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# Dysfunction of astrocytes in senescence-accelerated mice SAMP8 reduces their neuroprotective capacity

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## Summary

**Early onset increases in oxidative stress and tau pathology are present in the brain of senescence-accelerated mice prone (SAMP8). Astrocytes play an essential role, both in determining the brain's susceptibility to oxidative damage and in protecting neurons. In this study, we examine changes in tau phosphorylation, oxidative stress and glutamate uptake in primary cultures of cortical astrocytes from neonatal SAMP8 mice and senescence-accelerated-resistant mice (SAMR1). We demonstrated an enhancement of abnormally phosphorylated tau in Ser$^{199}$ and Ser$^{396}$ in SAMP8 astrocytes compared with that of SAMR1 control mice. Gsk3β and Cdk5 kinase activity, which regulate tau phosphorylation, was also increased in SAMP8 astrocytes. Inhibition of Gsk3β by lithium or Cdk5 by roscovitine reduced tau phosphorylation at Ser$^{396}$. Moreover, we detected an increase in radical superoxide generation, which may be responsible for the corresponding increase in lipoperoxidation and protein oxidation. We also observed a reduced mitochondrial membrane potential in SAMP8 mouse astrocytes. Glutamate uptake in astrocytes is a critical neuroprotective mechanism. SAMP8 astrocytes showed a decreased glutamate uptake compared with those of SAMR1 controls. Interestingly, survival of SAMP8 or SAMR1 neurons cocultured with SAMP8 astrocytes was significantly reduced. Our results indicate that alterations in astrocyte cultures from SAMP8 mice are similar to those detected in whole brains of SAMP8 mice at 1–5 months. Moreover, our findings suggest that this *in vitro* preparation is suitable for studying the molecular and cellular processes underlying early aging in this murine model. In addition, our study supports the contention that astrocytes play a key role in neurodegeneration during the aging process.**

**Key words: Astrocytes; glutamate uptake; neuroprotection; oxidative stress; senescence-accelerated mice; tau protein.**

## Introduction

The senescence-accelerated mouse (SAM) is comprised of a group of 12 strains developed from selective inbreeding of the AKR/J strain constructed by Takeda *et al*. (1981). The senescence-accelerated mouse prone-8 (SAMP8) strain manifests irreversible advancing senescence with pathological, biochemical and behavioural alterations, whereas the senescence-accelerated mice resistant-1 (SAMR1) strain presents a normal aging pattern (Takeda, 1999). For SAMP8 and SAMR1 strains, the median lifespans have been reported as 10 and 18.9 months of age, respectively (Takeda *et al*., 1994). Interestingly, the former exhibits age-related learning and memory deficits, as well as β-amyloid-like deposits in the brain (Flood & Morley, 1998; Chen *et al*., 2004). Furthermore, increases in hyperphosphorylated tau and cyclin-dependent kinase 5 (Cdk5) expressions and activation have also been detected in SAMP8 mice (Canudas *et al*., 2005). For these reasons, SAMP8 mice are regarded as a suitable rodent model for studying the molecular mechanisms underlying cognitive impairment in aged subjects.

The free radical theory of aging contends that oxidative alterations in biomolecules brought about by reactive oxygen species (ROS) contribute to cellular dysfunction during aging (Harman, 1956). Indeed, increasing evidence of elevated oxidative stress in the aged SAMP8 brain has been reported. Increased levels of lipoperoxidation, carbonyl proteins and ROS in the brain of SAMP8 mice have been observed, together with learning and memory deficits as early as 1–5 months of age (Liu & Mori, 1993; Sato *et al*., 1996a,b; Kurokawa *et al*., 2001; Yasui *et al*., 2002, 2003; Farr *et al*., 2003; Poon *et al*., 2004a; Alvarez-García *et al*., 2006). In addition, chronic administration of antioxidants, such as melatonin, α-lipoic acid, N-acetylcysteine and acetyl-L-carnitine, to SAMP8 mice not only reduced oxidative damage to neural lipids and proteins, but also lessened cognitive deficits (Okatani *et al*., 2002; Yasui *et al*., 2002; Farr *et al*., 2003; Poon *et al*., 2005). Decreases in superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase activities, as well as increases in acyl-CoA oxidase, which have been detected early in SAMP8 mice (1–12 months) compared with age-matched SAMR1 controls (Sato *et al*., 1996b; Kurokawa *et al*., 2001; Okatani *et al*., 2002; Alvarez-García

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*et al*., 2006; Sureda *et al*., 2006), may cause elevated generation of ROS. On the other hand, ROS generated by mitochondria or from other cell sites not only cause damage to mitochondrial components and DNA, but also trigger degradative processes that contribute to the aging process (Cadenas & Davies, 2000; Manczak *et al*., 2005). In fact, mitochondrial DNA deletions as well as inefficient mitochondrial hyperactivity were found in the brain of 4- to 8-week-old SAMP8 mice (Fujibayashi *et al*., 1998; Nishikawa *et al*., 1998). Differential gene expression profile in 12-month-old SAMP8 hippocampus revealed up-regulation of cytochrome *c* oxidase subunit I and III from the respiratory chain (Cheng *et al*., 2006).

Astrocytes play an essential role not only in maintaining normal brain physiology, but also in the brain's defence against oxidative damage (for a review, see Cotrina & Nedergaard, 2002). Astrocytes possess a great number of antioxidant systems, including glutathione, glutathione transferase, catalase and superoxide dismutase enzymes (Makar *et al*., 1994; Dringen *et al*., 2000). Although they are able to protect neurons from hydrogen peroxide ($H_2O_2$) toxicity (Deshager *et al*., 1996), astrocytes remain vulnerable to oxidative stress, since they react to low concentrations of $H_2O_2$ generating ROS and lipoperoxidation, and increasing antioxidant defences (Röhrdanz *et al*., 2001). Furthermore, astrocytes are primarily responsible for clearing extracellular glutamate, thereby preventing neuronal excitotoxiciy (for a review, see Anderson & Swanson, 2000). Thus, alterations in all these systems during aging may potentially reduce the neuroprotective capacity of astrocytes, further contributing to neurodegeneration. In fact, increasing evidence suggests that astrocytes play a significant role in neuronal aging. Heightened expression of glial fibrillary acidic protein (GFAP) and S100 β protein have been detected during aging in regions of the mouse and rat brain (O'Callaghan & Miller, 1991; Kohama *et al*., 1995; Amenta *et al*., 1998), as well as in mouse and rat cortical astrocytes aged in culture (Papadopoulos *et al*., 1998; Gottfried *et al*., 2002; Tramontina *et al*., 2002; Pertusa *et al*., 2007). Superoxide production, lipoperoxidation, protein oxidation, and iron staining remained elevated in aged astrocyte cultures, even though antioxidant defences had been maintained or increased (Papadopoulos *et al*., 1998; Gottfried *et al*., 2002; Klamt *et al*., 2002; Pertusa *et al*., 2007). Mitochondrial membrane potential in old astrocytes proved more depolarized than in young astrocytes (Lin *et al*., 2006). Furthermore, changes in glial glutamate uptake have been observed during brain aging (for a review, see Segovia *et al*., 2001). In aged astrocytes in culture, glutamate uptake was more vulnerable to inhibition by $H_2O_2$ exposition (Gottfried *et al*., 2002; Pertusa *et al*., 2007). Recent studies have shown that PC12 cells co-cultured with old astrocytes were more sensitive to the oxidant *tert*-buthyl $H_2O_2$ than those co-cultured with young astrocytes (Lin *et al*., 2006). In addition, we have shown that astrocytes aged *in vitro* have a reduced ability to maintain neuronal survival (Pertusa *et al*., 2007).

