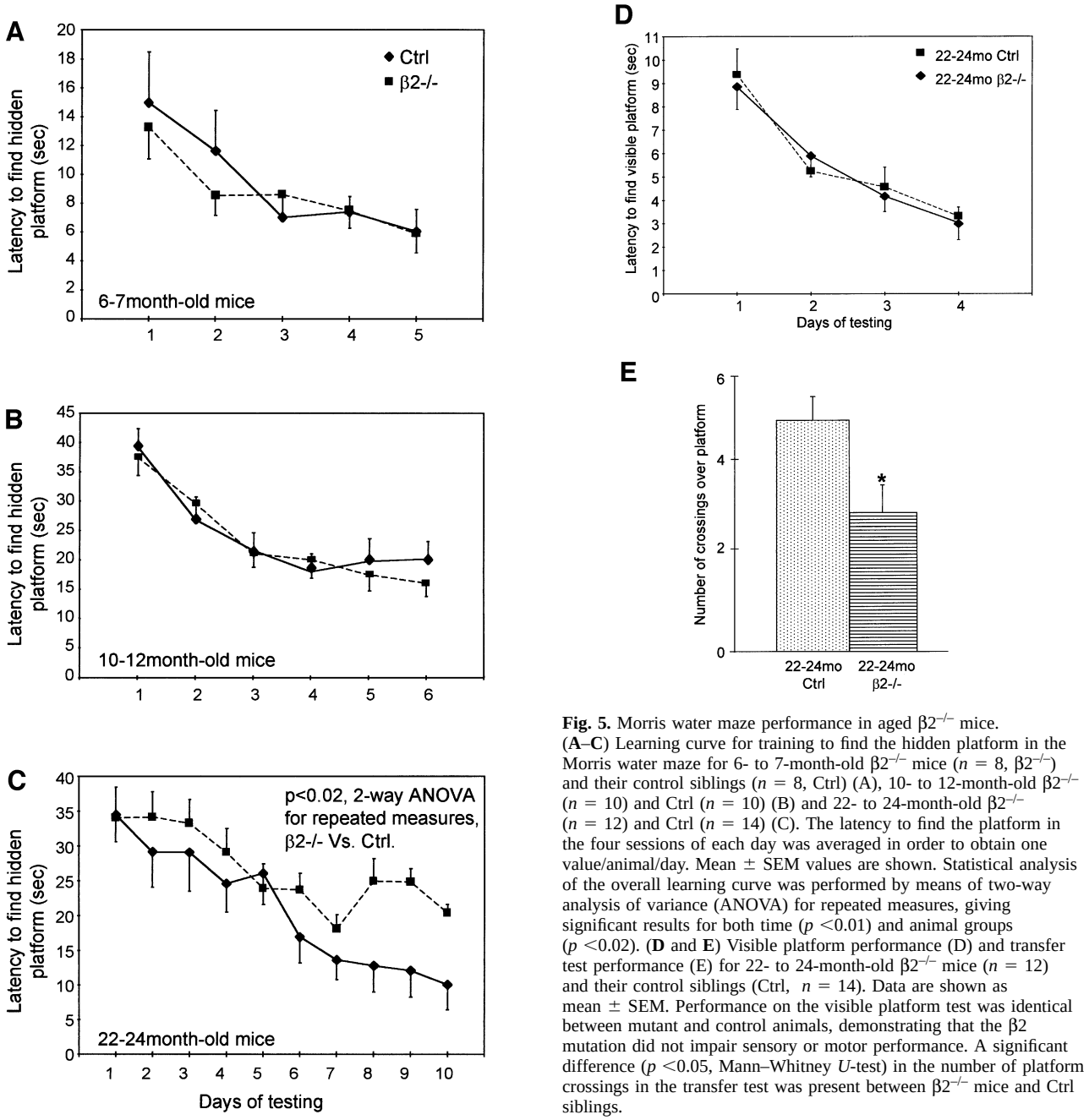

Fig. 5. Morris water maze performance in aged $\beta2^{-/-}$ mice. (**A**–**C**) Learning curve for training to find the hidden platform in the Morris water maze for 6- to 7-month-old $\beta2^{-/-}$ mice (n = 8, $\beta2^{-/-}$) and their control siblings (n = 8, Ctrl) (A), 10- to 12-month-old $\beta2^{-/-}$ (n = 10) and Ctrl (n = 10) (B) and 22- to 24-month-old $\beta2^{-/-}$ (n = 12) and Ctrl (n = 14) (C). The latency to find the platform in the four sessions of each day was averaged in order to obtain one value/animal/day. Mean ± SEM values are shown. Statistical analysis of the overall learning curve was performed by means of two-way analysis of variance (ANOVA) for repeated measures, giving significant results for both time (p <0.01) and animal groups (p <0.02). (**D** and **E**) Visible platform performance (D) and transfer test performance (E) for 22- to 24-month-old $\beta2^{-/-}$ mice (n = 12) and their control siblings (Ctrl, n = 14). Data are shown as mean ± SEM. Performance on the visible platform test was identical between mutant and control animals, demonstrating that the β2 mutation did not impair sensory or motor performance. A significant difference (p <0.05, Mann–Whitney U-test) in the number of platform crossings in the transfer test was present between $\beta2^{-/-}$ mice and Ctrl siblings.

whereas adult mutant animals were identical in performance to their control siblings (not shown). On days 12–15, training in the Morris maze was repeated using a visible platform. No significant difference in performance was observed between $\beta2^{-/-}$ and control mice, demonstrating that the animals had similar sensory–motor ability and motivation (Figure 5D).

Markers of cholinergic function are not dramatically altered in aged $\beta2^{-/-}$ mice