Until now, pathological neuronal dysfunction has been regarded as the main mechanism underlying the cognitive deficits in SAMP8 mice. However, some evidence suggests that altered astrocytes contribute to the accelerated senescence noted in this animal model. Increased astrogliosis and microgliosis have been reported in the hippocampus and cerebral cortex of SAMP8 mice (Nomura *et al*., 1996; Wu *et al*., 2005). In addition, D-serine, a glial modulator of nerve cells, is reportedly involved in the long term potentiation (LTP) dysfunction recorded in hippocampal slices from 12-month-old SAMP8 (Yang *et al*., 2005a). The aim of this study was to explore whether SAMP8 astrocytes exhibit aged-related characteristics consistent with a possible role in the biochemical and behavioural alterations observed in aged SAMP8 mice. The study was performed in cortical astrocytes since many senescence-related biochemical changes of SAMP8 mice have been described in this brain area, as mentioned above. In SAMP8 astrocytes, we found hyperphosphorylation in some forms of tau, protein kinase Gsk3β and Cdk5 activation, and increases in overall oxidative stress status. Glutamate uptake in primary cultures of cortical astrocytes from SAMP8 mice was decreased compared to that from SAMR1 mice. We also explored the neuroprotective capacity of SAMR1 and SAMP8 astrocytes in co-cultures with cortical neurons from both strains. Here, we have demonstrated that SAMP8 astrocytes suffer reduced neuroprotective abilities.

## Results

### Changes in tau, Gsk3β and Cdk5 protein expression in SAMP8 astrocytes

The hyperphosphorylation of tau was evaluated by Western blot using two phosphorylation-dependent and site-specific antibodies against tau in SAMR1 and SAMP8 astrocytes. Densitometric analysis of the immunoblots in SAMP8 astrocytes showed a statistically significant increase in phosphorylated tau at $Ser^{199}$/$Ser^{396}$ sites compared with SAMR1 controls (Fig. 1). The expression of two Ser/Thr protein kinases involved in regulating tau phosphorylation, Gsk3β and Cdk5, was also determined by Western blot. We detected a significant increase in phosphorylated Gsk3β at $Tyr^{216}$ (active form) and a significant decrease at $Ser^{9}$ (inactive form) in SAMP8 astrocytes compared with SAMR1 (Fig. 2a). These results indicated activation of Gsk3β kinase in SAMP8 astrocytes in culture. Figure 3(a) shows that Cdk5 immunoblots in SAMP8 and SAMR1 astrocytes were not significantly different, indicating similar Cdk protein expression for both strains. The induction of Cdk5 kinase activity was determined using p25/p35 labeling. The expression of p35 was decreased while that of p25 increased in SAMP8 compared with SAMR1. The calculated p25/p35 ratio showed increased Cdk5 activity in SAMP8 astrocyte cultures. When SAMP8 astrocyte cultures were treated for 48 h with 10 mM lithium, a specific inhibitor of Gsk3β activity, a significant increase and decrease of phosphorylated Gsk3β at $Ser^{9}$ and Tau at $Ser^{396}$, respectively, was detected (Fig. 2b). Roscovitine (15 μM), a non-specific Cdk5 kinase inhibitor, showed a tendency to decrease the p25/p35 ratio ($p = 0.2163$) and tau phosphorylation at $Ser^{396}$ ($p = 0.1375$)

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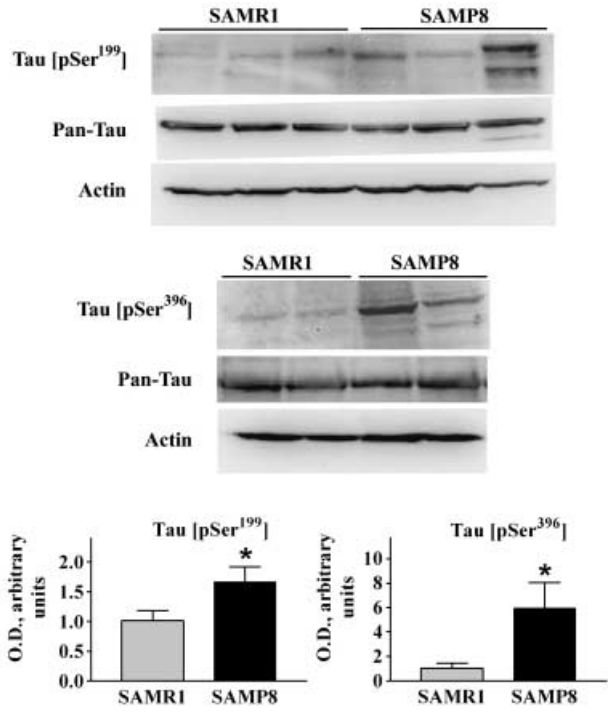


**Fig. 1** Representative immunoblots and densitometry analysis of tau protein phosphorylation in SAMR1 and SAMP8 astrocyte cultures. Tau phosphorylation was assessed using phosphorylation-dependent antibodies anti-tau [pSer$^{199}$] and anti-tau [pSer$^{396}$]. Graphs summarize the results from five separate experiments. The immunoreactivity levels of each band in the SAMR1 and SAMP8 cultures were first normalized to that of the corresponding Pan-Tau and then to β-actin. Both phosphorylated protein levels were increased in SAMP8 astrocyte cultures. Data were compared using the Student's *t*-test (*$p < 0.05$).

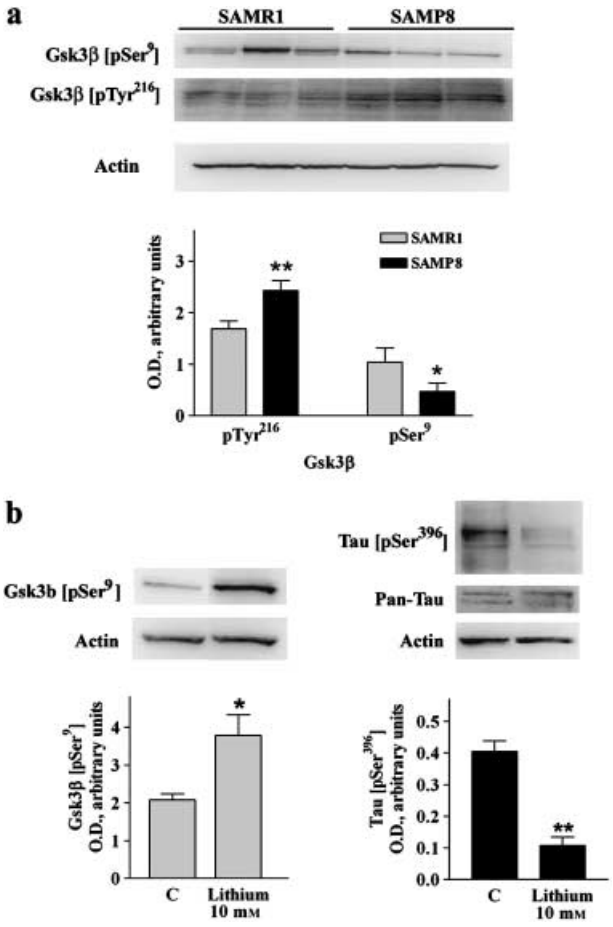


**Fig. 2** (a) Representative immunoblots and densitometry analysis of Gsk3β protein phosphorylation in SAMR1 and SAMP8 astrocyte cultures. Gsk3β phosphorylation was assessed using phosphorylation-dependent antibodies anti-Gsk3β [pSer$^{9}$] and anti-Gsk3β [pTyr$^{216}$]. Graphs summarize the results from three to seven separate experiments. The immunoreactivity levels of each band in the SAMR1 and SAMP8 cultures were normalized to that of the corresponding β-actin. The protein levels of Gsk3β [pTyr$^{216}$] and Gsk3β [pSer$^{9}$] increased and decreased, respectively, in SAMP8 astrocyte cultures vs. SAMR1. (b) Effects of the Gsk3β inhibitor lithium (10 mM, 48 h) on Gsk3β phosphorylation at Ser$^{9}$ and tau phosphorylation at Ser$^{396}$ in the SAMP8 astrocyte cultures. Graphs summarize the results from three to four separate experiments. The immunoreactivity levels of tau pSer$^{396}$ were first normalized to that of the corresponding Pan-Tau and then to β-actin. Lithium increased Gsk3β phosphorylation at Ser$^{9}$, indicating enzyme activity inhibition, and decreased tau phosphorylation at Ser$^{396}$. Data were compared using the Student's *t*-test (*$p < 0.05$, **$p < 0.01$).

in SAMP8 astrocytes (Fig. 3b). Roscovitine effects did not reach statistical significance due to the high variability found between samples from different cultures.

### Oxidative stress increase and mitochondrial membrane potential decrease in SAMP8 astrocytes

Basal levels of superoxide anion radical generated for 1 h in SAMR1 and SAMP8 astrocytes were determined via a hydroethidium probe. As shown in Fig. 4, SAMP8 cultures produced significantly more superoxide radical than did those of SAMR1. No differences were observed between the two strains in terms of hydroperoxide generation (data not shown). The main source of superoxide radical production is the mitochondrial electron transport chain that occurs during normal respiration. Mn-superoxide dismutase transforms superoxide radical into $H_2O_2$, which may diffuse through the mitochondrial membrane to the cytoplasm. As $H_2O_2$ can be transformed into harmful radicals, increased generation of these oxidants may cause lipid and protein oxidation. Figure 5(a) shows significantly increased production of lipoperoxidation, as measured by malondialdehyde and 4-hydroxyalkenal accumulation, in SAMP8 astrocytes vs. SAMR1. Protein carbonyl formation, which is indicative of protein oxidation and protein damage, was significantly elevated in SAMP8 compared with those of SAMR1 (Fig. 5b). Enhanced free radical production may be related to changes in mitochondrial membrane potential. Therefore, we measured the accumulation of the fluorescent dye rhodamine 123, which is dependent on membrane potential, in SAMR1 and SAMP8 astrocyte cultures. SAMP8 astrocytes exhibited a significant reduction in the basal uptake of rhodamine 123 compared with SAMR1 astrocytes, indicative of lower mitochondrial membrane potential in those cultures (Fig. 6).

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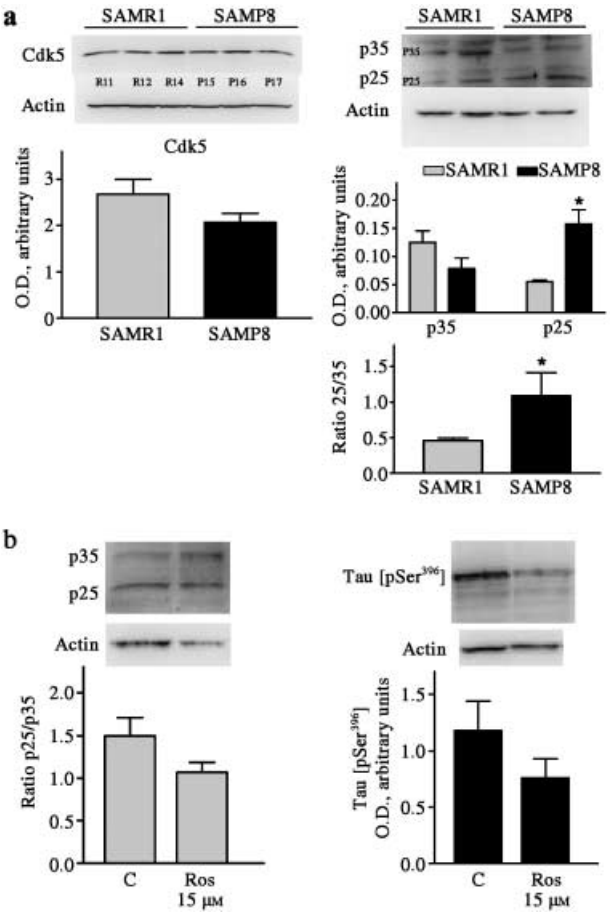