Several markers of the cholinergic system were examined to determine whether the behavioural and structural impairments observed in aged $\beta2^{-/-}$ mice were the consequence of a deficit in cholinergic neurotransmission secondary to the loss of high-affinity nicotine receptors (Table I). None of the markers examined, including acetylcholinesterase (AChE) staining, hemicholinium binding to assay choline transporter levels (a marker of overall cholinergic activity; Happe and Murrin, 1993), binding to α-bungarotoxin-sensitive nAChRs (a class of nAChRs which is spared in $\beta2^{-/-}$ mice; Zoli *et al.*, 1998) or binding to muscarinic M1- and M2-type receptors, were altered in 22- to 24-month-old $\beta2^{-/-}$ as compared with control mice. Other cholinergic markers, including soluble AChEs and choline acetyltransferase activities could not be measured in fixed tissue, thus it is possible that subtle alterations in cholinergic function could have been missed; however, these data imply that the overall structure and function of the cholinergic system is not substantially perturbed in aged $\beta2^{-/-}$ mice, and that the anatomical and behavioural changes observed result primarily from the deletion of the β2-containing nAChRs.

Table I. Cholinergic markers in aged $\beta2^{-/-}$ mice and their control siblings

	Genotype	Cg	Ctx	CPu	MS	Thal	CA1	CA3	DG
AChE	Ctrl	0.017±0.002	0.011±0.003	0.293±0.004	0.180±0.003	–	0.617±0.016	0.486±0.011	0.465±0.012
	$\beta2^{-/-}$	0.020±0.002	0.008±0.003	0.295±0.006	0.184±0.007		0.575±0.021	0.438±0.010	0.446±0.012
BTX	Ctrl	0.054±0.004	0.074±0.004	0.105±0.004	–	–	0.097±0.004	0.132±0.006	0.112±0.004
	$\beta2^{-/-}$	0.047±0.005	0.065±0.006	0.104±0.006			0.094±0.006	0.132±0.004	0.108±0.005
M1	Ctrl	0.106±0.006	0.087±0.004	0.171±0.004	–	–	0.186±0.005	0.103±0.003	0.132±0.005
	$\beta2^{-/-}$	0.118±0.007	0.092±0.007	0.172±0.008			0.182±0.002	0.101±0.002	0.131±0.003
M2	Ctrl	0.227±0.006	0.224±0.006	0.305±0.008	0.197±0.006	0.165±0.003	0.209±0.003	0.176±0.004	0.177±0.003
	$\beta2^{-/-}$	0.228±0.006	0.225±0.006	0.303±0.008	0.197±0.005	0.168±0.004	0.212±0.004	0.159±0.003	0.164±0.003
HEMI	Ctrl	0.123±0.004	0.098±0.004	0.120±0.005	–	0.080±0.004	0.123±0.004	0.112±0.004	0.123±0.004
	$\beta2^{-/-}$	0.132±0.007	0.102±0.004	0.127±0.003		0.086±0.004	0.122±0.005	0.114±0.004	0.123±0.005