**Fig. 3** (a) Representative immunoblots and densitometry analysis of Cdk5, p35 and p25 proteins in SAMR1 and SAMP8 astrocyte cultures. Graphs summarize the results from three to five separate experiments. The immunoreactivity levels of each band in the SAMR1 and SAMP8 cultures were normalized to that of the corresponding β-actin. SAMP8 and SAMR1 Cdk5 protein levels were not different. The p25/p35 ratio indicated a decrease in p35 band intensity, which resulted in an increase in p25 band intensity in SAMP8 astrocytes. (b) Effects of the Cdk5 inhibitor roscovitine (15 μM, 48 h) on p35 and p25 proteins and tau phosphorylation at $Ser^{396}$ in the SAMP8 astrocyte cultures. Graphs summarize the results from 3–4 separate experiments. The immunoreactivity levels of tau $pSer^{396}$ were first normalized to that of the corresponding Pan-Tau and then to β-actin. Roscovitine slightly decreased the p25/p35 ratio ($p = 0.2163$) and tau phosphorylation at $Ser^{396}$ ($p = 0.1375$). Data were compared using the Student's *t*-test (*$p < 0.05$).

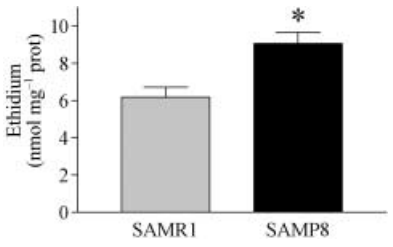


**Fig. 4** Intracellular superoxide anion radical generation after 1 h in SAMR1 and SAMP8 astrocyte cultures, as measured by dihydroethidium oxidation to ethidium. Results are expressed as nmol of ethidium $mg^{-1}$ of protein and represent the mean ± SEM of three independent cultures. SAMP8 cultures generated significantly more superoxide radicals than did SAMR1 cultures. *$p < 0.01$ compared with SAMR1 astrocytes by the Student's *t*-test.

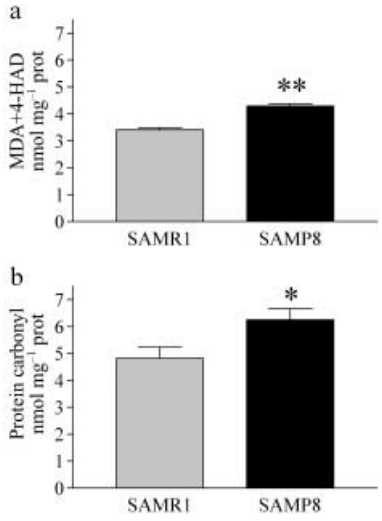


**Fig. 5** Lipoperoxidation and carbonyl proteins in SAMR1 and SAMP8 astrocyte cultures. Lipoperoxidation (a) was expressed as nmol of malondialdehyde plus 4-hydroxyalkenal $mg^{-1}$ of protein and carbonyl proteins (b) were expressed as nmol of protein carbonyl $mg^{-1}$ of protein. Results represent the mean ± SEM of six independent cultures. We observed significantly more lipid and protein damage in SAMP8 cultures than in SAMR1 cultures. *$p < 0.05$ and **$p < 0.0001$ compared with SAMR1 astrocytes using the Student's *t*-test.

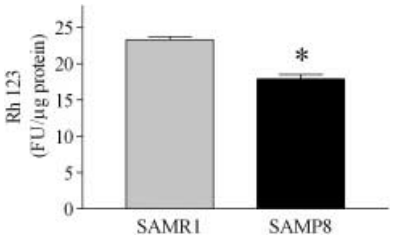


**Fig. 6** Mitochondrial membrane potential in SAMR1 and SAMP8 astrocyte cultures, as measured by the fluorescent dye rhodamine 123 (Rh 123). Results are expressed as fluorescence units (FU) of Rh 123 per μg of protein and represent the mean ± SEM of three independent cultures. SAMP8 astrocytes showed a reduced basal rhodamine 123 uptake. *$p < 0.01$ compared with SAMR1 astrocytes using the Student's *t*-test.

## Decreased glutamate uptake in SAMP8 astrocytes

The ability of astrocytes to uptake glutamate was evaluated in SAMR1 and SAMP8 cultures using an isotopic method with several glutamate concentrations. Figure 7(a) shows that glutamate uptake in SAMP8 astrocyte cultures was significantly lesser than in SAMR1 cultures. Kinetic parameters obtained from Eadie-Hofstee analysis revealed a statistically significant decrease in Km (92.78 ± 15.15 μM for SAMP8 and 222.2 ± 42.09 μM for SAMR1, $p < 0.05$) and Vmax (10.49 ± 0.67 nmol $mg^{-1}$ protein $min^{-1}$ for SAMP8 and 49.99 ± 5.09 nmol $mg^{-1}$ protein $min^{-1}$ for SAMR1, $p < 0.0001$) values in SAMP8 astrocytes (Fig. 7b). SAMP8 astrocytes treated with 100 μM trolox or 10 μM curcumin throughout the culture time period did not undergo a reversal of glutamate uptake inhibition (Fig. 7c). Expression of the glutamate-aspartate

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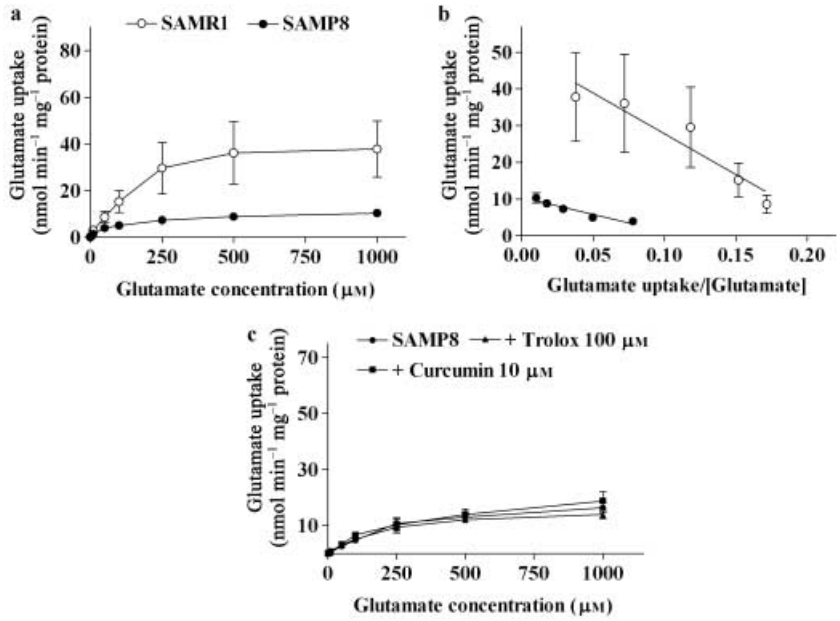