Specific optical density values ± SEM are shown. Statistical analysis according to Mann–Whitney *U*-test. No significant difference was found between Ctrl and $\beta2^{-/-}$. Abbreviations: AChE, acetylcholine esterase; BTX, α-bungarotoxin; CA1 and CA3, hippocampal fields CA1 and CA3; Cg, cingulate cortex; CPu, caudate-putamen; Ctx, neocortex; DG, dentate gyrus; HEMI, hemicholinium binding; M1 and M2, M1 and M2 muscarinic binding; MS, medial septum; Thal, thalamus.

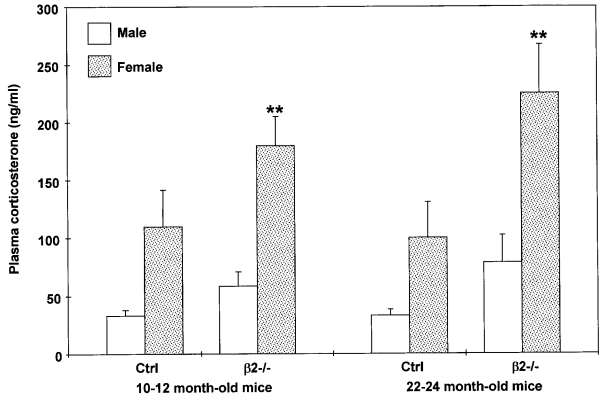

Fig. 6. Serum levels of corticosterone in $\beta2^{-/-}$ mice and their control (Ctrl) siblings. Mean values ± SEM are shown. Ctrl, 10 to 12 months old: $n = 6$ females + 10 males; 10- to 12-month-old $\beta2^{-/-}$: $n = 11$ females + 11 males; 22- to 24-month-old Ctrl: $n = 5$ females + 8 males; 22- to 24-month-old $\beta2^{-/-}$: $n = 5$ females + 6 males. Statistical analysis according to two-way ANOVA. At each age, sex and genotype were used as independent variables, $^{**}p < 0.01$, $\beta2^{-/-}$ versus age-matched control. A significant difference was also found for the variable sex ($p < 0.01$, not reported in the figure).

$\beta2^{-/-}$ mice show elevated circulating stress hormone levels

In both rodents and humans, ageing is often accompanied by changes in hormonal systems, including alterations in levels of sex steroid hormones, prolactin, corticosteroids and growth hormone (Cocchi, 1992). Alterations in corticosteroid levels may have a particularly important influence on brain ageing, since chronic elevation of corticosterone results in degeneration of pyramidal neurons in the hippocampal formation (Sapolsky, 1996). We measured serum levels of corticosterone, testosterone, prolactin and growth hormone in adult and aged $\beta2^{-/-}$ mice and their control siblings. Whereas 5- to 6-month-old $\beta2^{-/-}$ mice showed a slight, non-significant increase in corticosterone levels (not shown), aged $\beta2^{-/-}$ mice showed a significant increase (70 and 125% at 10 to 12 and 22 to 24 months of age, respectively, $p < 0.01$, $\beta2^{-/-}$ versus control, two-way ANOVA) in circulating corticosterone as compared with their control siblings (Figure 6). No significant change was observed in other hormones such as testosterone, growth hormone and prolactin (data not shown).

Discussion

Neuronal loss is confined to specific anatomical regions in the brains of $\beta2^{-/-}$ mice

In this study we have used neuroanatomical, biochemical, endocrinological and behavioural methods to assess whether lack of the β2 subunit of the nAChR affects brain ageing. These data indicate that the absence of β2-containing nAChRs leads to region-specific structural alterations, including cortical hypotrophy, neuronal cell loss in the CA3 field and gliosis in the cerebral cortex and CA3 and CA1 fields of the hippocampus, which are accompanied by a deficit in spatial learning and memory in aged animals. Structural alterations were not widespread, but rather localized to particular neocortical areas and some subregions of the hippocampal formation (i.e. CA1 and CA3 fields but not dentate gyrus). This selectivity does not reflect the regional distribution of β2-containing nAChRs, which are, for example, highest in the thalamus (Hill *et al*., 1993; Zoli *et al*., 1998), a region where no anatomical changes were observed. Rather, the observed structural alterations were mostly limited to the brain regions which are particularly vulnerable to the ageing process (Coleman and Flood, 1987). Loss of β2-containing nAChRs therefore aggravates some of the degenerative processes which are ongoing in the mouse brain during senescence and seems to mimic some markers of pathological ageing that have been documented in human brain.