**Fig. 7** (a) Glutamate uptake in SAMR1 and SAMP8 astrocyte cultures. Astrocytes were incubated with 9.8 nM [$^3$H]-glutamate and several concentrations of unlabelled glutamate as described in the Experimental procedures. Each point represents the mean ± SEM of five to eight determinations. Two-way analysis of variance followed by Bonferroni's test showed that strain ($F_{1,107} = 23.95$, $p < 0.0001$) and glutamate concentration ($F_{8,107} = 9.63$, $p < 0.0001$) had a significant effect. (b) Values from (a) (glutamate uptake and unlabelled glutamate concentrations from SAMR1 and SAMP8 astrocytes) were used to construct the Eadie-Hofstee representation to calculate Km and Vmax kinetic parameters. There was a significant decrease in the Km and Vmax values of glutamate uptake in SAMP8 astrocyte cultures (see Results). (c) Glutamate uptake in SAMP8 astrocytes treated during the culture period with 100 μM trolox or 10 μM curcumin. No significant effects were observed.

transporter (GLAST) was examined by Western blot and no changes were observed between SAMP8 and SAMR1 astrocytes (data not shown).

### SAMP8 astrocytes show reduced neuroprotective capacity

The neuroprotective capacity of astrocytes was evaluated in co-cultures with cortical neurons by determining neuronal survival. Living neurons were identified by staining in co-cultures of SAMR1 or SAMP8 neurons seeded on a monolayer of either SAMR1 or SAMP8 astrocytes for 12 days. Thereafter, the number of living cells, either in SAMR1 or SAMP8 neurons, proved to be less in SAMP8 astrocyte co-cultures than in those of SAMR1 (Fig. 8a). These results revealed that SAMP8 astrocytes suffered a reduced neuroprotective capacity. No differences were found in the number of living neurons from SAMR1 or SAMP8 mice co-cultured with SAMP8 astrocytes. When SAMR1 and SAMP8 neurons were cultured in the presence of astrocyte-conditioned medium from SAMR1 or SAMP8 mice, their survival was higher than in the absence of astrocytes but lower than in co-cultures, and was similar for both types of astrocytes (Fig. 8b). These results suggested that differences in neuroprotective capacity are not due to a soluble compound secreted by astrocytes to the medium.

Taking into account the high astrocyte enrichment of the cultures, alterations observed in this work were attributed to the astrocyte population. However, some small contribution of contaminant microglial cells cannot be discarded.

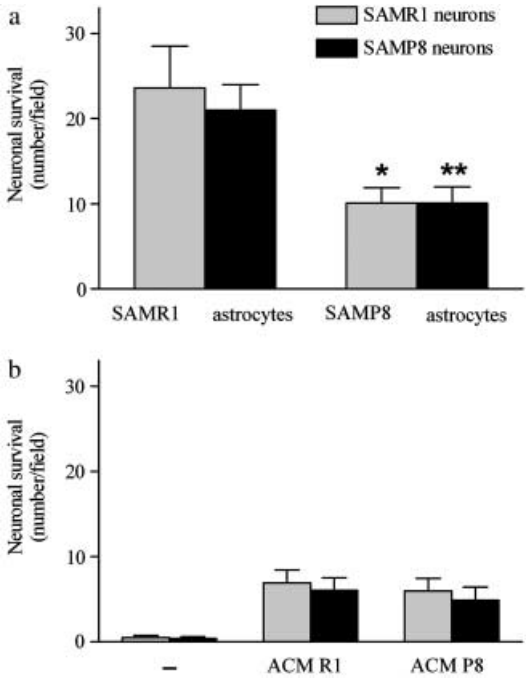


**Fig. 8** Neuronal survival of SAMR1 and SAMP8 neurons in the presence of (a) astrocytes or (b) astrocyte-conditioned medium (ACM) from SAMR1 and SAMP8 mice. Cortical neurons were cultured on a monolayer of SAMR1 and SAMP8 astrocyte cultures or with 48 h ACM for 12 days. Living neurons in both conditions were identified by cell staining using NeuN or NF antibody and propidium iodide. Results are expressed as the number of living neurons by field and represent the mean ± SEM of seven independent co-cultures. Number of astrocytes by field is 230 ± 13 and 237 ± 16 for SAMR1 and SAMP8 astrocytes, respectively. Data were compared using the Student's *t*-test (*$p < 0.05$, **$p < 0.01$ related to co-cultures of neurons and SAMR1 astrocytes).

## Discussion

Although mainly present in neuronal cells, tau protein has also been found in the astrocytes and oligodendrocytes of some neural diseases (for review, see Avila *et al.*, 2004). It has been largely demonstrated that the hyperphosphorylation of tau reduces its binding to microtubules, thereby resulting in neurofibrillary degeneration. This process occurs systematically in the neurons of human entorhinal and hippocampal regions during aging