The results presented here are consistent with recent evidence showing that selective lesion of the basal telencephalic cholinergic system results in degeneration of cortical areas (Robertson *et al*., 1998) which can be counteracted by long-term treatment with nicotinic agonists (Sjak-Shie and Meyer, 1993; Socci and Arendash, 1996). Further, nicotinic agonists have protective actions in models of β-amyloid- and excitotoxic-amino acid-mediated loss of hippocampal and cortical neurons (Marin *et al*., 1994; Borlongan *et al*., 1995; Semba *et al*., 1996; Kihara *et al*., 1998). The data presented here imply that high-affinity nicotine receptors may be involved in neuronal survival, suggesting that the loss of nicotine binding sites itself may contribute to development of neuronal degeneration during physiological, as well as pathological, ageing.

In contrast to what is seen in human dementias (Kasa *et al*., 1997) and animal models of cholinergic denervation

(see above), no cholinergic degeneration is associated with the loss of β2-containing nAChRs in aged $\beta2^{-/-}$ mice, indicating that lack of high-affinity nAChRs is sufficient to cause neuronal degeneration in cortical regions. This suggests that the decrease in nicotine binding sites in dementia may contribute to the development of neuronal degeneration in this pathological condition. Loss of high-affinity nicotine binding in $\beta2^{-/-}$ mice is complete and present throughout development, whereas in human dementias the loss of high-affinity nicotine binding reaches 50–80% of normal levels and is progressive through the course of the disease. Thus, the accelerated changes in markers associated with ageing in the $\beta2^{-/-}$ mice may reflect a long-term absence of these sites in this model.

Elevation of stress hormone levels has been correlated with neuronal degeneration

Lack of the β2 subunit of the nAChR also results in elevation of corticosterone serum levels. Whereas there was a trend to an increase in 5-month-old $\beta2^{-/-}$ mice as compared with their wild-type siblings, a significant 2-fold increase in circulating corticosterone was observed in 12- and 24-month-old $\beta2^{-/-}$ mice. Structural and functional impairment of hippocampal formation during normal ageing, as well as in some pathophysiological states, has been correlated with elevation of corticosteroid hormone levels in human and animal models. Indeed, hippocampal neuronal loss in aged rodents can be prevented if the animals are adrenalectomized during adulthood (for a recent review see Sapolsky, 1996). Specifically, it has been shown that chronic elevation of glucocorticoids can lead to selective degeneration of neurons in the CA3 region of the hippocampus and deficits in spatial learning (McEwen and Sapolsky, 1995). In turn, neuronal loss in the CA3 field contributes to maintenance of elevated levels of corticosteroids, since this brain region participates in the negative feedback of corticosterone on the hypothalamo pituitary–adrenal axis, thus initiating a self-maintaining pathological process (Sapolsky, 1996). It is therefore possible that the persistent increased glucocorticoid levels induced by loss of β2-containing nAChRs contribute to the progressive degeneration of hippocampal neurons as well as to the cognitive deficits observed in $\beta2^{-/-}$ mice during ageing.

The mechanism underlying the changes in circulating corticosterone in $\beta2^{-/-}$ mice is currently unknown, but several possible mechanisms can be proposed. One possibility is that loss of nAChRs primarily causes dysfunction and then loss of neurons in the CA3 field, which secondarily triggers corticosterone elevation and further lesion of the CA3 field (see above). Another possibility is that lack of β2-containing nAChRs leads to exaggerated endocrine responses to stressful stimuli. In fact, nicotine has been shown to act as an anxiolytic agent (Brioni *et al*., 1993) and to affect release of several neurotransmitters associated with stress pathways including both norepinephrine (Clarke and Reuben, 1996) and serotonin (Summers and Giacobini, 1995). Finally, an acute injection of nicotine increases corticosterone levels in the mouse (Caggiula *et al*., 1991). There is rapid tolerance to this response in wild-type animals, implying that adaptation to the chronic loss of nicotinic regulation in this pathway could result in increased baseline levels of corticosterone in β2 knockout mice. Any of these mechanisms could also result in alterations in circulating cortisol levels (Cocchi, 1992) following loss of high-affinity nicotine binding sites in human dementia.