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(Delacourte *et al*., 2002, 2003). Recently, several authors reported an age-dependent pattern of glial tau aggregation in both human and baboon brains (Schultz *et al*., 2000; Yang *et al*., 2005b). Previous studies have shown that hyperphosphorylation of tau occurs in the brain of SAMP8 mice at 5 months of age (Canudas *et al*., 2005; Sureda *et al*., 2006). Consistent with these data, the present study has demonstrated increased tau phosphorylation at $Ser^{199}$ and $Ser^{396}$ in SAMP8 astrocyte cultures obtained from neonate brain, as compared with those of SAMR1 mice. Both sites have been shown to be phosphorylated *in vitro* by several protein kinases, such as Cdk5 and Gsk3β (see the review by Gong *et al*., 2005). Our results showed an increase and decrease of the active ($pTyr^{279}$) and inactive ($pSer^{9}$) form, respectively, of Gsk3β in SAMP8 vs. SAMR1, thus indicating activation of this enzyme. In addition, an increase in the p25/p35 ratio in SAMP8 astrocytes also denoted activation of the Cdk5 kinase. It is worth noting that the presence of functional Cdk5/p35 in murine cortical astrocyte cultures was recently reported by He *et al*. (2007). Therefore, both protein kinases may be responsible for the hyperphosphorylation of tau in SAMP8 astrocytes. The increase of Cdk activity was in agreement with the results in early senescent SAMP8 brains reported in the above-mentioned study of Canudas *et al*. (2005). On the other hand, no changes were reported in the phosphorylation at $pTyr^{279}$ and $pSer^{9}$ sites of Gsk3β between SAMP8 and SAMR1 brain. We cannot discard an increase of Gsk3β kinase activity in the absence of Gsk3β phosphorylation changes in senescent mice *in vivo*, according to the findings in old p25 transgenic mice with constant Cdk5 over-activation (Plattner *et al*., 2006). Therefore, in brains of early senescent SAMP8, Gsk3β kinase activity might be enhanced through Cdk5 activation. If this were the case, Gsk3β phosphorylation changes in astrocyte cultures would be indicative of inconsequential differences in the pathways of tau hyperphosphorylation as related to the brain tissue. The hyperphosphorylation of tau in cultures of SAMP8 neonate astrocytes may be considered as an early signal of an aging-related pathological process. The biological significance of astrocytic tau pathology in aging remains largely unknown. In certain diseases characterized by tau-based neurofibrillary pathology (tauopathies), tau aggregation in astrocytes appears to have no link with gliosis and, in fact, has been regarded as a degenerative process (Togo & Dickson, 2002). Moreover, a transgenic mouse model of human tau expression in astrocytes has shown an age-dependent pathology with abnormal tau phosphorylation leading to a focal neuron degeneration (Forman *et al*., 2005).

The regulation of tau hyperphosphorylation at different sites by protein kinases is a complex mechanism not well known, that probably requires the synergistic action of two or more kinases (Sengupta *et al*., 2006). Li *et al*. (2006) reported that Cdk5 phosphorylates tau at $S^{404}$ and enhances Gsk3β-catalysed tau phosphorylation at $S^{400}$ and $S^{396}$, suggesting that Cdk5 primes tau for Gsk3β in the brain. In our work, tau hyperphosphorylation at $S^{396}$ was reduced by lithium and roscovitine, indicating a directly Gsk3β- and Cdk5-mediated tau hyperphosphorylation at this site in SAMP8 astrocytes. However, we cannot discard that Gsk3β-mediated tau hyperphosphorylation at $Ser^{396}$ in SAMP8 astrocytes were a consequence of Cdk5-mediated phosphorylation at other sites in tau, as has been reported by Li *et al*. (2006) in rat brain. Further studies are necessary to identify the precise mechanisms underlying the hyperphosphorylation of tau in SAMP8 astrocytes.

There is wide consensus that free radicals are involved in the damaging processes associated with brain aging (Poon *et al*., 2004b). Interestingly, there is significant evidence that SAMP8 mice (age 1–5 months) possess a more elevated oxidative status compared with SAMR1 controls (Liu & Mori, 1993; Sato *et al*., 1996a,b; Kurokawa *et al*., 2001; Yasui *et al*., 2002, 2003; Farr *et al*., 2003; Poon *et al*., 2004a; Alvarez-García *et al*., 2006). In the current study, we demonstrated that SAMP8 astrocyte cultures present increased superoxide radical generation, lipoperoxidation and carbonyl proteins vs. SAMR1, which is consistent with *in vivo* studies. Elevated oxidative stress and abnormally phosphorylated tau in SAMP8 astrocytes suggest that neonatal astrocytes maturated in culture acquire characteristics similar to those observed *in vivo* in the brains of senescent SAMP8 mice. Thus, SAMP8 astrocyte cultures offer an effective model for studying the molecular and cellular processes underlying aging. Furthermore, these results support previous hypotheses contending that glial oxidative stress is involved in the aging process. In this context, several authors have demonstrated increased superoxide production, lipoperoxidation, protein oxidation and iron staining in astrocytes aged in culture (Papadopoulos *et al*., 1998; Gottfried *et al*., 2002; Klamt *et al*., 2002; Pertusa *et al*., 2007). It has been proposed that ROS generated by mitochondria accumulate during aging and are not only responsible for the damage present in mitochondrial components, but also cause degradative processes (Cadenas & Davis, 2000; Lenaz *et al*., 2006). Accordingly, we have detected lower mitochondrial membrane potential in SAMP8 astrocytes, which may stem from the elevated oxidative stress detected in these cultures. These results are in agreement with studies carried out by Lin *et al*. (2006) in which astrocytes cultured from older mice exhibited lower mitochondrial membrane potential than astrocytes cultured from younger mice. Age-related correlations between increases in ROS production and decreases in mitochondrial membrane potential have also been detected in hippocampal neurons cultured from old rats (Parihar & Brewer, 2007). On the other hand, our results are in agreement with those authors who observed altered mitochondrial dysfunction in SAMP8 mice. For instance, Nishikawa *et al*. (1998) detected an early stage mechanism underlying the age-associated mitochondrial dysfunction present in SAMP8 mouse brain, and Xu *et al*. (2007) reported that platelet mitochondrial membrane potential as well as hippocampal and platelet ATP content in SAMP8 mice decreased at early age compared with SAMR1. Thus, our results support the contention that mitochondrial alterations may play a key role in the aging process.

The consequences of tau pathology on astrocyte function have not been thoroughly investigated. Recently, a transgenic mouse model used to investigate tau pathology in astrocytes

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revealed some loss of function as a consequence of increased tau expression and abnormally phosphorylated, ubiquitinated and filamentous tau (Forman *et al*., 2005; Dabir *et al*., 2006). These transgenic mice manifested neurodegeneration and reduced glutamate transport in those cerebral regions with robust astrocytic tau expression. In our own SAMP8 astrocyte cultures, we also noted decreased glutamate uptake in tandem with the above-mentioned increases in abnormally phosphorylated tau and oxidative stress. Alterations in glial glutamate transport may result from oxidative injury. Trotti *et al*. (1997) reported that glutamate transporters are vulnerable to oxidants due to the fact that they possess a redox regulatory mechanism. We were unable to reverse the inhibition of glutamate uptake in SAMP8 astrocytes using trolox and curcumin antioxidant treatments, even though we did reverse $H_2O_2$-induced glutamate uptake inhibition in astrocyte aged in culture (Pertusa *et al*., 2007). It is possible that a different antioxidant pattern treatment is required to reverse long-time oxidative effects. On the other hand, reduced glutamate uptake in the transgenic mouse of tau pathology has been associated with a decreased expression of glial glutamate transporters (Dabir *et al*., 2006). However, we could not detect any reduction in GLAST transporter expression in SAMP8 astrocytes. It has been suggested that alterations in the trafficking of glutamate transporters by tau pathology may lead to reduced glutamate uptake (Dabir *et al*., 2006). Additional studies are needed to evaluate the functional consequences of tau alterations in glial glutamate transport.

The role played by astrocytes in neuronal antioxidant defence is now widely understood (Deshager *et al*., 1996). Previous studies have demonstrated that functional alterations in astrocytes aged *in vitro* (Pertusa *et al*., 2007) or in astrocytes cultured from old mice (Lin *et al*., 2006) lead to reduced neuronal survival. We examined the survival of cortical neurons from SAMR1 and SAMP8 mice 12 days after co-culturing with SAMR1 and SAMP8 astrocytes. The latter were less able to protect cortical neurons from both strains than were SAMR1 astrocytes. When SAMR1 and SAMP8 neurons were cultured with astrocyte-conditioned medium from SAMR1 or SAMP8 astrocytes, similar neuronal survival was attained. Therefore, differences in neuroprotective capacity seem not to be mediated by soluble compounds released from astrocytes to medium. The attained neuroprotection was lower than that observed in co-cultures. Accordingly, it is well known that survival of neurons cultured in conditioned medium from astrocytes is significantly prolonged in relation to pure neuronal cultures, but is lower than that obtained when neurons and astrocytes are co-cultured (Bauer & Tontsch, 1990; Walsh *et al*., 1992). This suggests that several cellular mechanisms can underlie the neuroprotective role of astrocytes in addition to soluble factors. It can be suggested that alterations in oxidative stress, mitochondrial function, glutamate uptake and/or tau phosphorylation in SAMP8 astrocytes are underlying factors in the lost of neuroprotection. For instance, reduction in glial glutamate uptake capacity may elevate extracellular glutamate levels leading to neuronal excitotoxicity (Rohstein *et al*., 1996). Thus, changes in astrocyte function may play a key role in determining neuronal survival in the aged SAMP8 brain. Indeed, increases in GFAP expression and PK-11195 binding activity (a marker of gliosis), together with neurodegeneration, have been reported in the hippocampus and cerebral cortex of SAMP8 mice (Nomura *et al*., 1996; Wu *et al*., 2005; Sureda *et al*., 2006). Altered astrocytic function may contribute not only to brain neurodegeneration, but also to the premature learning and memory deficits observed in this murine model of early aging.

In summary, we have shown that astrocytes cultured from neonatal SAMP8 mice present similar alterations to those described in the whole brains of SAMP8 mice at 1–5 months of age. Similar to what occurs during brain aging, increased ROS generation in SAMP8 astrocytes can lead to lipid and protein oxidation, which in turn can cause changes in mitochondrial activity, tau phosphorylation and glutamate transport. All of these alterations can compromise astrocyte functionality. In this context, we have shown that SAMP8 astrocytes have reduced neuroprotective ability. This finding is in agreement with our previous studies showing decreased neuroprotection in astrocytes aged in culture (Pertusa *et al*., 2007). The present study demonstrates the dysfunction present in SAMP8 astrocytes and also supports the hypothesis that age-related alterations in astrocytes contribute to neurodegeneration.

## Experimental procedures

### Materials

SAMP8 and SAMR1 mice were provided by Harlan Interfauna Ibérica (Barcelona, Spain). Dulbecco's modified Eagle medium (DMEM), gentamycin and fetal bovine serum (FBS) were purchased from Gibco-BRL (Invitrogen, Paisley, UK). Culture plates, chamber slides and flasks were from NUNC (Roskilde, Denmark). L-[$^3$H]-glutamate (specific activity, 51 Ci mmol$^{-1}$) was purchased from PerkinElmer Inc. (Wellesley, MA, USA). The fluorescent probes 2′-7′-dichlorfluorescin diacetate (DCFH-DA), dihydroethidium and rhodamine-123 were obtained from Molecular Probes (Leiden, The Netherlands). L-glutamic acid, $H_2O_2$, thiazolyl blue tetrazolium bromide (MTT), trolox, curcumin, roscovitine and lithium chloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-tau [pSer$^{199}$], anti-tau [pSer$^{396}$] and anti-Pan-Tau antibodies were from Biosource International Inc. (Camarillo, CA, USA), anti-Cdk5, anti-p35 and anti-p25 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), anti-Gsk3β [pTyr$^{216}$] and anti-Gsk3β [pSer$^9$] from Bioreagents (Golden, CO, USA), anti-GLAST antibody from Alpha Diagnostic International (San Antonio, TX, USA), anti-NeuN from Chemicon International (Temecula, CA, USA) and anti-neurofilament (NF) from DAKO (Glostrup, Denmark). All chemicals were of analytical grade.

### Astrocyte cultures

Primary cultures enriched in astrocytes were established using cerebral cortical tissue from 2-day-old SAMP8 and SAMR1 mice.

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Brains were dissected free of the meninges, diced into small cubes and dissociated by incubation with a 0.5% trypsin-EDTA solution (Gibco) for 25 min. Cells were seeded at $0.25 \times 10^6$ cells $mL^{-1}$ ($0.75 \times 10^5$ cells $cm^{-2}$) in multiwell plates, chamber slides or flasks in DMEM supplemented with 2.5 mM glutamine, 100 μg $mL^{-1}$ gentamycin and 20% FBS at 37 °C in a humidified atmosphere of 5% $CO_2$/95% air. The culture medium was changed every 3–4 days and FBS was progressively lowered to 10% during the first 2 weeks. Experiments were routinely carried out at 21 days *in vitro*. Established astrocyte cultures of both SAMR1 and SAMP8 consisted of 85–90% astrocytes, 10–15% microglia and 0.1–1% oligodendroglia. Neither cell death nor morphological alterations were observed in SAMP8 cultures.