Spatial learning deficits only occur during ageing in $\beta2^{-/-}$ mice

Loss of high-affinity nicotine-binding sites is not sufficient to cause deficits in spatial learning on its own, as young and adult mice lacking any high-affinity nicotine binding sites perform normally on the Morris water maze task (Picciotto *et al*., 1995). Instead, these experiments demonstrate that over time, lack of high-affinity nicotine binding sites results in structural alterations of cortical areas that can ultimately contribute to the decreased performance in a memory task during ageing. These results are consistent with recent experiments showing that following highly selective cholinergic lesions, there is little or no impairment in spatial learning (Chappell *et al*., 1998) unless other neurotransmitter systems are impaired (Gallagher and Colombo, 1995). Therefore, the role of β2-containing nAChRs in modulation of cognitive processes might be compensated by other systems in a normal adult brain. Accordingly, only in the presence of other deficits would the contribution of β2-containing nAChRs to a spatial learning task become apparent.

Aged $\beta2^{-/-}$ mice as an animal model of accelerated ageing

Significant cortical atrophy and hippocampal neuronal loss was observed in aged control mice. The aged control animals showed a decrease of 5–15% in cortical thickness or neuronal number as compared with adult control animals, in substantial agreement with studies of neuronal loss during normal ageing in the literature (Coleman and Flood, 1987). $\beta2^{-/-}$ mice show two to three times more cortical atrophy and hippocampal cell loss than age-matched control animals; in other words, a 15–30% decrease was noticed with respect to adult control values. These figures are in line with the amount of neuronal loss or cortical atrophy observed in other models of accelerated or pathological ageing, such as transgenic mice expressing mutated forms of amyloid precursor protein (15% decrease in number of hippocampal neurons; Calhoun *et al*., 1998), senescence-accelerated mice SAM-P/10 (10% decrease in neocortical area; Shimada *et al*., 1994) and rats subjected to postnatal lesion of telencephalic cholinergic system (10% decrease in neocortical area; Robertson *et al*., 1998). In addition, as in most of these models, a marked gliosis (40–60% increase in GFAP or MAC1 IR) was observed in aged $\beta2^{-/-}$ mice. Aged $\beta2^{-/-}$ mice may therefore represent a new animal model for some of the cognitive deficits and degenerative processes which take place during physiological ageing and in Alzheimer's disease, particularly those associated with a dysfunction of the cholinergic system.

Conclusions

The cholinergic hypothesis for Alzheimer's disease, in which cholinergic dysfunction is considered to be a primary event in the aetiopathogenesis of this disease, has been superseded by demonstration of the involvement

of amyloid proteins (Selkoe, 1994), apolipoprotein E (Strittmatter and Roses, 1996), the presenilins (Hardy, 1997) and inflammation (Breitner, 1996). Still, there remains a consensus that cholinergic impairments, including loss of nAChRs, contribute to the pathogenesis of cognitive deficits associated with Alzheimer's disease (Robbins *et al*., 1997). For example, AChE inhibitors and nicotine itself have been reported to delay cognitive decline in Alzheimer's disease (Wilson *et al*., 1995). However, new evidence is accumulating that the cholinergic system may also have a specific role in preventing the development of age-related neuropathological lesions (see above). The demonstration that lack of high-affinity nicotine receptors accelerates the development of structural, as well as cognitive, deficits associated with ageing supports the hypothesis that nAChR loss in the course of the ageing process can contribute to the development of neuronal degeneration. $\beta2^{-/-}$ mice may thus serve as an animal model for the investigation of cognitive deficits and degenerative phenomena which take place during physiological ageing and in Alzheimer's disease.

Materials and methods

Animals

Mice used for this study were generated by an F_1 cross of heterozygous β2 mutant mice with a mixed DBA/2 and C57Bl/6 background. In all these experiments, no significant difference was seen between $\beta2^{+/+}$ and $\beta2^{+/-}$ siblings. The data for +/+ and +/– animals were therefore pooled and are referred to in the text as the 'control siblings'. Both male and female animals were used. Apart from corticosterone serum levels (see below), no significant sex-related difference in the parameters studied was observed in either control mice or $\beta2^{-/-}$ mice.

All animals were sacrificed in the late morning by decapitation. Trunk blood was collected on ice and centrifuged. After removal of clots, serum was frozen and stored at –70°C until use in hormone assays. Brains were rapidly dissected out and frozen on dry ice.

Histological staining

Neuronal and glial cells were stained using two classical histological methods, the Feulgen and Nissl methods. Frozen sections (14-μm thick) were cut using a cryostat and processed according to established protocols (Bancroft and Stevens, 1982). Morphometric (measurement of the area/section of several brain regions and thickness of cortical regions) as well as stereological (count of profile number) analysis of the sections was performed by an image analyser (KS300 software, Zeiss-Kontron, München, Germany) as described in Zoli *et al*. (1992). Cell count in the pyramidal layer was peformed on nuclei stained using the Feulgen method in 150×120 μm rectangular fields. Neuronal nuclei were discriminated from glial nuclei on the basis of their morphological and staining features (neurons = large, pale with some dark spots; glia = little, uniformly dark). Positive profiles were encircled manually and their diameter was measured and used for the Abercrombie formula.

Immunocytochemistry and enzymohistochemistry

Astrocytes and microglia were evaluated by semi-quantitative immunocytochemistry. Frozen sections, 14 μm thick, were cut using a cryostat, postfixed for 30 min in ice cold 4% paraformaldehyde and processed for immunocytochemistry as described in Zoli *et al*. (1997). Microglia and astrocytes were detected using a mouse monoclonal antibody against the MAC1 antigen (Boerhinger Mannheim, Mannheim, Germany, antibody dilution 1:50) and a bovine polyclonal antiserum against GFAP (DAKO, Denmark; antibody dilution 1:500), respectively. Acetylcholinesterase (AChE) staining was performed on frozen sections as described in Franklin and Paxinos (1997). Microdensitometry of MAC1 and GFAP immunoreactivity (IR), as well AChE staining, was performed using image analysis (KS300 software, Zeiss-Kontron, München) as described in Zoli *et al*. (1990). In the case of GFAP and MAC1 IR, rectangular fields (150×120 μm) were selected in the stratum oriens of CA1 and CA3 fields as well as in the polymorph layer of the dentate gyrus. Manual cell count of GFAP IR astrocytes was performed using a microscope (40× objective) by two researchers blind to genotype.

Receptor autoradiography

Radioligand binding was performed on 14-μm coronal brain sections cut using a cryostat and mounted on gelatinized slides. Sections were incubated with the appropriate ligand [^{125}I]α-bungarotoxin, [^{3}H]hemicholinium, [^{3}H]pirenzepine and [^{3}H]AF-DX384 to identify α7-containing nAChRs, choline transporter, M1 and M2 muscarinic receptors respectively) and washed as described in Happe and Murrin (1993) and Zoli *et al*. (1998). Displacement to determine specificity of binding were performed with 1 mM nicotine for [^{125}I]α-bungarotoxin, 10 μM hemicholinium for [^{3}H]hemicholinium and 1.5 μM atropine for the muscarinic receptor ligands. Quantitative evaluation of autoradiographic films was performed by image analysis as described in Benfenati *et al*. (1986).

Morris water maze

The Morris task was modified from Morris (1989). To assess spatial learning animals were placed in an 85 cm pool filled with room temperature milky water and allowed to swim for 60 s or until they found the location of a hidden 4-cm square platform. Mice were trained with four trials per day (one per quadrant) for 10 days and escape latency was recorded and averaged across the four trials for each day. On day 11 the platform was removed and the animals were allowed to swim for 60 s. Time spent in each quadrant and crosses of the previous platform location were measured. On day 12 the platform was moved to a new location and marked with a flag. Training was repeated as above for days 12–15 using the visible platform.

Hormone assays

Serum hormone levels were measured by radioimmunoassay (ICN Biomedicals, Costa Mesa, USA, for corticosterone and testosterone. Reagents for growth hormone and prolactin were kindly provided by NIADDK Bethesda, MD).

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