### Neuronal survival in co-culture with astrocytes

To study the neuronal protective response of SAMR1 and SAMP8 astrocyte cultures, co-cultures of neurons and astrocytes were plated on 12-mm coverslips. Fresh neurons disaggregated from the cerebral cortical tissue of SAMR1 and SAMP8 15-day-old fetuses were directly seeded at $1.5 \times 10^6$ cells $mL^{-1}$ ($4.5 \times 10^5$ cells $cm^{-2}$) on a confluent monolayer of SAMR1 and SAMP8 astrocyte cultures. Cultured medium was DMEM supplemented with 0.2 mM glutamine, insulin B 100 mU $L^{-1}$, pABA 7 μM and 10% FBS. Neuronal survival was assessed at 12 DIV. Dead cells were detected by staining the cultures with 7.5 μM propidium iodide (red fluorescence) for 30 min. Cultures were subsequently washed with PBS and fixed with 4% paraformaldehyde. Living neurons were identified by immunostaining with NeuN or NF antibodies. Cells were permeabilized with 0.25% Triton in PBS for 30 min, washed with PBS, incubated with goat serum to block unspecific binding sites at room temperature, and incubated with mouse Neu-N or NF antibodies (diluted 1 : 200) overnight at 4 °C. Cultures were then washed with PBS and incubated with anti-mouse Alexa Fluor 488 (green fluorescence) for 1 h at room temperature. After washing with PBS, nuclei were stained with 5 μM bisbenzimide. Finally, cultures were mounted on microscope slides. Randomly chosen fields were examined using a fluorescence microscope (Nikon Eclipse E1000, Nikon, Tokyo, Japan) and digitally photographed with a ColorView camera (Soft Imaging Systems, Stuttgart, Germany). Neuronal survival was determined by counting immunoreactive neurons to NeuN or NF that were not propidium iodide stained, using the ANALYSIS software (Soft Imaging System). Astrocyte conditioned media were prepared from SAMR1 and SAMP8 astrocyte cultures at 21 DIV. For that purpose, astrocyte medium was changed to the neuronal medium used for co-cultures (see above). Conditioned media were collected after 48 h, centrifuged for 5 min at $300 \times g$ and used immediately. Neurons were seeded in astrocyte-conditioned media supplemented with 30% of fresh neuronal medium to provide a complete nutrient provision. Media were partially changed every 4 days with fresh conditioned media. At 12 DIV, neuronal survival was measured by the same procedure as in co-cultures.

### Reactive oxygen species generation

Intracellular generation of hydroperoxides and superoxide anion radicals was determined using DCFH-DA and dihydroethidium, respectively, as previously described (Sebastià *et al.*, 2004). Briefly, cultures in 96-well plates were washed in HBSS and loaded with 10 μM DCFH-DA for 20 min at 37 °C. Wells were then washed with HBSS and basal 2′-7′-dichlorfluorescein (DCF) fluorescence was measured after 1 h incubation at 37 °C in a fluorescence plate reader (Spectramax Gemini XS, Molecular Devices, Wokingham, UK) at 485 nm excitation/530 nm emission. To measure superoxide anion generation, cultures in 96-well plates were loaded with 4.8 μM dihydroethidium and basal ethidium fluorescence was measured at 485 nm excitation/590 nm emission after 1 h at 37 °C.

### Lipid and protein damage

Lipid peroxidation and protein damage were determined as described previously (Alvarez-García *et al.*, 2006). Lipid peroxidation was measured by determining malondialdehyde and 4-hydroxyalkenal using a Lipid Peroxidation Assay Kit from Calbiochem (EMD Biosciences Inc., Darmstadt, Germany). Protein damage was determined as carbonylated proteins. The chromogene 2,4-dinitrophenylhydrazine reacted with the carbonyl groups of the damage proteins. Protein carbonyls were determined at 366 nm.

### Mitochondrial membrane potential

Changes in mitochondrial membrane potential were measured using rhodamine 123. Cultures in 96-well plates were incubated with 13 μM rhodamine 123 at 37 °C for 1 h. The cells were washed twice with HBSS, and fluorescence was determined at 507 nm excitation/529 nm emission.

### Western blot analysis

After shaking the cultures for 4 h to eliminate microglial cells, the medium was removed and astrocytes were rinsed twice with PBS. Cells were lysed for 10 min on ice in RIPA buffer (10 mM PBS, 1% Igepal AC-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing a protease inhibitor cocktail (Complete) and 1 mM orthovanadate. They were then collected and frozen at –20 °C until assay. Protein extracts were quantified following the Bradford method. Subsequently, 15 μg of the protein extracts were denatured at 100 °C for 5 min, loaded onto a 15% sodium dodecyl sulfate–polyacrylamide gel and electrophoresed. Proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA). This was incubated overnight at 4 °C with the following primary polyclonal antibodies: anti-tau p[$Ser^{199}$] and anti-tau p[$Ser^{396}$] diluted to 1 : 2000; anti-Gsk3β p[$Tyr^{216}$], anti-Gsk3β p[$Ser^9$], anti-Cdk, and anti-p35/p25 diluted to 1 : 1000; anti-GLAST diluted to 1 : 500. Membranes were incubated for 1 h at room

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temperature with horseradish peroxidase-conjugated secondary antibodies. Proteins were detected with a chemiluminiscence detection system based on the luminol reaction. Protein loading of the gels was controlled by staining blots with an antibody against β-actin (diluted 1 : 10 000). The immunoreactive bands were digitalized and a densitometry analysis was performed using Quantity One software (Bio-Rad). The levels of protein immunoreactivity were normalized to that of β-actin. In some experiments, SAMP8 astrocytes were treated with 10 mM lithium and 15 μM roscovitine for 48 h.

### Glutamate uptake

Astrocyte glutamate uptake was determined as previously described (Pertusa *et al.*, 2007). Glutamate culture medium was removed from 96-well cultures and astrocytes were washed with warm HEPES-buffered saline solution (HBSS) (136 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.0 mM $NaH_2PO_4$ and 10 mM HEPES) containing 9 mM glucose at pH 7.3. Astrocyte cultures were then incubated for 10 min at 37 °C in HBSS with 9.8 nM (500 nCi $mL^{-1}$) of [$^3$H]-glutamate and several concentrations of unlabelled glutamate in the range of 1 μM to 1000 μM. Uptake was terminated by removing the medium and washing the cells three times with ice-cold HBSS. This was immediately followed by cell lysis in 0.2 N NaOH. Aliquots were taken for liquid scintillation counting (with Optiphase 'Hisafe' cocktail) and for Bradford's protein assay using bovine serum albumin as the protein standard. Radioactivity was analysed by scintillation counting in a Wallac 1414 Liquid Scintillation Counter (PerkinElmer).

### Statistical analysis

Experiments were performed with astrocytes from three to eight primary cultures (*n* for each experiment is indicated in the corresponding figure legend). Data were pooled and the results given as mean ± SEM. Statistical significance ($p < 0.05$) was determined by the Student's *t*-test and two-way analysis of variance followed by Bonferroni's multiple comparison test.

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